ZINC FINGER, AN1-TYPE DOMAIN 2A---A NOVEL TARGET FOR LUNG CANCER THERAPY

by

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ABSTRACT

Arsenic is a commonly toxin found in the natural environment and from anthropogenic sources. Arsenic exposure can produce multiple adverse effects including chronic lung disease and lung cancer. The Ubiquitin Proteasome System (UPS) is involved in disease development and aging, its dysfunction can cause improper degradation of misfolded and unfolded proteins. UPS is regulated, in part, by zinc finger gene, thus zinc finger gene can mediate protein degradation, disease development and aging. Zinc finger AN1-type domain 2A (*ZFAND2A*) can be induced by arsenic and heat shock, and it plays an important role in maintaining cellular homeostasis and longevity. Here, we present a short review about arsenic-induced diseases, the UPS protein degradation machinery, and *ZFAND2A* and a preliminary study addressing *ZFAND2A* expression in NCI-H441 cell (human lung adenocarcinoma epithelial cell line) following treatment with heat shock or sodium arsenate. H441 cells appear to be resistant to the arsenic cytotoxicity and *ZFAND2A* increased markedly in these cells in response to heat stress or arsenic exposure. Because *ZFAND2A* is thought to improve UPS function and thus could be viewed as protective to cancer cells, it has significant public health relevance, future studies targeting *ZFAND2A* may be useful in the development of novel therapies to lung cancer.

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1.0 INTRODUCTION

1.1 ARSENIC AND LUNG DISEASE

Arsenic is an environmental toxicant present in food (e.g., seafood or rice), water, air, and soil (Earth's crust component). Arsenic exposures result from anthropogenic processes including use in pesticides, herbicides, wood preservatives, and dye-stuffs. Ingestion and dermal contact are the main routes of exposure. Drinking water contaminated by arsenic has been a major caused of extensive chronic poisoning, thus remains a major public health concern worldwide.

1.2 ARSENIC EXPOSURE

Arsenic is a metalloid, which means that it can display properties of both a metal and a nonmetal. There are several structural forms and oxidation states of arsenic because it can form alloys with metals and covalent bonds with hydrogen, oxygen, carbon, and other elements. Environmentally relevant forms of arsenic are inorganic and organic existing in the trivalent (III) or pentavalent (V) state. Inorganic arsenic persists in human body for longer time than organic forms due to different metabolic mechanisms. The more potent arsenic compounds are trivalent inorganic forms, such as arsenic trioxide (As_2O_3) and sodium arsenite ($NaAsO_2$).

1.2.1 Current Exposure Limits

The No Observed Adverse Effect Level (NOAEL) for inorganic arsenic is 0.009 mg/L (0.0008 mg/kg · day), the Lowest Observed Adverse Effect Level (LOAEL) is 0.17 mg/L (0.014 mg/kg day) (Environmental Protection Agency 1998), and the reference dose (RfD) is 3E-4 mg/kg day. The maximum contamination level (MCL) suggested by the World Health Organization (WHO) and the U.S. Environmental Protection Agency (EPA) is 10 μg/L currently (Environmental Protection Agency 2001), which was 50 μg/L prior to 2001 (National Research Council 2001). This level could be reduced in the future as technology and research allows, because 10 μg/L of arsenic may not have an adequate margin of safety. Cancer-death risk associated with consumption of 1.6 liters of water per day with inorganic arsenic 50μg/L· day has been estimated to be 21/1,000 (Bates et al., 1992).

1.2.2 Extend of Human Exposure

Worldwide, 160 million people are estimated to have been exposed to arsenic contaminated drinking water at concentration above the recommended exposure limits recommended by the WHO and EPA (IARC Working Group 2004). These exposures occur in geological areas where the Earth's crust composition is rich in arsenic. Especially in developing countries, such as Bangladesh, Chile, Argentina, Mexico and Taiwan, high levels of arsenic are naturally present in drinking water and cause significant public health concerns. In some regions, well water arsenic concentrations can exceed 1 mg/L and in these areas the crude lung cancer specific mortality is higher than the average level of the world.

1.3 ARSENIC NON-LUNG DISEASES

Arsenic can cause cell damage and cell death via arsenic-induced oxidative stress, and chronic exposure of arsenic-contaminated drinking water has been shown to link with various kinds of diseases in different body systems.

1.3.1 Dermal disease

The skin, a major target organ in chronic arsenic exposure, can develop skin lesions including hyperkeratosis and both hyperpigmentation, and hypopigmentation (Mazumder et al., 1998). Dermal effects are hallmarks of early stages of arsenic poisoning. In southwest Taiwan, Blackfoot disease is commonly caused by chronic exposure of high arsenic-contained drinking water. An epidemiological study conducted in the Gangetic plain of West Bengal, India and neighboring Bangladesh where the population was exposed to water with arsenic concentrations in excess of 50 μg/L, found that males tend to have a higher prevalence rate of arsenic skin lesions than females (Chowdhury et al., 2000). Skin cancer can be caused by chronic exposure to arsenic in drinking water and it is one of the most common form of neoplasm associated with arsenic ingestion (Mead 2005).

1.3.2 Cardiovascular disease

Epidemiological studies has have noted that cardiovascular system is particularly sensitive to arsenic exposure. In Taiwan, elevated mortality has been reported for ischemic heart disease (ISHD) (Chen et al., 1988), indicating that an increased risk of coronary heart disease is associated with long-time exposure to inorganic arsenic (Tchounwou et al., 2003). Hypertension, peripheral atherosclerosis, cardiac arrhythmias and other cardiovascular conditions can also be induced by chronic ingestion of arsenic-contaminated drinking water.

1.3.3 Neurological Disorders

Exposure of inorganic arsenic can cause nervous system injury, characterized by both central and peripheral neuropathy (Vahidnia et al., 2007). A reduced nerve conducting velocity in peripheral nerves is a hallmark of arsenic-induced neurotoxicity (Mathew et al., 2010). For children, the adverse effect is even worse and impaired cognitive effect have been reported in adolescents following by chronic arsenic exposure of drinking water (Tsai et al., 2003).

1.3.4 Other diseases

In addition, to dermal, cardiovascular, and neurological effects, arsenic can produce adverse hepatic, renal, reproductive, and immunological effects. Chronic arsenic exposure can cause liver injury, which is presented as jaundice, abdominal pain, and hepatomegaly (NRC, 2001: Mazumder, 2005), and progress to cirrhosis and ascites, and even liver cancer (Liu and Waalkes, 2008; Straif et al., 2009; IARC, 2011). Inorganic arsenic compounds do not cause any

significant renal injury in humans, while organic forms can cause renal and bladder effects following oral exposure in animal models, including bladder cancer (Jomova et al., 2011). Arsenic exposure has reproductive health effects, including reduced weights of the uterus and ovary and reduced ovarian and uterine peroxidase activities in mice model (Chattopadhyay et al., 2001). Arsenic exposure also has immunotoxic effects (ATSDR, 2005), thus people with chronic arsenic exposure are more susceptible to inappropriate inflammatory and wound healing responses.

1.4 ARSENIC LUNG DISEASE

1.4.1 Cancer

Arsenic has been classified as a class I human carcinogen by the International Agency of Research on Cancer (IARC), meaning that there is sufficient evidence of carcinogenicity to humans.

1.4.1.1 Molecular mechanism of lung cancer

i) The development of cancer is associated with arsenic-induced oxidative stress via reactive oxygen species (ROS) and reactive nitrogen species (RNS).

Arsenic-induced oxidative stress can cause cell damage and death through activation of oxidative sensitive signaling pathways (Roy et al., 2009). Many studies confirmed the generation of various types of ROS during arsenic metabolism in cells (Valko et al., 2005). Major arsenic-induced ROS include superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide

(H_2O_2), singlet oxygen ($^1O_2$), and peroxyl radicals (Flora et al., 2011). Superoxide anions are considered "primary" ROS. They can further interact directly, through enzyme or metal-catalyzed processes, with other molecules to generate "secondary" ROS (Valko et al., 2005). ROS have been demonstrated to be involved in the signaling of the cell transformation response (Li et al., 1998) and can affect cytoplasmic and nuclear signal transduction pathways that regulate gene expression (Sen et al., 1997), thus cause cell damage and death. These effects are important in tumor promotion. In addition to ROS, RNS are also thought to be directly involved in oxidative damage to lipids, proteins and DNA in cells exposed to arsenic (Shi et al., 2004).

ii) Arsenic alters gene expression patterns, causes DNA damaging, and inhibits DNA repair by altering histone modification.

Although arsenic may not be a classic mutagen which can cause point mutations (Huang 2004), it can induce large deletion mutations (Hei 1998). Also, arsenite inhibits the completion of DNA excision repair, perhaps via effects on DNA ligase, which is especially sensitive to arsenite in the cell (Li 1989). Histone post-translational modification is a key step that may result in an epigenetic mark that regulates chromatin structure and gene transcriptional activity thereby impacting many fundamental aspects of human biology (Zou 2014). Arsenic can modify H3K4, H3K9, and H3K27 histone methylation patterns in both malignant and nonmalignant lung cell lines, leading to decreased expression of genes associated with histone acetylation and DNA methylation changes (Jensen 2008; Zhou 2008).

1.4.1.2 The association between arsenic and lung cancer

High-level, chronic arsenic exposure (i.e. >100 μg/L in water), lung cancer mortality rate is dose dependent (Hopenhayn-Rich et al., 1998). In Northern Chile, increasing arsenic concentration

exposure in drinking water was associated with increased lung cancer incidence (Nicolli et al., 1985). Furthermore, cigarette smoking can increase the risk of lung cancer when combined with ingestion of arsenic (Begum et al., 2012). There is a substantial heterogeneity in lung cancer risk among people with similar exposure levels worldwide, which may due to genetic variation, diet habit, and the exposure patterns (arsenic species) (Celik et al., 2008).

Medium- or low-level chronic arsenic exposure do not demonstrate a similar concentration depend association with lung cancer mortality, which has been shown in studies conducted in Denmark where arsenic concentration in drinking water ranged from 0.05 to 25 μg/L and in Belgium where arsenic concretion ranged from 20-50 μg/L (Baastrup et al., 2008; Buchet et al., 1998).

1.4.2 Chronic obstructive pulmonary disease (COPD)

Arsenic-induced stress can cause a misfolded and unfolded protein response, which are significant for disease development, including COPD. An epidemiological study in Chile (Smith et al., 1998) found that young people living in areas of high arsenic exposure have high relative rates for COPD mortality. Among people aged from 30 to 39, 4 deaths from COPD occurred in males (0.8 expected), and 6 in females (0.1 expected) with men and women combined standard mortality ratio (SMR) = 11.1, 95% confidence interval (CI): 5.3-20.4 ($p<0.001$). The author suggested that early exposure to arsenic was important and led to the elevated COPD mortality rates in younger adults, since the mortality rate was not increased in older age groups (Mazumder et al., 2000).

1.4.3 Bronchiectasis, bronchitis

The prevalence of bronchiectasis was found to be 23-fold greater among children who have arsenic-induced skin symptoms onset (skin lesion) living in Antofagasta than children live in the rest of Chile (Zaldivar et al., 1980). An association between chronic arsenic ingestion and chronic bronchitis was reported in a small cross-sectional study among 94 individuals (Milton et al., 2001). In Bangladesh, another study was conducted in a population of 218, and results showed that the crude prevalence ratio for chronic bronchitis was 10.3 (95% CI: 2.4-43.1) for females and 1.6 (95% CI: 0.8-3.1) for males (Mazumder Guha 2007).

1.4.4 Other respiratory disorders

Chronic arsenic exposure induced respiratory effects was first noted in a arsenic-affected village, West Bengal, in 1995, when 57% of the 156 patients were reported to have chronic cough (Mazumder Guha et al., 1997). Chest sounds also could be caused by chronic arsenic ingestion.

2.0 THE UBIQUTIN PROTEASOME SYSTEM (UPS)

Ubiquitin proteasome system (UPS) functions to degrade unneeded or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds. Enzymes that carry out such reactions are called proteases. Proteasomes are part of a major mechanism by which cells regulate the concentration of particular proteins and degrade damaged or misfolded proteins. UPS is also involved in cell cycle controlling and the arsenic-induced alteration process of DNA expression and repair, it is also associated with aging. Properly functioning proteasome is essential for numerous cellular processes, including protein turn over and quality control, cell growth and signaling, immune response and antigen presentation. Dysfunction of UPS can cause the accumulation of misfolded and unfolded proteins, thus associated with disease development and aging. In addition, accumulation of damage proteins can lead to apoptosis. Apoptosis is programmed cell death which is controlled by cell signal transduction. Properly functioning apoptotic mechanisms are crucial for the removal of damaged cells and the prevention of cancer development (Leonard 2004), an imbalance in cell proliferation and cell death can lead to cancer development.

2.1 THE UBIQUITIN ENZYME CHAIN

2.1.1 E1, E2, E3 Ubiquitin Protein Ligases

Ubiquitination is needed for substrate (target protein) to be recognized by the proteasome before degradation, it is a complicated process in an ATP-dependent fashion and involves many enzymes. First, the terminal carboxyl group of ubiquitin is joined in a thioester bond to a cysteine residue on ubiquitin activating enzyme (E1), then transferred to a sulfhydryl group on a ubiquitin-conjugating enzyme (E2), and last to a specific target protein, a critical event that is catalyzed by a ubiquitin E3 ligase (Weathington et al., 2013a). The last step promotes transfer of ubiquitin from E2 to the ε-amino group of a lysine residue of a protein recognized by that E3, and forms an isopeptide bond. Skp1–Cullin–F-box protein (SCF), a multi-protein E3 ubiquitin ligase complex, catalyzing the ubiquitination of proteins destined for proteasomal degradation (Weathington et al., 2013 b) and can be inhibited by arsenic through PI3K/Akt pathway (Zhang et al., 2008).

2.1.2 E3 ligases are most important

E3 is crucial in orchestrating the ubiquitination of target proteins. After the target gene has undergone some post-translational modification and generates a “degron” (a specific structural motif) (Glickman and Ciechanover, 2002), E3 shuttles the target gene for degradation. The whole processes of targeting, ubiquitination, and degradation of proteins are specific and highly regulated (Weathington et al., 2013b).

There are two major E3 ligase families, the RING (really interesting new gene) and the HECT (homologous to the E6-AP carboxy terminus) proteins. The RING finger and RING-related proteins are the largest E3 ligase family, whose members can ubiquitinate a broad range of substrates, either independently as monomers or dimers, or in a multisubunit complex. The HECT domain-containing proteins are a smaller E3 family, whose members are significant in regulating proteins, including the transmembrane surface proteins (Weathington et al., 2013a).

2.2 PROTEASOME STRUCTURE AND FUNCTION

After the substrate is "marked" through polyubiquitination, it is degraded by the 26S proteasome protein complex, which is composed of one 20S (a catalytic core) and two 19S proteolytic subunits (Glickman et al., 2002).

2.2.1 Structure

2.2.1.1 20S proteasome (28-subunint)

Two outer rings are composed of 7 alpha subunits [(prosome, macropain) subunit, alpha type (PSMA 1-7)] and 2 inner rings are composed of 7 beta subunits (PSMB1-7).

The 20s has a barrel-like structure, with two-fold symmetry (Meiners and Eickelberg, 2012). The proteasome's proteolytic active sites are sequestered within the largest internal space of it. Once substrates access into the proteolytic chamber of the free core, it is blocked through an essentially topological mechanism (Finley 2009).

2.2.1.2 19S proteasome

The 19S regulator is composed of a base, which contains 6 ATPase subunits and 2 non-ATPase subunits, and a lid, which contains up to 10 non-ATPase subunits. This component deubiquitylates, unfolds, and translocates the recruited protein to the 20S catalytic chamber.

It unfolds target protein before the protein is translocated into the core particle. The "lid", serves regulatory functions, including polyubiquitinated proteins binding and substrates de-ubiquitination. And the "base" mediates energy-dependent unfolding of substrates and makes the access to the 20s core particle chamber open (Meiners and Eickelberg, 2012).

2.2.2 Protein degradation function

2.2.2.1 Protein to amino acids

Although many studies have focused on the initial steps in the protein degradation process, such as the ubiquitination of polypeptides and their degradation in proteasomes, very few have been focusing on the degradation of the proteasome's products to amino acids, and the enzymes involved in this process remain to be unknown (Goldberg et al., 2002). For large proteasome products in cytosolic degradation, it proceeds through two steps. First, large peptides are cleaved by thymet oligopeptidase (TOP) and probably other unidentified endopeptidase(s). Then, the products generated from the first step can be further degraded by bestatin-sensitive di- or tri-peptidases to single amino acids. For shorter proteasome products, they are degraded directly by various aminopeptidases to amino acids (Goldberg et al., 2002).

2.2.2.2 Protein to peptides

The proteasome produces highly heterogeneous peptides mixture from a particular target protein. These peptides serve as raw material for adaptive cell-mediated immunity in mammals (Kisselev et al., 1999). Proteasomes containing proteasome (prosome, macropain) 26S subunit, non-ATPase, 8 (PSMD8), PSMD9, and PSND10 subunits are call the immunoproteasomes. This unit generates 8-10 amino acid peptides for docking with major histocompatibility (MHC) class I. These peptide-MHC complexes form in the endoplasmic reticulum after the exportation of the peptides from cytoplasm via the peptide-specific TAP transporter. The peptide-MHC complex may be recognized by epitope-specific T cell receptor after it routes to the cell surface. If the presented epitope is derived from "foreign" sources, such as viruses and tumors, this process can initiate apoptosis (Finley 2009).

2.2.3 Regulatory protein degradation by the UPS

2.2.3.1 Example 1: Hypoxia Inducible Factor 1, alpha subunit (basic helix-loop-helix transcription factor) (HIF1A) and von Hippel-Lindau Tumor Suppressor, E3 Ubiquitin Protein Ligase (VHL).

The UPS is mediates cellular functions via HIF1A, which is a potent transcriptional activator of many stress response proteins, such as growth factors, chemokines and proteases (Weathington et al., 2013 b). HIF1A protein hydroxylation is regulated differently under normoxic and hypoxia conditions. HIF1A protein is important in both normal growth and neoplasia, where cancer cells with limited blood supplies. An E3 ligase, VHL mediates HIF-1α polyprotein ubiquitination and disposal by the proteasome (Jaakkola et al., 2001). This process regulates HIF-1α protein concentrations to modulate cell growth and survival responses, it also controls

edema formation during lung injury. The HIF1A-VHL signaling axis is associated with oncogenesis (Weathington et al., 2013b).

2.2.3.2 Example 2: Nuclear Factor of kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor, alpha (NFKBIA) and Beta-Transducin Repeat containing E3 Ubiquitin Protein Ligase (BTRC) protein-protein interaction.

The Skp1–Cullin–F-box protein (SCF) family is the largest family of Culling-RING E3 ligases that mediates ubiquitination of post-translationally modified target proteins or substrates often for proteasomal degradation (Weathington et al., 2013b). In this family, FBP (F-box proteins) is crucial for affecting changes in cellular states, including cell cycle progression, it is also related to critical pathways of pulmonary homeostasis and disease. One F-box protein is beta-transducin repeat containing E3 ubiquitin protein ligase (BTRC aka FBXW1).

Innate immune responses are triggered by the detection of pathogen-associated molecular patterns (PAMPs) that bind to cell membrane receptors including toll-like receptors (TLRs). TLRs recruit a set of adaptor proteins with TIR domains by homophilic interaction of their TIR domains. These interactions result in triggering of downstream signaling cascades leading to the activation of transcription factor nuclear factor-kappa B (NFKB), which controls induction of proinflammatory cytokines and chemokines as well as the modulation of co-stimulatory molecules on dendritic cells that are essential for T-cell activation. NFKB is typically held inactive by a family of IκB inhibitor proteins including NFKBIA. The WD40 repeats in BTRC can bind phosphorylated NFKBIA, which induces polyubiquitylation and, subsequently, degradation of NFKBIA. This is an essential step for the release of NFKB from the NFKB-NFKBIA complex, and it leads to the nuclear translocation and transcriptional activation of NFKB targets. In addition, BTRC can mediate degradation of CD4 via its interaction with HIV-

1 Vpu. Other F-box protein species also have been identified to be related with pulmonary inflammation at level of cytokine receptor stability, surfactant homeostasis, and inflammatory signaling (Weathington et al., 2013b).

3.0 PROTEOSOME ASSOCIATED PROTEINS

As mentioned before, the proteasome has a cylindrical structure with a “lid” and a “base”, and the active sites in α and β subunits face the interior of the cylinder. The ubiquitin-independent substrates must pass through an open core particle channel to be degraded after being unfolded. The CP gate is in closed status by default, and the modulation of the gate is regulated by several proteasome activators (Schmidt and Finley 2014).

3.1 CLASSIC ACTIVATORS (11S/REG/PA28)

The PA28/11S/REG proteasome activator family is expressed in eukaryotes. There are three isoforms in PA28: PA28α, PA28β and PA28γ, they utilize the activation loop (internal loop structure) to promote opening the CP gate by contacting the 20s alpha subunits (Schmidt and Finley 2014). PA28α and PA28β form hetero-heptameric rings in vertebrates, and induced by interferon-γ, which indicates that PA28α and PA28β in MHC Class I may mediate antigen presentation (Realini et al., 1997). However, the cellular function of them has not fully understood yet. PA28γ forms homo-heptameric rings and is found in the nuclei of both vertebrates and invertebrates (Dubiel 1992; Ma 1992). It promotes the degradation of specific small regulatory proteins in an ATP-and ubiquitin-independent way.

3.2 ZINC FINGER, AN1-TYPE DOMAIN PROTEIN FAMILY

3.2.1 Zinc Finger, An1 Domain Protein Structure

The zinc finger, AN1-type domain (ZFAND) protein family consists of 6 proteins: ZFAND1, 2A. 2B, 3, 4, 5, and 6. A related protein is immunoglobulin mu binding protein 2. To date the most studied are *ZFAND2A*, ZFAND2B, and ZFAND5. ZFAND proteins contain a N-terminal ubiquitin (Ub)-like domain C-terminus a zinc finger, AN1 domain, which was original found in Xenopus laevis (Linnen et al. 1993). The AN1-type zinc finger consists of 6 conserved cysteines and 2 histidines that could potentially coordinate 2 zinc atoms: C-X2-C-X(9-12)-C-X(1-2)-C-X4-C-X2-H-X5-H-X-C: Where X can be any amino acid, and numbers in brackets indicate the number of residues. This domain was originally thought to have a role in determining cell fate, but now is contained in proteins associated with stress responses.

The uneven distribution of maternal mRNAs in unfertilized eggs and the unequal inheritance of these molecules by dividing blastomeres may be one mechanism for determining cell fate during embryogenesis. Complementary DNA (cDNA) clones corresponding to maternal mRNAs localized to specific regions of the Xenopus laevis egg was identified by Rebagliati et al. (1985). The maternal mRNA, An1, was originally identified as being localized to the animal hemisphere of X. laevis eggs and early embryos. Unlike Ub polyproteins and most Ub fusion proteins, Linnen et al. (1993) found that the N-terminal Ub-like domain found in the An1 proteins does not undergo proteolytic processing, and that An1 transcripts are present in later embryonic stages and in all adult tissues. Moreover, AN1 domain in combination with A20 zinc finger domains is found in a number of stress associated proteins (SAP). For example SAP gene family in rice,

whose members were all inducible by abiotic stress, indicating that this gene help to build tolerance under stress in plants (Vij 2006).

3.2.2 Zinc finger, AN1-type domain 5 (ZFAND5 aka Znf216)

ZFAND5A is protein that contains an AN1 zinc-finger at the C terminus and an A20 zinc-finger at the N terminus and can negatively regulate the NFKB activation pathway by interacting with immune response proteins including receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIPK1 aka RIP), inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma (IKBKG aka IKKγ) and TNF receptor-associated factor 6, E3 ubiquitin protein ligase (TRAF6) (Huang 2004). The interaction of ZFAND5 with RIPK1 and IKBKG is mediated by the A20 zinc-finger domain, while its interaction with TRAF6 is mediated by the AN1-type domain; therefore, both zinc-finger domains are involved in regulating the immune response.

Very little is known about human ZFAND5 protein, however, this protein is similar to sequestosome 1 (SQSTM1 aka p62), also is a protein with A20 zinc finger domain, which can inhibit NFKB activation triggered by overexpression of RIPK1 and TRAF6 (Komatsu et al. 2012). A stress-inducible intracellular protein, SQSTM1 regulates various signal transduction pathways involved in cell survival and cell death. Analysis of microtubule-associated protein 1 light chain 3 alpha (MAP1LC3A aka LC3 (an autophagosome localizing protein)-binding proteins resulted in the recognition of autophagy and SQSTM1. Although autophagy modulates SQSTM1 protein levels, SQSTM1 can suppress autophagy via activation of mechanistic target of rapamycin (serine/threonine kinase) (MTOR). SQSTM1also directs ubiquitinated cargos toward autophagy as well as compaction of those cargos. Furthermore, this protein functions as a signaling hub for various signal transduction pathways, such as NFKB signaling, apoptosis, and

nuclear factor, erythroid 2-like 2 (NFE2L2 aka Nrf2) activation. It also inhibits tumor necrosis factor (TNF)- , interleukin-1- , and Toll-like receptor 4-induced NFKB activation in a dose-dependent manner. The protein functions as a scaffolding/adaptor protein in concert with TNF receptor-associated factor 6 to mediate activation of NF-kB in response to upstream signals.

4.0 ZFAND2A

Arsenicals activate multiple stress pathways resulting in the induction of specific genes whose identity and role in the adaptation to arsenical-induced cellular stress are poorly understood. Sok et al. (2001) identified *ZFAND2A*, which was initially called arsenite-inducible, cysteine- and histidine-rich RNA-associated protein, AIRAP, and reported that is conserved among mammals, Drosophila, and Caenorhabditis elegans. Subsequently, Kimura and colleagues (2010) determined that *ZFAND2A* mRNA increased within 3 h in the kidney of BALB/c and C57BL/6 mice injected with sodium arsenite (NaAs; 13.5 mg/kg).

Suspecting that protein misfolding caused by exposure to arsenite could be associated with transcriptional activation of the AIRAP gene, Stanhill et al. (2006) found that ZFAND2 an arsenite-inducible subunit of the proteasome's 19S cap that binds near PSMD2 at the 19S base. Arsenite activates the electrophile responsive transcription factors NFE2L2 (Alam 1999; Gong 2002) and their C. elegans homolog skn-1 (An 2003). In mouse fibroblasts, *ZFAND2A* was induced both by arsenite (25 μM, 4 h) and the electrophile, methyl methanesulphonate (50 μg/ml, 6 h), but it was not induced by the amino-acid analog azetidine (5 mM, 7 h), the endoplasmic recticulum (ER) stress-inducing agents tunicamycin (2.5 μg/ml, 6 h), or thapsigargin (400 nM, 6 h). In C. elegans, interfering RNA (RNAi) to skn-1inhibited the arsenite-induced increased *ZFAND2A* promoter: green fluorescence protein reporter activity. In addition, skn-1 RNAi treatment augmented the heat-shock response of arsenite-treated worms and increased the levels

of polyubiquitylated proteins. Thus, both direct interference with ZFAND4A expression and blocking an upstream activator skn-1, impaired the ability of C. elegans to cope with arsenite-mediated protein misfolding. Stanhill et al. (2006) also generated mouse embryo fibroblasts from gene targeted *ZFAND2A* mice, and transient arsenite exposure (50 μM, 2 h) accumulate polyubiquitylated proteins, whose levels subsequently decline, coincidental with AIRAP induction. Under the same conditions, *ZFAND2A* deficient embryo fibroblasts accumulate more polyubiquitylated proteins than the wild-type cells, a feature that was reversed by constitutive expression of AIRAP from a ‘‘rescuing’’ transgene.

Because protein misfolding may contribute to aging, Yuns et al (2008) tested the role of AIP-1, a C. elegans homologue of mammalian *ZFAND2A*, and determined that loss of *ZFAND2A* decreased the lifespan and hypersensitivity to misfolding-prone proteins in C. elegans. NFKB is a ubiquitous transcription factor involved in several biological processes, especial stress response and induction of inflammatory mediators (Karin 2000). NFKB is retained in the cytoplasm in an inactive state by specific inhibitors including NFKBIA. Upon degradation of the inhibitor, NFKB moves into the nucleus and activates transcription of specific genes. NFKB transcriptional complex is composed of NFKB1 or NFKB2 bound to either rel avian reticuloendotheliosis viral oncogene homolog (REL), RELA, or RELB. The most abundant protein combination in the NFKB complex contains NFKB1 and RELA. Examining transcripts that are increased with stress-inducing copper (400 μM) treatment in HepG2 cells, McElwee et al. (2009) found that *ZFAND2A* was induced 10- and 33 fold at 3 or 6 h, respectively. These increases were inhibited with the IMD-3054 (which inhibits NFKB activation) or with siRNA directed at RELA, suggesting the involvement of NFKB signaling in the transcriptional activation of *ZFAND2A*.

Heat shock transcription factor-1 (HSF1) is the central regulator of heat-induced transcriptional responses leading to rapid expression of molecular chaperones that protect mammalian cells against proteotoxic stress (Anckar 2007). HSF1 targets specific promoter elements (HSE) located upstream of heat shock genes encoding a variety of heat shock proteins, including HSP70, HSP90, HSP27, and other proteins of the network. *ZFAND2A* expression also was found to be temperature-dependent and controlled by HSF1 (Rossi 2010). Transcription is triggered at temperatures above 40^{o}C. For example, human keratinocytes (HaCaT cells) and two human cancer cell lines (HCT116 colon carcinoma and Jurkat T-cell lymphoma) were incubated at 43°C for 40 min and allowed to recover at 37 °C for 90 min. Heat treatment resulted in an increase in *ZFAND2A* and HSP70 mRNA levels in all cell lines. Similar results were obtained in human primary blood monocytes subjected to heat stress (43 °C, 40 min) and analyzed at 1.5 and 3 h after recovery at 37 °C, suggesting that AIRAP expression represents a general response of human cells to temperature increase. Chromatin immunoprecipitation (ChIP) analysis demonstrated that, HSF1 was recruited to the *ZFAND2A* promoter rapidly after heat treatment, with a kinetics that parallels HSP70 promoter HSF1-recruitment. HSF1-silencing abolished heat-induced *ZFAND2A* promoter-driven transcription, which was rescued by exogenous Flag-HSF1 expression. The HSF1 binding HSE sequence in the *ZFAND2A* promoter critical for heat-induced transcription was identified. In a follow-up study, Rossi et al. (2014) treated human umbilical vein endothelial cells with a proteaosome inhibitor, bortezomib and measured an increase in HSF DNA binding activity by electrophoretic mobility shift assay (EMSA) and in ZFAND2 protein levels by Western-blot. *ZFAND2A* protein increased in bortezomib-treated perpherial blood monocytes and lymphocytes. In borteezomib treated cell HSF1and HSF2

formed a heteromeric complex that was recruited to the *ZFAND2A* promoter, with the HSF1 positively and HSF2 negatively regulating transcription.

Additional stress studies involved cultures of human renal epithelial cells derived from cortical tissue obtained from nephrectomies treated with cadmium (Garrett 2013). These cultures exhibit many of the characteristics of proximal tubule cells and were treated with by various Cd^{2+} concentrations (4.5-45 μM) and multiple times (1-13 days). The transcriptome (microarray) analysis revealed transcriptional dynamics and gene interaction using a mutual information-based network model. The most prominent network module consisted of a set of transcripts that included *ZFAND2A* along with AKAP12, AKR1B10, AKR1C1, DNAJA4, INHBA, KIF20A, and SCL7A11.

Similarly, *ZFAND2A* was found to be a part of a gene dysregulation network (GDN) with a classifier to assign compounds to their appropriate class (Pronk 2013). Gene pairs in the GDN are dysregulated in the sense that they are linked by a common expression pattern in one class and differ in this pattern in another class. The classifier gives a quantitative measure on this difference by its prediction accuracy. Gene pairs were selected microarray data obtained with human keratinocytes (HaCaT cells) treated with 8 sensitizers or 6 irritants. Pairs with known and novel markers were found such as HMOX1 and *ZFAND2A*, ATF3 and PPP1R15A, OXSR1 and HSPA1B, ZFP36 and MAFF.

5.0 LABORATARY RESEACH PROJECT

5.1 OBJECTIVES

The Ubiquitin Proteasome System is involved in cell cycle controlling and aging. The accumulation of unfolded protein caused by dysfunction of UPS is significant to disease development. *ZFAND2A* is a zinc-finger protein that is inducible by arsenite (Sok 2001), which serves as a regulator of the UPS, associates with 19s-regulatory particle (Stanhill 2006). Thus this response regulates the protein degradation machinery and could influence cell apoptosis process to counteract proteotoxicity induced by an adverse environment. Thus it has a protective function and help to keep cells maintenance and long longevity (Yun 2008).

Currently, the role that *ZFAND2A* gene plays in the lung or in cancer cells is uncertain. In this project, we explore how *ZFAND2A* transcripts are altered by heat shock and arsenic-induced cell stress. Human epithelial cancer cells were used to observe the performance of this gene, and the molecular mechanism and stress-induced change in protein level will be conducted in further study to understand the role of this gene in human lung health better. Given that the *ZFAND2A* plays an important role in cell homeostasis, if we could inhibit transcriptional activation of this gene or inhibit the function of the encoded protein in injured or cancer cells, cell death will be induced in those cells only, we could find a way to promote lung health with high specificity.

5.2 METHODS

This is a laboratory based research project on *ZFAND2A* expression level in NCI-H441 cell (human lung adenocarcinoma epithelial cell line) under cell stress induced by heat shock and sodium arsenite.

a. Reagents

Complete growth medium: The base medium for this cell line is Dulbecco's Modified Eagle's Medium (DMEM) (ATCC® 30-2002™) (Manassas, VA). Complete growth medium included the following components to the base medium: fetal bovine serum to a final concentration of 10% and 1% penicillin-streptomycin.

Serum-free medium: Dulbecco's Modified Eagle's Medium (DMEM) without Phenol Red (ATCC® 30-2601™) RPMI-1640, purchased from ATCC. Add 1% penicillin-streptomycin and 2% glutamin (100×).

PBS: Phosphate Buffered Saline (D-PBS), 1X (ATCC® 87571™), purchased from ATCC.

DPBS: Dulbecco's Phosphate Buffered Saline (D-PBS), 1X (ATCC® 30-2200™), purchased from ATCC.

0.05M Sodium arsenic solution: Product number: 35000. Brand: Fluka. Purchased from Sigma-Aldrich (Saint Louis MO,).

RNA isolation solution: SIGMA TRI Reagent®, Product number: 93289. Purchased from Sigma-Aldrich..

Gene probe: ribosomal protein L32 (RPL32), Catalog No. 4453320. Zinc finger, AN1-type domain 2A (*ZFAND2A*), Catalog No. 4351372. Activating transcription factor 4 (ATF4),

Catalog No. 4331182. Vascular endothelial growth factor A (VEGFA), Catalog No. 4453320, all of them were purchased Life Technology (San Diego, CA).

DNase I treatment kit: Bio-Rad (Hercules, CA)...

iScript™ cDNA synthesis reaction kit: Bio-Rad (170-8890).

qRT-PCR master mix: TaqMan® PCR Master Mix, (Life Technologies Cat. No. 4364344).

b. Cell culture

NCI-H441 cells (human lung adenocarcinoma epithelial cell line) were cultured in complete medium at 5% CO_2 and 37°C.

c. Treatment Protocol

Heat shock:

Dose-response

H441 cells were seeded in 4×12-well plates (0.4×10^6 cells/well). After 24h incubation, the medium was removed, washed twice with DPBS and then serum-free RPMI was added. After 20h serum-starvation, plates were treated at 37℃, 39℃, 41℃, and 43℃ for 30 min, and recover at 37℃ for 1.5 h. Then 0.2ml/well Trireagent was added to extract RNA for qRT-PCR analysis.

Temporal analysis

H441 cells were seeded in 4×12 well plates (0.4×106 cells/well). After 24h incubation, the medium was removed, washed twice with DPBS and serum-free medium RPMI

1640 added. After 24h serum-starvation, plate for treatment groups were treated at 43℃ for 40 min, and recovery at 37℃ for 1.5, 3, and 5.5h. Then 0.2ml/well TriReagent was added to extract RNA for qRT-PCR analysis.

Sodium arsenite:

Dose-response

H441 cells were seeded in 3×12-well plates (0.4×10^6 cells/well). After 24h incubation, the medium was removed, washed twice with DPBS, and 1 ml/well serum-free medium RPMI 1640 added. After 24h serum-starvation, the medium was removed, and 0.8 ml/well serum-free medium was added. After 30 min pre-incubation, 0.2 ml/well sodium arsenic solution at 0, 0.1, 0.5, 1, 5, 10, 20, 50, and 500 μM was added. After 24h treatment, the medium was removed, washed once with PBS and added 0.2 ml/well TriReagent to extract RNA for analysis.

Temporal Analysis

H441 cells were seeded in 3×12 well plates (0.4×10^6 cells/well). Plate 1: control 1 with 2h and 4h groups. Plate 2: control 2 with 24h group. Plate 3: control 3 with 48h group. After 24h incubation, the medium was removed, washed 2· with DPBS, and 1 ml/well serum-free medium RPMI 1640 added. After 24h serum-starvation, the medium was removed, and 0.8 ml/well serum-free medium was added. After 30 min pre-incubation, 0.2 ml/well sodium arsenic solution at 20 μM was added. After 2, 4, 24 and 48h treatment, the medium was removed, washed once with PBS and added 0.2 ml/well TriReagent to extract RNA for analysis.

d. RNA isolation, cDNA synthesis, Real-time PCR

Total RNA was harvested and isolated using TriReagent and quantified by measuring absorbance (260 nm).

Use iScript cDNA synthesis reaction kit to make cDNA synthesis reaction mix, 5×iScript reaction buffer: reverse transcriptase: H_2O: DNase 1 treated RNA = 4: 0.5: 12.5: 3. Then run the iScript cDNA Synthesis program, 25℃ for 5min, 42℃ for 30min, 85℃ for 5min.

Use Taqman real-time PCR reaction kit to make real-time PCR reaction mix, Taqman 2×mastermix: probe for each gene (ATF4, *ZFAND2A*, VEGFA, and the housekeeping gene RPL32): H_2O: cDNA = 10: 1: 7: 2. Place the mix into a 384-well PCR plate, and run by RQ Manager in Applied Biosystem PCR machine.

e. Statistical analysis

Heat shock:

All changes in *ZFAND2A* and ATF4 cDNA levels were normalized to changes in RPL32. One-way ANOVA was conducted to determine whether the mean of each treatment group was significantly ($p<0.05$) different from the untreated group (control group). All statistics were performed using EXCEL and SigmaPlot (Systat Software Inc.). Data are represented as mean ± SEM or as fold control.

Sodium arsenite:

All changes in *ZFAND2A* and VEGFA cDNA levels were normalized to changes in RPL32. One-way ANOVA was conducted to determine whether the mean of each treatment group was significantly ($p<0.05$) different from the untreated group (control group). All statistics were

performed using RQ Manager (Applied Biosystems Software Informer 2.2) and SigmaPlot (Systat Software Inc.). Data are represented as mean ± SEM or as fold control.

5.3 RESULTS

a. Heat shock cell stress

ZFAND2A transcript increased in cells treated with 43℃ group (log 2 = 1.76 or 3.4-fold) as compared to cells maintained at 37℃ (Control) (Figure 1). Cells treated with 39℃ or 41℃ were not significantly different from cells maintained at 37℃. ATF4 mRNA levels are not statistically different from control.

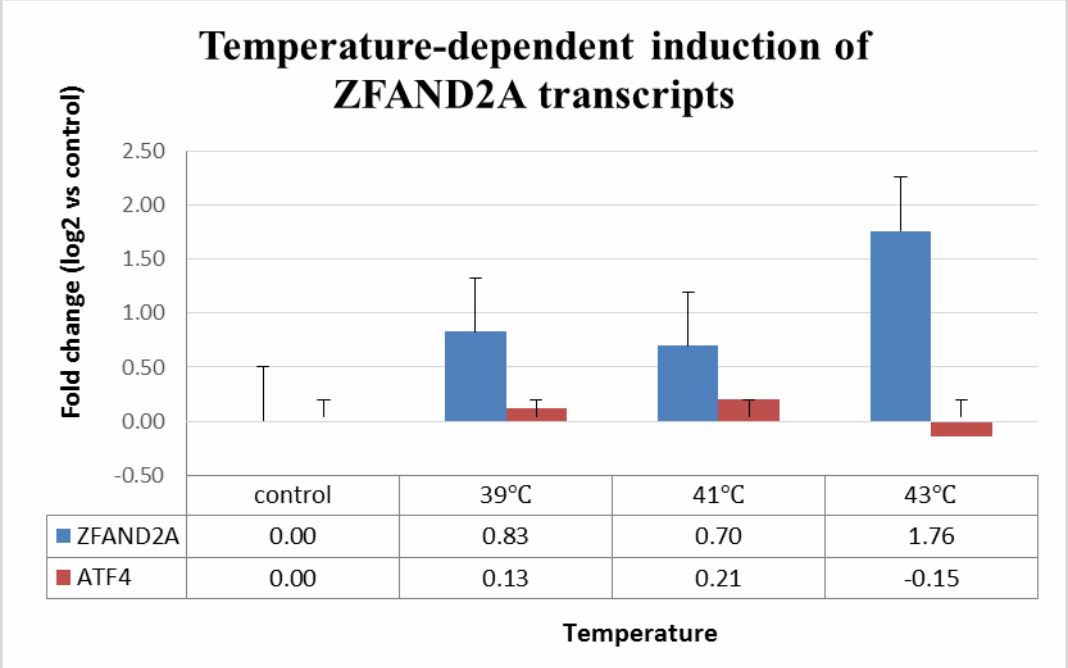

Figure 1. Temperature-dependent increase in zinc finger, AN1-type domain 2A (ZFAND2A) transcript in H441 cells treated for 30min with a 1.5h recovery (at 37℃).

ZFAND2A transcripts significantly increased after 43oC whereas activating transcription factor 4 (ATF4) transcripts were not significantly different from 37oC control. Transcript level were measured by qRT-PCR and normalized to RPL32. Values are the mean of 3 trials with standard error and statically assessed using ANOVA.

Heat-induced increased *ZFAND2A* transcripts in H441 cells peaked at 1.5 h recovery after treatment with 43℃ for 40min (Figure 2). The changes in 1.5h recovery following 40 min was log2 = 3.47-fold, which was greater than that produced by 30 min treatment (Figure 1). *ZFAND2A* remain greater than control with the 3 h recovery but was not statistically different from control at 5.5 h. ATF4 transcript levels were not statistically different from control.

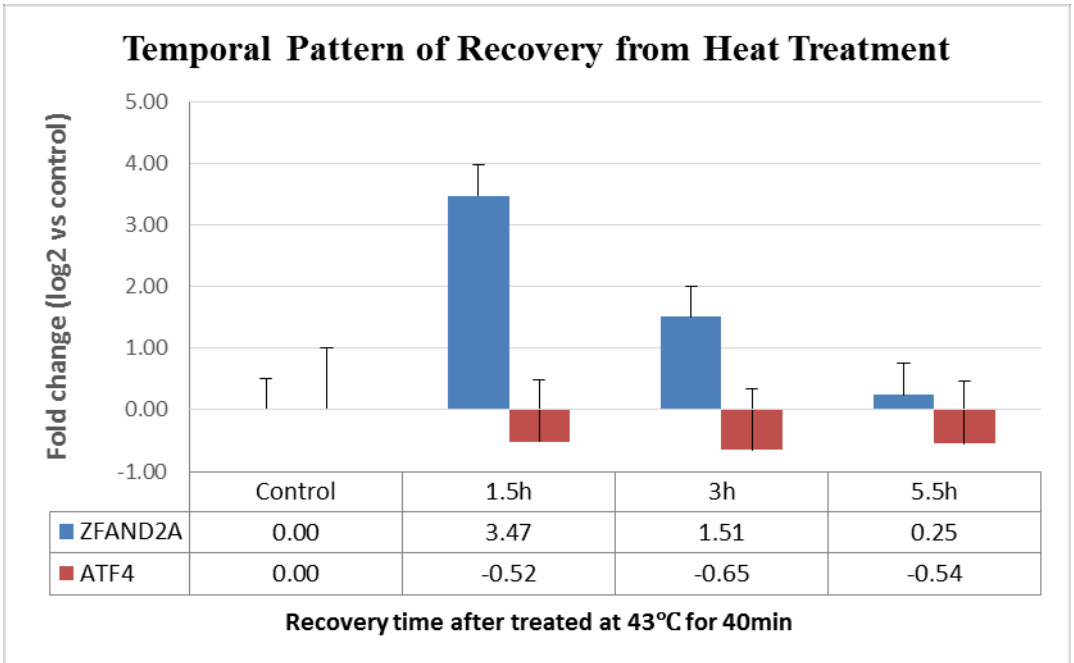

Figure 2. Recovery for heat shock treatment at 43℃ for 40 min of zinc finger, AN1-type domain 2A (ZFAND2A) transcripts in H441 cells.

ZFAND2A transcripts significantly increased following recovery periods of 1.5 h and 3 h after 43^{o}C whereas activating transcription factor 4 (ATF4) transcripts were not significantly different from 37^{o}C control. Transcript level were measured by qRT-PCR and normalized to RPL32. Values are the mean of 3 trials with standard error and statically assessed using ANOVA.

b. Arsenic-induced cell stress

ZFAND2A transcripts increased in a concentration dependent manner with sodium arsenite treatment in H441 cells (Figure 3). The change relative to control is statistically significant at doses of $\geq$10 μM sodium arsenite. The response continued to increase with increasing doses and 500 μM sodium arsenite induced the maximal response of log 2 = 5.30 fold. H441 cells appear to be relatively resistant to arsenic exposure because this dose can produce cytotoxicity in other cells (Dodmane et al. 2013).

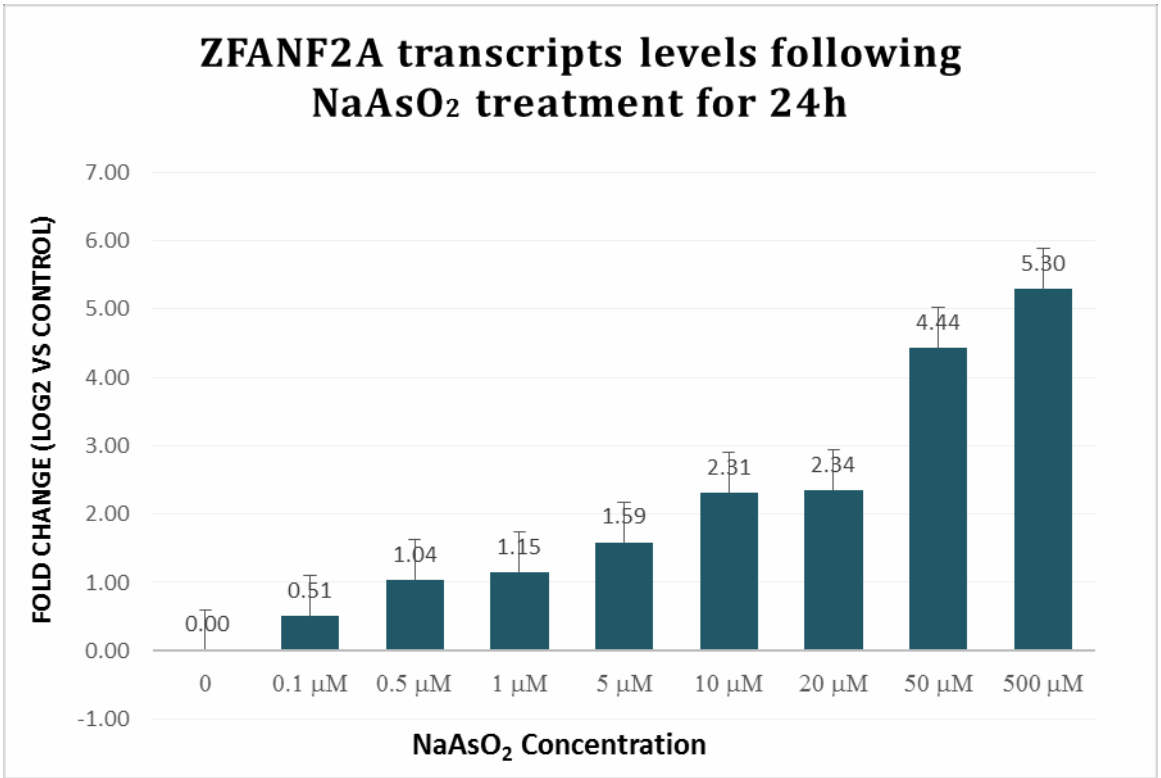

Figure 3. Concentration dependent increases in ZFAND2A transcripts in H441 cells treated with sodium arsenite for 24 h.

Doses ≥ 10 µM were statistically different from control. Transcript level were measured by qRT-PCR and normalized to RPL32. Values are the mean of 3 trials with standard error and statically assessed using ANOVA.

ZFAND2A transcripts increased with increasing time of 20 µM sodium arsenite treatment (Figure 4). The increase for each time is statistically significant compared to untreated control. The increase at 48 h was greater than that at 24 h, again suggesting that H441 cells are resistant to sodium arsenite treatment.

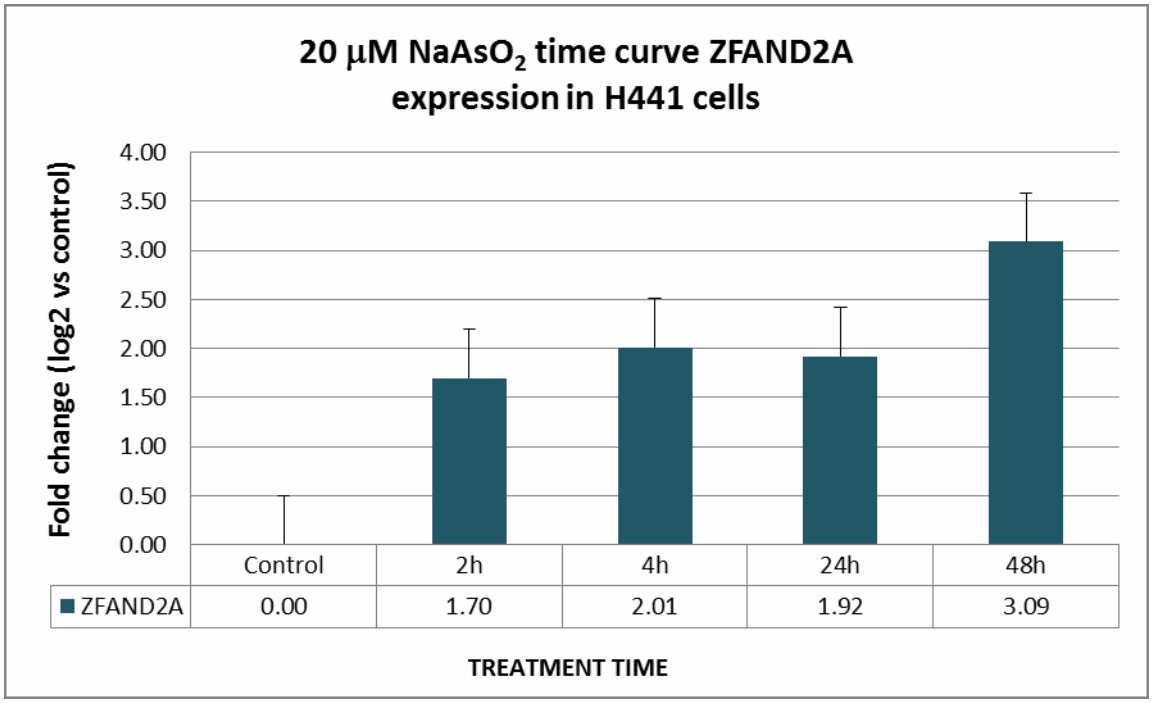

Figure 4. Concentration dependent increases in NaAsO2 dose response for 24h treatment ZFAND2A expression transcription H441 cell, in H441 cells treated with sodium arsenite for 24 h.

Transcript level were measured by qRT-PCR and normalized to RPL32. Values are the mean of 3 trials with standard error and statically assessed using ANOVA.

Vascular endothelial growth factor A (VEGFA), a member of the vascular endothelial growth factor (VEGF) family, encodes a protein that is often found as a disulfide linked homodimer. Epithelial cell can be a major source of this protein in the lung (Acarregui 1999; Christou 1998). VEGFA is also important for normal lung development (Acarregui 1999) and airway epithelial cell proliferation (Brown 2001). VEGFA plays a role in angiogenesis and vascularization, however, its role in lung injury and diseases remains controversial (Mura 2004). In the initial phase of inflammation lung diseases, such as asthma and acute lung injury, the expression level of this gene is increased and cause pulmonary edema (Kanazawa 2003; McColley 2000; Mura 2004). While in the later phases, elevated VEGFA is associated with the

resolution of acute lung injury (Thickett 2002). VEGFA transcripts are known to increase with arsenite treatment primary vascular smooth muscle cells (Soucy et al. 2004). After 48 h, sodium arsenite also increased VEGFA transcripts in H441 cells (Figure 5).

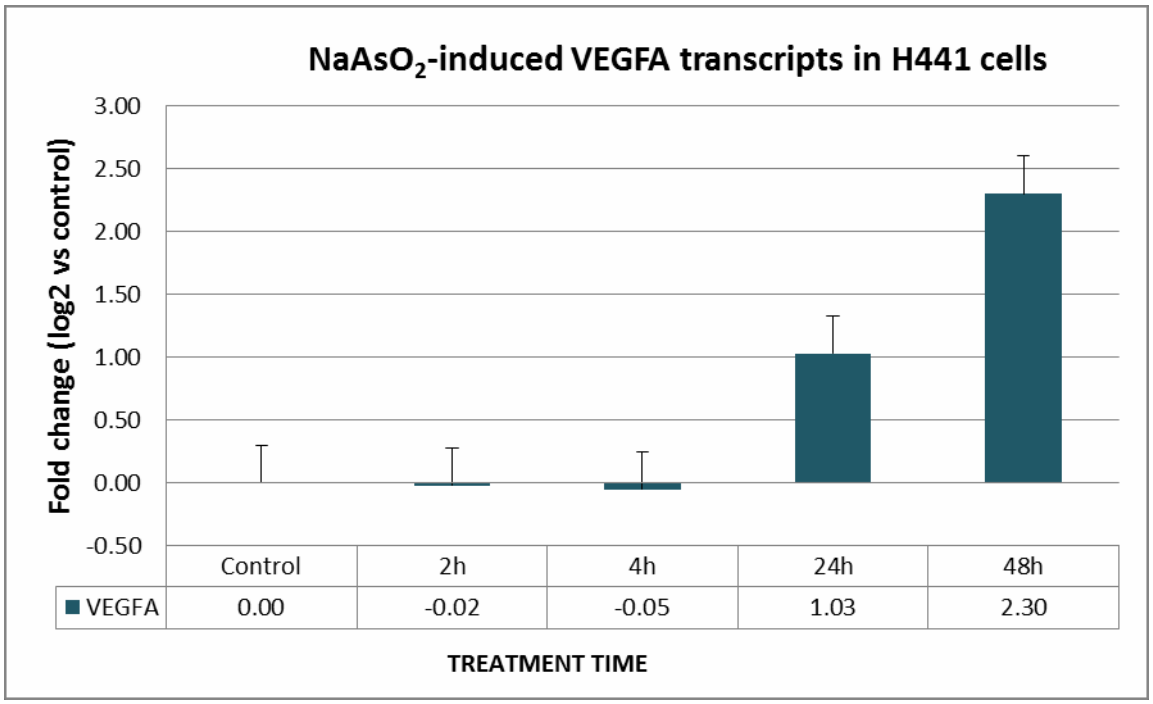

Figure 5. Temporal pattern of increases in vascular endothelial growth factor A (VEGFA) in H441 cells treatment with 20 μM sodium arsenite.

Transcript level were measured by qRT-PCR and normalized to RPL32. Values are the mean of 3 trials with standard error and statically assessed using ANOVA.

5.4 DISCUSSION

Epidemiological studies have associated arsenic exposure in drinking water with lung diseases (Smith 2012, Dauphiné 2011). In this study we investigated whether *ZFAND2A* is regulates by heat- and arsenic-induced cell stress along in human lung cancer cells. We observed increases in *ZFAND2A* following either treatment, suggesting this response may serve to detoxify cell stress. Previously, temperature-dependent increases in *ZFAND2A* mRNA were detected in human cervical carcinoma cell line treated at 41℃, 42℃, and 43℃ for 40 min followed by a 1.5 h recovery with 43℃ producing the maximum level (Rossi et al., 2010). When HeLa-Ps cells were subjected to heat shock at 43℃ for 40 min *ZFAND2A* mRNA levels were greater after a recovery time of 1.5 h than after 3 h (Rossi et al., 2010). Our results are consistent with this temporal pattern (Figure 2). However, only 43℃ for 30 min (Figure 1) or 40 min (Figure 2) produced a statistically significant increase in *ZFAND2A* in H441 cells. This may be due to a small sample size and a relatively large standard error. Nonetheless the response was notable in that the magnitude of the response was approximately 11-fold increase (log 2 = 3.47) following 40 min.

In this study we found that 24 h treatment with $\geq$10 μM sodium arsenite increased *ZFAND2A* transcripts (Figure 3). This is a relatively high dose of arsenic, in that other have observed cytotoxicity in lung epithelial cells at these levels (Dodmane et al. 2013). Although lower doses 0.5 μM $\leq X \leq$ 5 μM produced essentially a 2 fold increase in *ZFAND2A* transcripts, these value were not statistically significant. This may be due to the shorter duration of exposure of 24 h, because a greater response was noted when cells were treated for 48 h (Figure 4). In addition, the sample size (n =3) was relative small. Thus, the evaluation of the threshold dose of

sodium arsenite that induces *ZFAND2A* should be conducted with longer exposure and in a larger sample size. It would be prudent to attempt exposures greater than 48 h, because it is unknown whether *ZFAND2A* transcripts will continue to increase with exposure of 72 or even 96 h. Thus, to better understand this response under arsenic stress, exposure with lower concentration and longer time should be conducted both in and in animal models.

Former study have reportted that VEGFA could be induced following 1 μM or 5 μM arsenite treatment for 4h in HMVEC (Human lung microvascular endothelial cells) (Klei 2008). In H441cells treated at a much higher concentration 20 μM for even longer treatment time 24h, the change was not significant. Thus, H441 cells appear to be resistant to sodium arsenite. Cancer cell lines are considered to be much more resistant than primary cell lines under varies of cell stresses, thus cancer cells could maintain themselves in a good condition and proliferate well even in a poor environment, while primary cells are going to be injured or died. Besides, these proteins may act differently in cancer cells than primary cells, VEGFA involves in arsenic-induced injury in primary cells, but its function could be varied in cancer cells. Again, these preliminary studies were only conducted with a small sample size, which limits the statistical power of our study. Nonetheless, it seems worthwhile to conduct additional studies comparing the responsiveness of *ZFAND2A* and VEGFA in primary epithelial cells and in animal models in future studies. Lastly, Given that ZFAND2A may play an important role in cell growth and repair mechanism, if the pathways of its protective reaction in cancer cells can be demonstrated clearly, then selective inhibition of these proteins may be useful in cancer therapy.

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