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Assessing the role of arsenite in disrupting the EGFR signaling axis.

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ASSESSING THE ROLE OF ARSENITE IN DISRUPTING THE EGFR SIGNALING AXIS

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This thesis is dedicated to my parents Seong Dong Kim and HyunJa Choi for their unconditional love and support in all my endeavors**.** They are my forever role models. I am who I am today because of their sacrifices and prayers. Thank you for always believing in me. I'd like to thank my sibling, John. I'm always thankful for having a brother like you. You're the best.

ABSTRCT

ASSESSING THE ROLE OF ARSENITE IN DISRUPTING THE EGFR SIGNALING AXIS

Christine Kim

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The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase localized on the cell surface. Overexpression of EGFR has been used as biomarkers for many different types of cancers, including lung cancer. There is a strong association between arsenic and lung cancer development, although the mechanism is unclear. **We hypothesize that chronic exposure of "a physiologically relevant" level of arsenite disrupts the EGFR endocytic trafficking.** The goal of this project is to identify molecular mechanisms and roles of chronic arsenite-induced EGFR overexpression in lung cancer development. A non-malignant human bronchial epithelial cell line, Beas-2B cells were exposed to 100 nM sodium arsenite for 24 weeks. The chronic arsenite-treated cells had increased EGFR protein expression levels and activity, increased transcription levels of TGFα, and altered the distribution of the EGFR. In conclusion, the impact of chronic arsenite exposure on the EGFR signaling axis can explain arsenite-induced overexpression of the EGFR.

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CHAPTER 1

INTRODUCTION

Arsenic

Environmental contamination of heavy metals is a global health hazard. Among heavy metals, arsenic is ranked number one on the Agency for Toxic Substances and Disease Registry (ATSDR) Substance Priority List (ATSDR, 2017), and in 1973, arsenic was classified as group I "carcinogenic to humans" by the International Agency for Research on Cancer (IARC) based on epidemiological carcinogenicity evidence in human and in animal models [1-3]. Arsenic is ubiquitous in the environment, and it has been used by of human civilization since ancient times, both for constructive and destructive purposes. Arsenic is a powerful poison, owing to its lack of color, taste and odor. Moreover, the symptoms from arsenic poisoning are very similar to the symptoms of food poisoning, thus rendering it nearly untraceable without sophisticated analytical procedures.

i. Arsenic Uses in Medical Applications

Arsenic is a double-edged sword, as it has been used for medical applications (Fig. 1). In the 18th century, Thomas Fowler, an English physician, produced Fowler's Solution, a potassium bicarbonate-based arsenic solution, which was widely used to treat many conditions such as asthma, convulsions [4] and psoriasis [5]. Also known as a cancer of the blood cells, leukemia is associated with abnormally high number of white blood cells. Arsenic was also used to treat leukemia; with the Fowler's Solution, the number of white blood cells declined dramatically in leukemia patients over 10-weeks

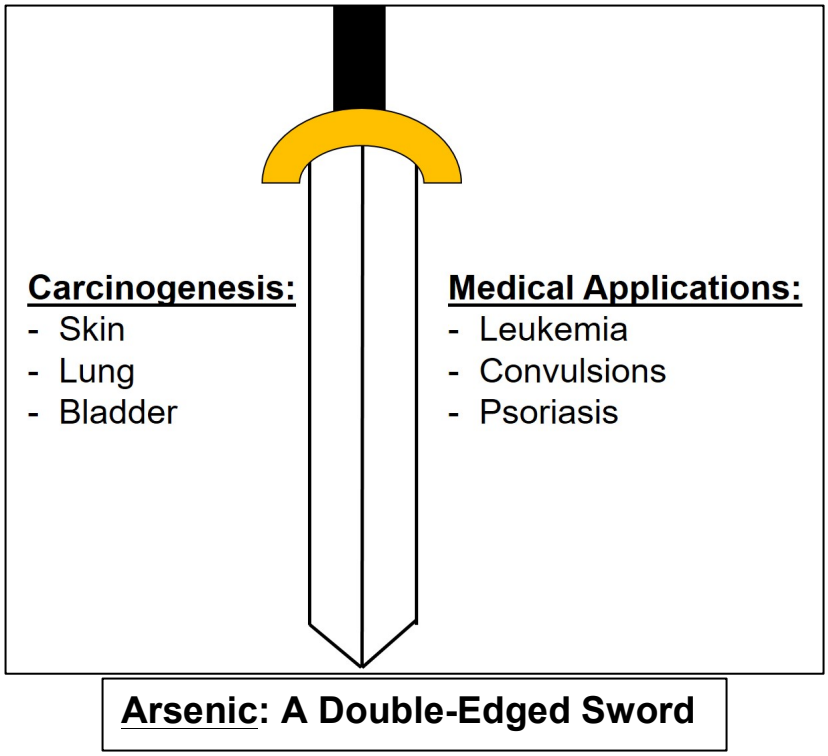

Figure 1. Arsenic is a double-edged sword. Arsenic is now a well-established carcinogen and is prominently associated with skin, lung, and bladder cancers. Arsenic is also used in medical applications, especially as a treatment for leukemia. Despite its effectiveness, over time, carcinogenicity was observed in patients who had received arsenic treatment. Thus, its usage declined, but due to its potency in medical applications, it is under investigation for the treatment for other diseases.

treatment period [6]. Discontinuation of the therapy, however, led to clinical relapse within few weeks. Because of its anti-leukemic ability, arsenic was approved by Food and Drug Administration (FDA) in 2001 for the treatment of acute promylelocytic leukemia (APL) [7], and is under investigation for the treatment of other cancer types [8-10].

Arsenic hinders repair processes of UV-induced photoadducts [11-14], and its role in repressing DNA repair processes also contributes to chemotherapeutic effect. Wang et al. showed combination treatment of arsenic with cisplatin is more effective in treating hepatocellular carcinoma than cisplatin treatment alone [15]. Further, Neher et al. observed cisplatin treatment alone induces xeroderma pigmentosum group C (XPC), which is an important protein in the global genomic nucleotide excision repair pathway [16], suggesting cisplatin-induced XPC expression is a part of the resistance mechanism in cisplatin-resistant cells. Interestingly, co-treatment of arsenic with cisplatin suppressed cisplatin-induced XPC expression by sensitizing wild-type p53, inhibiting the repair pathway [17]. This study highlights the enhanced chemotherapeutic effect of cisplatin when it is given with arsenic, and suggests a potential role of arsenic in enhancing sensitivity towards chemotherapeutic agents. Overall, such characteristics of arsenic support its contribution not only to carcinogenesis, but also to chemotherapy.

Health Effects of Acute and Chronic Arsenic Exposure

Arsenic toxicity is highly dependent on its dose and duration of exposure time, as its acute toxicities are different from chronic toxicities. Usually acute toxicity results from accidental ingestion of high levels of arsenic. Ingestion of large amounts of arsenic will require treatment, such as decontamination, administration of intravenous fluids, and chelation therapy. The symptoms, including diarrhea, vomiting, dehydration, and hypotension, are commonly observed in workers who ingest high levels of arsenic in their workplaces from dust and fumes. In severe cases, the symptoms can lead to death,

primarily due to dehydration and shock [18]. To determine the acute exposure to arsenic, several tests can be performed, including blood and urine tests. Blood test, however, is accurate only for 2-4 hours after initial exposure due to rapid clearance from the blood [19]. More accurate measurement would be determined by performing urine test. Twenty-four hour urine arsenic levels greater than 50 μg/L are considered acute arsenic toxicity [20].

Chronic toxicity is also commonly seen in workers who are exposed to low levels of arsenic over a long period of time. The skin, lungs, and liver are the main target sites, as arsenite, especially, readily interacts with thiol or sulfhydryl groups in tissue proteins of the organs [21]. The reaction between arsenic and thiol groups can hinder critical biochemical events that lead to major toxicities, and symptoms include skin lesions, pulmonary disease, hypertension, cardiovascular disease, diabetes, neurological disorders, cancer, and death [21-23]. Because epithelial cells have high content of cysteine residues, arsenic tends to accumulate at those locations due to its reactivity with thiol groups of cysteine residues [24, 25]. Thus, for chronic arsenic exposure, usually hair and nail analysis are useful to measure the time of exposure. The concentration of arsenic in hair in individuals who have no known arsenic exposure ranges from 0.02 to 0.2 mg/kg [26-32]. In areas with high concentrations (>50 μg/L) of arsenic in drinking water, the arsenic hair level ranges from 3 to 10 mg/kg [33]. Chelation therapy can be used to mitigate chronic arsenic toxicity, but chronic arsenic exposure can cause many irreversible changes in organs and tissues. Unfortunately, there is no effective treatment, which increases mortality rate [34, 35]. It is difficult to deduce whether acute and chronic toxicities result from two distinct mechanisms, because 1) different biochemical mechanisms may occur in different tissues and organs, and/or, 2) different duration time at the same organ may result in different biochemical mechanisms. Therefore, depending on dose and duration of exposure, arsenic can

target many different types of tissues and organs, causing adverse health effects [36]. The acute and chronic arsenic toxicological effects on human health are summarized in Table 1.

Arsenic in Drinking Water

As mentioned above, due to its toxicity and the frequency of human exposure, arsenic is ranked number one on the ATSDR Substance Priority List, and its environmental contamination is a global issue. Although the Environmental Protection Agency (EPA) has set the standard for arsenic in municipal drinking water of 10 ppb (parts per billion), which is approximately 133 nM, we are still exposed to low levels of arsenic on a daily basis. Exposure to arsenic is associated with an increased risk for a variety of health problems, such as skin and nerve damage, and cancer. More than 200 million people worldwide are chronically exposed to drinking water that is contaminated with arsenic that exceed 10 μg/L, the limit of arsenic contaminant level in drinking water [37], and suffer from different types of diseases from drinking arsenic-contaminated water. The major countries that suffer from arsenic contamination are Taiwan, Bangladesh, India, and Chile, where the arsenic concentrations in drinking water range from 40 μg/L to 2 mg/L [38, 39], which corresponds to 532 nM and 27 μM, respectively. In the United States, the maximum arsenic contaminant level in drinking water is 10 ppb. However, approximately 15% of the U.S. population rely on private wells for their water supply that were found to have arsenic levels greater than 10 ppb [40-43]. Ingesting arsenic in naturally contaminated food and water is the most common route of arsenic exposure. Unfortunately, it is difficult to obtain an accurate measurement of the absorbed dose of arsenic in specific tissues, mainly due to variation among individuals [44], including both endogenous and exogenous factors, such as gender and occupational exposures, respectively. In 2000, Pi et al. demonstrated the average blood arsenic level in people who were exposed to high levels of arsenic in their drinking water

Acute Arsenic Exposure (<24 hours)	Chronic Arsenic Exposure (>3 months)
Vomiting, diarrhea, dehydration, hypotension, abdominal pain, renal failure	Cancer in many organs, skin pigment changes, cardiovascular disease, respiratory disease, diabetes, hypertension, skin lesions, neurological disorders

Table 1. Acute and chronic arsenic toxicological effects on human health.

approximately for 18 years to be about 100 nM [45], which is the concentration that we used in this thesis and from this point forward referred to as “a physiologically relevant” level of arsenic. The lethal human adult dose for ingested arsenic is about 600 µg/kg/day [19], which is about 60 times higher than the standard set by the EPA.

Arsenic Absorption, Distribution, Metabolism, and Excretion (ADME)

Ingestion is the main route of arsenic exposure, and arsenic is readily absorbed from the gastrointestinal-tract (GI tract). The liver is the main site of arsenic metabolism, and historically, methylation steps have been characterized as a critical arsenic metabolism process. This arsenic methylation process was initially studied by Frederick Challenger and his colleagues in the 19th century [46, 47]. In the liver, arsenic metabolites including, monomethylarsonous acid (MMA^{III}), monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{III}), and dimethylarsenic acid (DMA^{V}) are produced (Fig. 2). When arsenate (pentavalent arsenic) enters the cells, it gets rapidly reduced to arsenite (trivalent arsenic) [48, 49]. Arsenite, then, gets methylated by arsenic (III) methyltransferase (AS3MT) as it attaches a methyl group. AS3MT uses S-adenosylmethione (SAM) as the methyl donor, to generate the metabolites [50]. The average relative distribution of arsenic metabolites in the urine varies among population groups, which suggests genetic polymorphism of human methyltransferases [51-53]. Because the methylation process is important in elimination of arsenic from the systemic circulation, it is easy to assume that methylation is just a detoxification process of arsenic, as it enhances excretion of arsenic from the systemic circulation. However, all arsenic metabolites are toxic to different degrees. For example, DMA^{V}, MMA^{III} and DMA^{III} are observed to be more cytotoxic and genotoxic than arsenite [54-57]. Thus, arsenical metabolites are not just the by-products of inorganic arsenic metabolism; they, too, exert toxic effects.

Alternative schemes for arsenic metabolism were proposed. First,

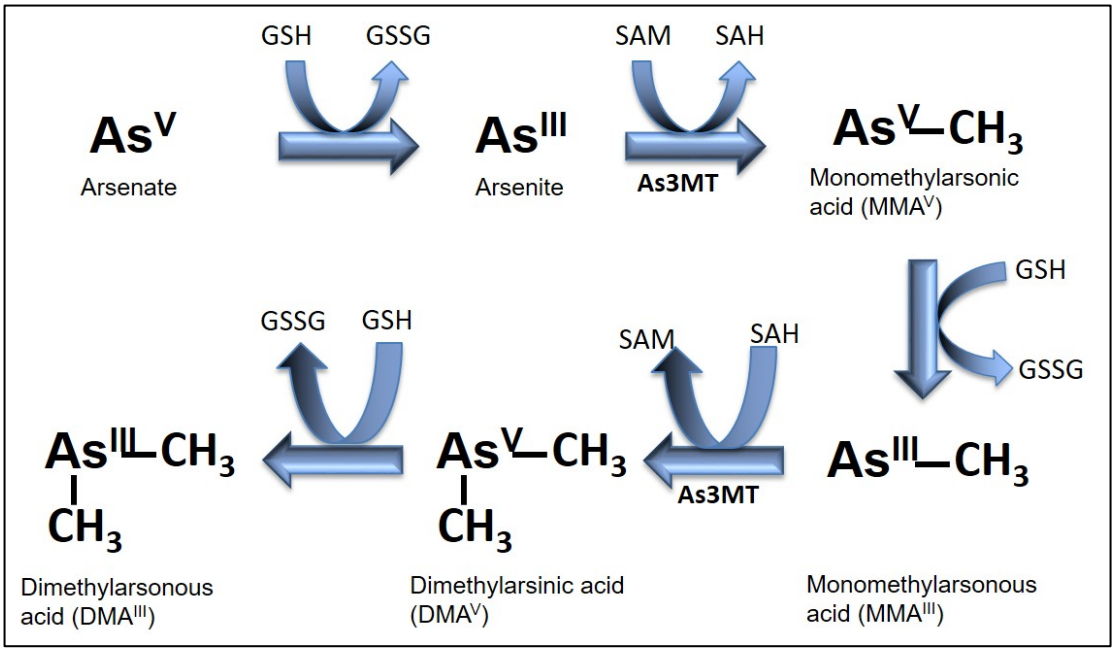

Figure 2. The metabolism pathway of inorganic arsenic. The metabolism pathway of arsenate undergoing reduction and methylation to produce metabolites in pentavalent and trivalent forms. The products of inorganic arsenic metabolism include MMA^{III}, MMA^{V}, DMA^{III} and DMA^{V}. Unlike humans, rats can undergo another round of methylation from DMA^{III} to trimethylarsine oxide (TMAO or TMA^{V}).

Hayakawa et al. focused on the toxicological effects of the metabolites produced by the conventional metabolic pathway of arsenic, and the abundance of DMA from inorganic arsenic than from MMA [58]. The group proposed the reduced form of glutathione (GSH) is critical, and As-GSH complexes are directly involved in inorganic arsenic methylation and important substrates for human arsenic methyltrasnferases [58]. Second, Rehman et al. proposed a reductive methylation pathway [59]. The group proposed the methylation reaction occurs simultaneously, instead of stepwise oxidative methylation, by detecting trivalent form of inorganic arsenic bound to both soluble and non-soluble proteins [59]. Nonetheless, both the conventional and the alternative pathways support the liver serves as the major site of arsenic methylation.

Though many toxicological effects of arsenic are studied using rodent models, the ADME of arsenic is significantly different between rodents and humans. Unlike humans (Fig. 2), rats can metabolize further to TMA^V [60]. Thus, the metabolite ratios are different between the two species, indicating differences in metabolism. Not only species, but also strain differences in rodents also alter sensitivity to arsenic. Thus, to design experimental model to study human toxicological effects of arsenic using rodents requires many considerations including, routes of exposure, dose, and strains, to make the study applicable as possible to human exposure. Nonetheless, rodent models are great tools to study carcinogenicity of arsenic, because the target sites of arsenic carcinogenic effects in rodents are strongly concordant with most of the human targets of arsenic [61].

Arsenic and Lung Cancer

i. Arsenic in Drinking Water and Lung Cancer Development

A common type of cancer in the U.S. that is induced by chronic exposure of arsenic is lung cancer [62-64]. The two major forms of lung cancer are non-small cell lung carcinoma (NSCLC), and small cell lung carcinoma (SCLC). NSCLC accounts for

more than 80% of all lung cancer. NSCLC can be divided into three major histological subtypes: squamous-cell carcinoma, adenocarcinoma, and large-cell lung cancer. Adenocarcinoma is the most common type of lung cancer in patients who have never smoked. Squamous-cell carcinoma is also frequently observed in non-smokers, and is highly associated with chronic exposure to arsenic [65, 66]. Previous studies reveal a direct correlation between arsenic in drinking water and cancer development [39, 63, 65-80]. To investigate a direct environmental factor that is associated with lung cancer development in individuals who have never smoked, Putila and Guo identified a positive correlation between arsenic levels and lung cancer incidence [63]. This study underscored the association between arsenic and lung cancer, independent of smoking and socioeconomic status in the United States [63]. Particularly, people who reside in the Appalachian portion of Kentucky have a higher incidence of lung cancer due to coal mining in Appalachia [81], as arsenic is naturally found in rocks and coal. This direct relationship between arsenic and lung cancer was also observed in countries that have high levels of arsenic in drinking water that can range up to 300 μg/L, which approximately corresponds to 4 μM, and these countries had some of the highest incidences of lung cancer in the world [74, 79, 82-84]. In Bangladesh, 1 in 16 cancer deaths are attributable to arsenic exposure in drinking water [85].

ii. Early Life Exposure to Arsenic and Lung Cancer

Not only post-natal arsenic exposure, but also pre-natal arsenic exposure can lead to lung cancer as well. As pre-natal stage is a critical period of development, young individuals in Chile, who had *in utero*-only and/or early-life arsenic exposure concentrations nearly up to 1,000 μg/L, developed lung cancer [86]. Consistently, when pregnant mice received 85 ppm arsenic-contaminated water only during pregnancy (gestation days 8 to 18), the offspring had lung tumor formation in adulthood [87]. These studies highlight the lethality of pre-natal exposure to arsenic, as the placenta cannot

serve as a barrier to arsenic. A study compared the cancer progression in mice with whole-life 24 ppm arsenic exposure (exposure after birth and termination months before a full life span [88]) and *in utero*-only 85 ppm arsenic exposure (gestation days 8 to 18), and observed development of lung adenocarcinoma and hepatocellular carcinoma in both groups of mice with whole-life and *in utero*-only exposure [89]. However, there were higher incidences of cancer development in offspring that had whole-life arsenic exposure. This result suggests whole-life arsenic exposure, or post-natal arsenic exposure, can aggravate the tumor progression [90-92].

iii. *In vitro* and *In vivo* Studies of Arsenic and the Effects on Lung

Both *in vitro* and *in vivo* models are used to measure toxicological effects on lung and to understand arsenic-induced lung cancer development. The majority of *in vitro* studies use non-malignant cell lines with chronic arsenic exposure, and the reported toxicological effects include increased cell proliferation, colony formation, cell transformation, and ROS induction [93-96]. Furthermore, lung adenocarcinoma development is predominantly observed in *in vivo* model in response to chronic arsenic exposure [87, 89, 91, 97]. The *in vitro* and *in vivo* studies of arsenic and the effects on lung are summarized in Table 2.

iv. Proposed Mechanisms of Arsenic-induced Carcinogenesis

Proposed mechanisms of arsenic-induced carcinogenesis include oxidative stress [98-104], epigenetic changes including histone modification, miRNA expression, and DNA methylation [105-116], aneuploidy [117], and activation of oncogenic pathways [118-136], such as the epidermal growth factor receptor (EGFR). The EGFR is a well-established biomarker of cancer [137-140], and studies have found the EGFR is overexpressed in a variety of tumors and cancer cells, which correlates with poor patient prognosis [141-156], including NSCLC [157-159]. Both previous studies and our preliminary data have shown acute arsenic exposure induces overexpression of EGFR

Type of Study	Target Organ	Cell type or Animal Strain	Arsenic Exposure	Pathological Phenotype	References
In vitro	Lung	Beas-2B	10 μM for 24 hours	Overexpression of EGFR	[160][§*]
		Beas-2B	0.25, 1, and 5 μM for 26 weeks	Increased cell proliferation, anchorage-independent growth, ROS levels, and colony formation	[93]
		Beas-2B	5 μM for 6 hours, 0.25 μM for 24 weeks	Increased ROS levels, cell transformation	[94]
		Beas-2B	0.125, 0.25, and 0.5 μM for 6 months	Increased cell transformation, ROS levels, activation of STAT3,	[95]
		16-HBE	2.5 μM for 13 weeks	Increased cell transformation, percentage of cells in G2/M and S phases, colony formation, chromosome aberration	[96]
		HELF	1 μM for 15 weeks	Activation of Erk, NF-κB, increased cell transformation	[161]
		HBE	1 μM for 3 hours	Activation of STAT3	[162]*
In vivo		C3H, CD1	Gestation day 8 to 18 (42.5, 85 ppm), Gestation day 8 to 18 (85 ppm).	Lung adenoma, carcinoma incidence	[89, 91]
		CD1	Whole-life exposure	Lung adenocarcinoma, bronchiolo-alveolar tumors formation	[87, 91, 97]

Table 2. *In vitro* and *In vivo* studies of arsenic and the effects on lung

*= acute arsenic exposure; [§]= Effect on EGFR expression

in human bronchial epithelial cells [160] (Fig. 3). *Despite a direct association between supraphysiological levels of arsenic and lung cancer, how chronic exposure to "a physiologically relevant" level of arsenic affect EGFR expression and signaling are not known.*

EGFR Biology

i. Structure and Activation of EGFR

EGFR is a receptor tyrosine kinase (RTK) localized on the cell surface (Fig. 4). As one of the ErbB family receptors (EGFR, ErbB2, ErbB3, and ErbB4), EGFR is activated by specific ligands, and there are 7 endogenous EGFR ligands (epidermal growth factor (EGF), transforming growth factor-α (TGFα), HB-EGF, amphiregulin (AREG), betacellulin (BTC), epigen (EPGN), epiregulin (EREG)). Of these ligands, HB-EGF and BTC are known to have high affinity for the receptor and have relatively high downstream effects [163-165]. The EGFR and the other ErbB family members can be divided into three main domains: extracellular domain, transmembrane domain, and intracellular domain, which consists of kinase domain and tyrosine residues on the C terminus that serve as docking sites for many downstream proteins. Ligand binding to the extracellular domain of the EGFR leads to a conformational change that allows receptor dimerization and activation of the intrinsic kinase activity. Once activated, the kinase from one receptor trans-phosphorylates tyrosine residues on the intracellular carboxy-terminus of its receptor pair (Fig. 4). These newly formed phosphotyrosines then serve as docking sites for downstream signaling proteins (effectors) that mediate cell proliferation, survival, tumorigenesis, and differentiation.

ii. EGFR Function in Epithelial Development

The EGFR is a critical component in development. The EGFR expression was observed in embryogenesis, and its ligands, specifically EGF and TGFα, were also expressed from 4- to 8-cell stage of embryogenesis [166-171]. The co-localization of the

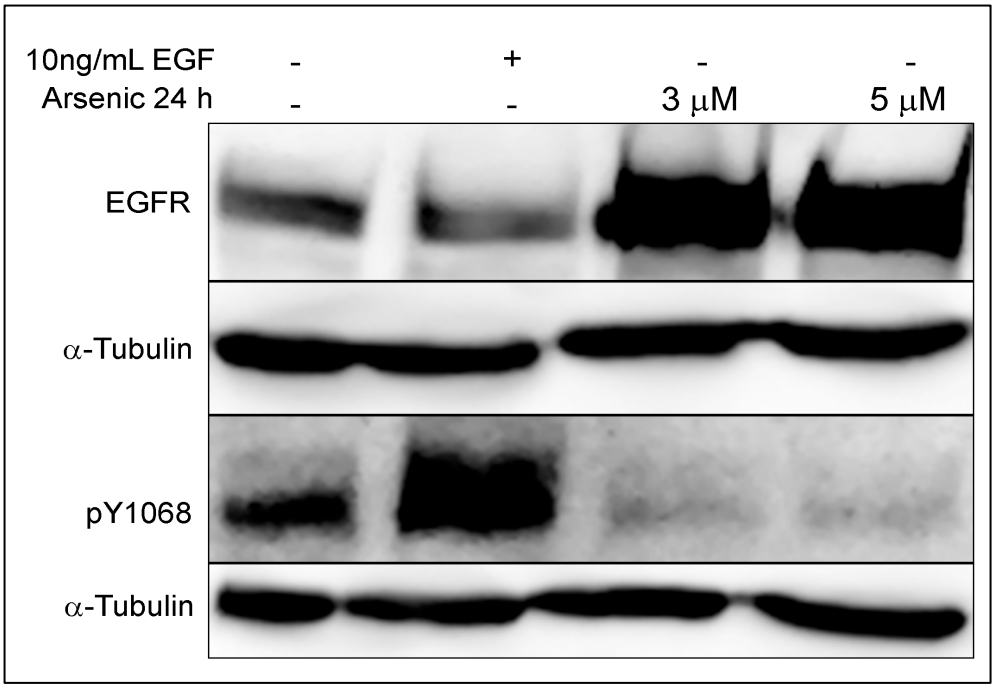

Figure 3. Acute arsenite induces overexpression of EGFR. Immunblot of EGFR and phosphotyrosine 1068 (pY1068) in Beas-2B cells treated with arsenite at the indicated concentrations for 24 hours. 10 ng/mL of EGF treatment in Beas-2B cells was used as a positive control. EGF was stimulated after 2 hours of serum-starvation, and it was stimulated for 7 minutes. The samples were resolved on 7.5% SDS-PAGE.

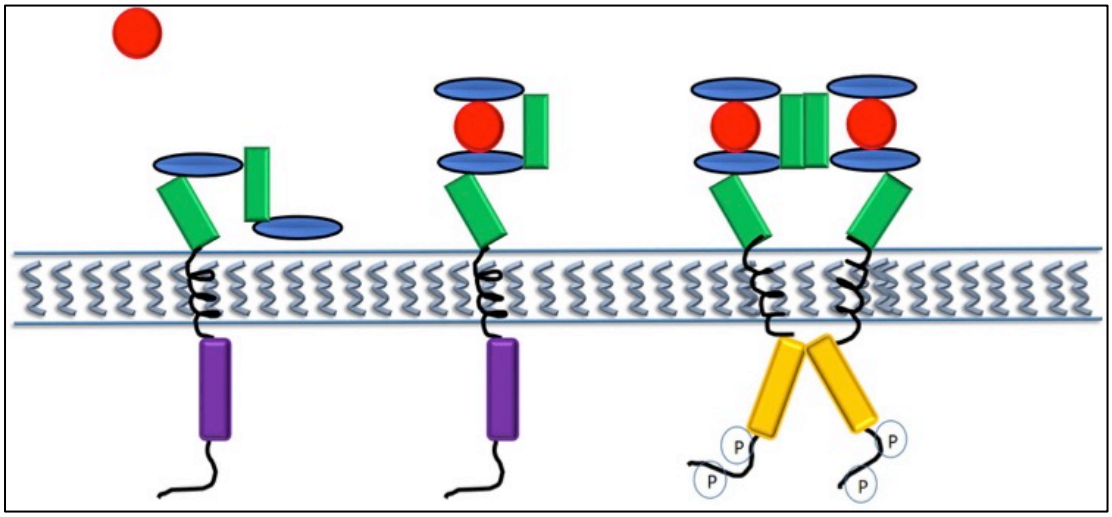

Figure 4. EGFR structure. When the receptor is not occupied by a ligand, the cysteine-rich regions of the extracellular domain of the receptor interact with each other and maintain a “closed” conformation. When a ligand binds to the ligand binding domains of the extracellular domain, the EGFR undergoes a conformational change, exposing cysteine-rich regions. These regions, then, interact with other exposed cysteine-rich regions of another ErbB family receptor. This allows receptor dimerization and activation of the intrinsic kinase activity. Once activated, the intracellular kinase from one receptor trans-phosphorylates tyrosine residues on its receptor pair. These newly formed phosphotyrosines then serve as docking sites for downstream signaling proteins that mediate cell proliferation, survival, tumorigenesis, and differentiation. Red=ligand; green=cysteine-rich regions; blue=ligand-binding domains; purple=inactive kinase domains; yellow=active kinase domains.

ligands with the EGFR throughout the critical stages of embryogenesis underscores the requirement of full activation of EGFR for proper development. Therefore, EGFR null and/or EGFR mutations are deleterious. Earlier studies have found EGFR null mice were either embryonically lethal or died shortly after birth [172]. The mice that had EGFR mutation manifested changes in hair and skin, which support the roles of EGFR in epithelial development [173, 174].

iii. The Endocytic Trafficking Pathway of EGFR

When a ligand binds to the EGFR, the activated EGFR gets internalized via clathrin-coated pits into the cell. Once the clathrin is shed, the vesicle then fuses with the early endosomes [175]. From the early endosomes, the receptors can have two different fates; the receptors can move back to the plasma membrane from the early endosomes (recycling), or the receptors can be degraded via lysosomes. For receptors to degrade, the receptors-containing early endosomes increase acidity to “mature” into the late endosomes [176]. The late endosomes then fuse with lysosomes to degrade the receptors (Fig. 5). The degradation of EGFR is tightly regulated to control the downstream signaling events, such as proliferation. Thus, improper regulation of degradation of EGFR can lead to overstimulation of proliferative signaling and contribute to cancer development. When the receptors are activated, the tyrosine residues of the kinase domain of the receptors get phosphorylated and get internalized into the cell. This receptor activation triggers c-Cbl, which is an E3 ubiquitin ligase, to be recruited and binds to a specific phosphorylated tyrosine residue (pY1045) of the kinase domain of the receptor [177]. This binding causes ubiquitination of the receptors and the receptors get marked for degradation via lysosomes [178, 179]. As mentioned earlier, the other fate of the internalized receptors is to recycle back to the plasma membrane. Previous studies have found even without any ligand stimulation, about 10% of the EGFR constitutively internalize and recycle back to the plasma membrane [180], predominantly by the basal

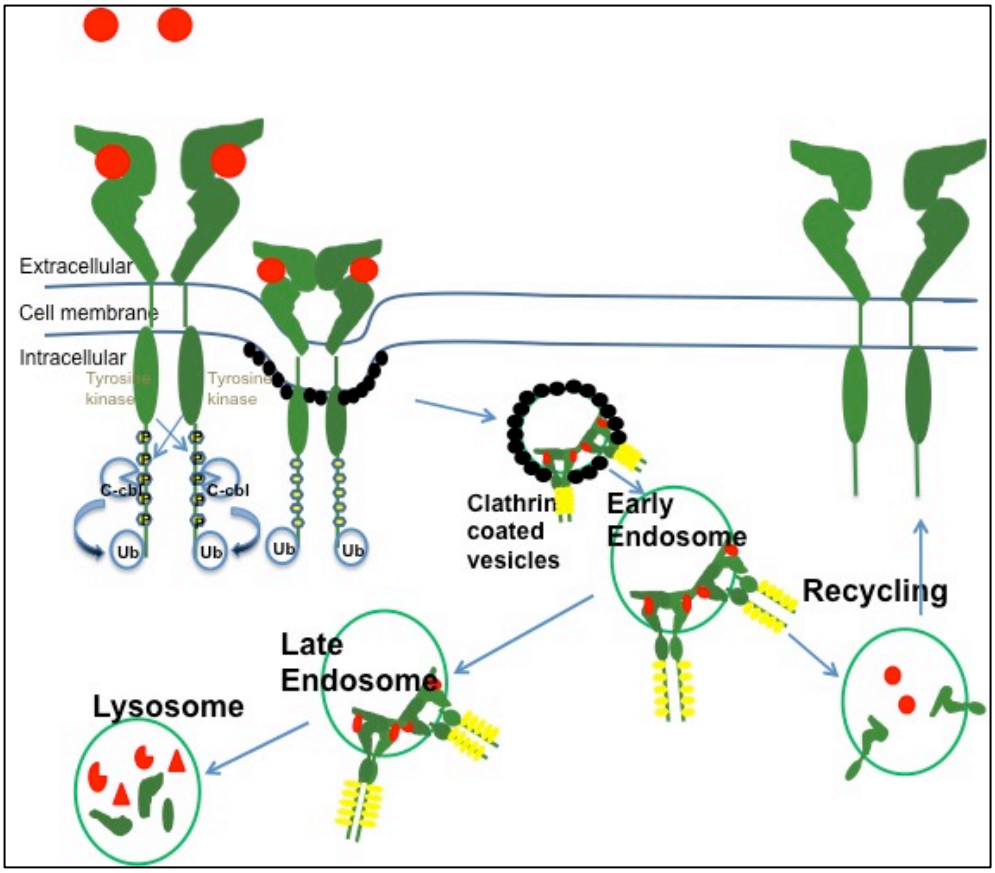

Figure 5. The endocytic trafficking pathway of EGFR. When a ligand binds to the receptor, the receptors become activated. The activated tyrosine kinase domains then induce trans-phosphorylation of the tyrosine residues on the carboxy-terminus of its receptor pair. This phosphorylation triggers c-Cbl to be recruited to the activated receptors and ubiquitinates the receptors. The activated receptors get internalized via clathrin-coated pits into the cell. Once the clathrin is shed, the vesicle then fuses with the early endosomes. From the early endosomes, the receptors can have two different fates; the receptors can move back to the plasma membrane from the early endosomes (recycling), or the receptors can be degraded via lysosome.

expression of TGFα, an EGFR ligand that is involved in the recycling of the receptors [181]. EGF, on the other hand, is a well-known EGFR ligand, and EGF-stimulated EGFR undergo degradation. The different effects between TGFα and EGF ligands are mainly due to their sensitivity to the acidic environment [182]. TGFα is pH sensitive, and it is known to dissociate from the receptors at pH of about 6.8, whereas EGF get dissociated from the receptors at pH of about 5.8 [183-185]. Thus, TGFα gets dissociated from the early endosome compartment (pH of about 6.8), which allows the EGFR to recycle back to the plasma membrane, whereas EGF:EGFR complex endure the acidic environment until they get degraded [185]. The ErbB2 expression levels also affect the recycling pathway of the receptors. Previous studies have observed decrease in EGF-stimulated EGFR degradation in cells that overexpress ErbB2 [186, 187]. Further studies supported these observations by demonstrating ErbB2 overexpression preventing clathrin-mediated endocytosis of EGFR [186, 188]. However, a clear mechanism for ErbB2-induced impairment of EGFR endocytosis remains elusive.

EGFR and Cancer

There are four main events that can perturb the EGFR regulatory mechanisms, which all contribute to cancer development: 1) overexpression of EGFR, 2) overproduction of its ligands, 3) improper receptor trafficking, and 4) the EGFR kinase domain mutations.

i. Overexpression of EGFR

The overexpression of EGFR (as compared to adjacent non-cancerous tissue) is seen in many cancers, including colorectal cancer, pancreatic cancer, NSCLC [159, 189-195], and gliomas [196-201], and is associated with a poor prognosis. Under pathological conditions, such as cancer, the normal regulatory mechanisms of the signaling pathways are perturbed, resulting in hyperactivation of the signaling pathways. The overexpression of EGFR and its association with a poor prognosis can be explained

by increased sites for the ligands to bind to the receptors, leading to enhanced downstream signaling events, such as proliferation.

ii. Overproduction of the EGFR Ligands

An increase in synthesis of ligands above the basal levels also triggers enhancement of the EGFR-induced activation of proliferative pathways. High levels of EGFR ligands, including EGF, TGFα, AREG, and BTC, were observed in cancers [202, 203]. The frequency of overexpression in protein levels of the EGFR and TGFα, has been observed in NSCLC patients, and predicts a poor outcome [190]. All ligands function similarly to EGF; they bind to the ligand binding domain of the receptors, and induce a conformational change in the receptors that leads to dimerization of the receptors and increased tyrosine kinase activity. However, their tissue distribution, expression regulation, and binding affinity and preferences on the receptors differ from one another, which all can alter the EGFR signaling by changing the endocytic trafficking itinerary [204-208].

iii. Improper Receptor Trafficking

Disruption of proper EGFR trafficking is known to contribute to cancer development [209-216] mainly due to poor downregulation of the receptors and sustained downstream proliferative signaling. Without any ligand stimulation, the unstimulated receptors predominantly localize on the surface of the cells. However, when the cells are stimulated with ligand, such as EGF, the internalized receptor co-localizes with the early endosomes within 10 minutes post-stimulation [217]. Over time, the EGF-stimulated cells will have reduced staining of the EGFR in the early endosome compartment, due to its lysosomal degradation. However, when the EGFR endocytic trafficking pathway is perturbed, specifically between the early and the late endosomes, the EGFR will remain in the early endosome compartment and still active, prolonging the signaling as the EGFR accumulate in the early endosome compartment [218].

Consistently, a previous study observed an increase in the EGFR protein expression level in response to prevention of the EGFR degradation [213], which suggests improper EGFR endocytic trafficking can contribute to both overexpression and hyperactivation of EGFR, leading to carcinogenesis. Thus, abnormalities in the endocytic trafficking of signaling receptors are now well-established hallmarks of malignant cells [219, 220].

iv. Kinase Domain Mutations.

Not only overexpression of the wild type EGFR, but also expression of the mutant EGFR kinase domain can contribute to cancer development. The most common EGFR kinase domain mutation is EGFRvIII, which is a deletion of residues from 6 to 273. Despite the inability to bind to the ligands, EGFRvIII is known to induce constitutive activation and trigger downstream signaling [221]. This form of receptor is known to enhance tumorigenesis, proliferation, and metastasis of tumors [222-225]. Such mutation is commonly observed in about 40% of glioblastoma cases [226-228]. Even though the overexpression of EGFR is commonly observed in NSCLC cases, there are only about 10 to 30% of NSCLC patients who are EGFR mutant positive [229]. The most common EGFR kinase domain mutations in NSCLC patients are exon 19 deletion, which is near the ATP-binding pocket that tyrosine kinase inhibitor (TKI) targets, and a mutation in exon 21 by substituting leucine 858 with arginine, or L858R [221, 230]. These kinase mutations cause constitutive activation of the EGFR by destabilizing the inactive conformation of EGFR [231, 232]. Therefore, NSCLC patients with the EGFR kinase domain mutations have better clinical response to TKI, as the mutants are more sensitive to TKI than the wild type EGFR [233, 234]. Unfortunately, patients with mutant EGFR kinase domain acquire resistance to the TKI therapy. A common EGFR kinase domain mutation that leads to resistance is T790M (substitution of threonine 790 with methionine). The location of T790 is significant, as it is located in the ATP binding pocket [235]. This substitution allows structural conformation of the receptors to gain near wild

type levels of ATP affinity and allow ATP to bind to the kinase domain with higher affinity than the drug [235, 236], which supports T790M-induced resistance to TKI. Additionally, Shtiegman et al. observed decrease in endocytosis and downregulation of L858R- and T790M-containing EGFR, and continuous phosphorylation status of the receptors several hours following EGF stimulation, unlike the wild type receptors [211], which suggests prolonged downstream signaling and predisposition to lung cancer. Particularly, L858R-containing EGFR mutant had impaired recruitment and phosphorylation of c-Cbl with EGF stimulation [211].

v. EGFR-targeted Chemotherapy

Because of the importance of EGFR in cancer development, there have been several chemotherapeutic agents that target EGFR. The EGFR-targeting chemotherapeutic agents can be divided into two main classes: monoclonal antibodies and small molecule kinase inhibitor. An example of the monoclonal antibody is Cetuximab, which targets the extracellular domain of the receptor to prevent ligand:receptor interactions. This drug is approved for treatment of cancers, such as colorectal cancer and squamous cell carcinoma of the head and neck (NCI, 2018). Erlotinib and getfitnib are examples of TKI, which bind to the kinase domain of the receptor to prevent activation of the downstream proteins and their signaling. Erlotinib is widely used as first-line therapy to treat particularly NSCLC patients [237]. As mentioned earlier, patients with mutant EGFR, such as L858R, have good responses to TKI, as they induce constitutive activation of the kinase, and such activity is found to increase sensitivity to the drug [233, 234, 238]. These specific mutations have higher affinity for TKI than does the wild type EGFR, and they have lower affinity for ATP, as compared to the TKI [231, 239]. Consistently, NSCLC cells with the mutant EGFR kinase domain have lower IC_{50} value of TKI, as compared to the wild type EGFR [240]. Furthermore, TKI inhibited both EGFR activation and proliferation, and induced apoptosis in NSCLC

cells with the mutant EGFR kinase domain, but it did not induce apoptosis in NSCLC cells with the wild type EGFR [233, 241, 242]. Interestingly, when the ErbB2 expression was blocked by monoclonal antibodies, enhancement of the mutant receptors' downregulation was observed [211], which highlights the importance of ErbB2 regulation. Thus, these studies suggest the complexity of the mutant receptors, and more specific targets-driven chemotherapeutic agents are needed. There are several irreversible inhibitors that target these mutations, and they act through covalent binding, but they are yet to be approved [235].

EGFR and Arsenic-induced Carcinogenesis

As mentioned above, both previous studies and our preliminary data have shown acute high levels of arsenite exposure induces the overexpression of EGFR in human bronchial epithelial cells [160] (Fig. 3). Understanding the molecular mechanism of such phenomena will contribute to developing new drug target. This thesis examines the effect of "a physiologically relevant" level of arsenite on the EGFR signaling.

Arsenic readily accumulates in epithelial cells as they have high content of thiol groups, and EGFR plays a critical role in epithelial development. The interaction between arsenic and thiol groups supports arsenic role in lung cancer development through EGFR signaling axis in epithelial cells. In this thesis, we suggest a potential role of chronic arsenite exposure in the regulation of components of the EGFR signaling axis. A previous study used micromolar range of arsenite, and observed increased level of EGFR ligand mRNAs, specifically HB-EGF [119]. HB-EGF is seen in a variety of cancers, such as colorectal, cervical, breast and gastric cancers [243-247]. Consistently, an earlier study demonstrated increase in TGFα mRNA expression levels in mice chronically exposed to arsenic-contaminated drinking water [248]. Also, overexpression of TGFα was observed in the tumor from nearly 50% of primary NSCLC patients [190]. As mentioned earlier, TGFα is an EGFR ligand that is involved in the constitutive

recycling of EGFR, which can delay the EGFR lysosomal degradation [181], resulting overexpression of the receptors. Thus, these studies support the hypothesis that arsenic has an impact on regulation of the ligands, resulting in overexpression of the EGFR.

There is a scant amount of studies that observed the effect on the receptor trafficking upon chronic toxicant exposure. A study has shown arsenic increases protein levels of Rab4, a protein involved in the recycling of EGFR [249]. This study suggests a potential role of arsenic in altering the endocytic trafficking of EGFR. Under normal conditions, EGFR internalizes via clathrin-mediated endocytosis, but at high doses of EGF, the EGFR undergoes clathrin-independent endocytosis, including caveolin-mediated endocytosis [250]. This interplay between clathrin-mediated endocytosis and caveolin-mediated endocytosis controls the expression of EGFR in the cells to prevent overstimulation, as caveolin-mediated endocytosis preferentially couples to the EGFR degradation [251]. In our preliminary study, we observed overexpression of EGFR in response to chronic arsenic exposure (Fig. 7), suggesting a possible role of arsenic in dysregulating the interplay between the two distinct endocytic trafficking pathways, which contributes to arsenic-induced carcinogenesis by allowing overstimulation of EGFR signaling. Additionally, acute high levels of arsenic exposure is known to prevent microtubule disassembly by losing the integrity of the tubulins and mircotubules [252], and microtubules are critical component in movement of proteins between the organelles [253]. Thus, the EGFR endocytic trafficking pathway is a potential target site of chronic arsenic to induce overexpression of EGFR in the cells. Disruption of proper EGFR trafficking is known to contribute to cancer development, such as lung, pancreatic, and breast cancers [211, 213, 254]. Despite of the importance of proper EGFR trafficking in cancer development, there has been no study that tested alterations in the route of EGFR trafficking from chronic arsenic exposure.

Importance of the study

There are several chemotherapies for lung cancer; however, cancer cells acquire resistance to therapy, as they find alterative routes to survive. More specific cellular targets are needed to be found, and an understanding of the underlying mechanisms to cancer development is critical. The present study was achieved by using Beas-2B cells, which are non-malignant human lung bronchus epithelial cells generated by SV40 transfection [255]. They have been widely used to study heavy metal-induced carcinogenesis [256-260], and they are commonly used in arsenic studies because the lungs are known to be the major target of inorganic arsenic carcinogenesis [62, 261]. *The long-term goals are to understand the molecular mechanisms of arsenite-induced overexpression of EGFR and to identify novel roles of chronic arsenite exposure in the EGFR endocytic trafficking.*

CHAPTER 2

MATERIALS AND METHODS

Cell culture

Human bronchial epithelial cells, Beas-2B cells obtained from ATCC were grown in LHC-9 media (Gibco) supplemented with or without 100 nM of sodium arsenite (Fisher Scientific) on a matrix of 10 µg/mL of fibronectin and 35 µg/mL of collagen (FNC Coating Mix, AthenaES). This concentration of sodium arsenite (arsenite) was selected based on the average blood arsenic level in people who were exposed to high levels of arsenite in drinking water [45]. Cultures were grown in 5% CO_2 at 37°C atmosphere. Multiple cultures of cells (4 with and 4 without 100 nM sodium arsenite) were maintained separately for 24 weeks. Cells were propagated by splitting at 1 x 10^6 cells/ 10 cm dish every 3-4 days. Once a week, the arsenite treated cells were frozen down at 1 x 10^6 cells/vial and stored in liquid nitrogen.

Immunoblotting

Cells were washed with phosphate-buffered saline (PBS), and kept in EDTA/PBS solution for 15 minutes at 37°C to allow non-enzymatic dissociation of cells. Then the cells were harvested in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 50 mM Tris (pH 8.0)] containing protease inhibitor, PMSF (EMD Millipore)]. The samples were diluted in the 6X SDS buffer containing 10% βME, then the samples were boiled at 95°C for 3 minutes prior to gel loading. The lysates were separated by 7.5% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was blocked with

5% milk/TBST before probing with primary antibody overnight at 4°C. The following antibodies were used for protein detection: EGFR (Santa Cruz, sc-03) (1:1000 dilution in 5%milk/TBST), pY1068 (Cell Signaling, 2234L) (1:500 dilution in 5%milk/TBST), and α-tubulin (Sigma-Aldrich, T6199-200UL) (1:4000 dilution in 5%milk/TBST). After incubation with the horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit, Thermo Fisher), we used enhanced chemiluminescence (ECL) to visualize the proteins using a Fotodyne imaging system. The immunoblot was analyzed and quantified using ImageJ software.

Indirect Immunofluorescence

Beas-2B cells were cultured on sterile 12mm round #1 glass cover slips for 2 days. The cells were serum-starved for 2 hours prior to EGF stimulation. After 15 minutes of EGF stimulation, cells were washed with PBS++ (PBS with 0.5 mM Ca^{2+}, 0.5 mM Mg^{2+}). The cells were fixed in 4% paraformaldehyde/PBS++ for 5 minutes at room temperature followed by 15 minutes incubation on ice. After PBS++ washes, the cells were permeabilized in blocking buffer (0.1% saponin, 5% FBS in PBS++) for 15 minutes at room temperature. After washes with PBS++, the cells were incubated with the indicated primary antibody [EGFR (Ab-1, EMD Millipore, GR01)] in the blocking buffer (1:1000 dilution) for 60 minutes at room temperature. After 3 washes with PBS++, the cells were then incubated with fluorophore-conjugated secondary antibody (Alexafluor-488, Life-technologies) in the blocking buffer (1:250 dilution) for 60 minutes at room temperature in the dark. After 6 washes with PBS++, the glass cover slips were dipped into beaker with ddH_2O to remove associated salts. Kimwipes were used to carefully remove excess liquid on the cover slips. The coverslips were mounted onto slides using Prolong Gold Antifade Mountant with DAPI (Thermo-Fisher Scientific). The slides were cured in the dark overnight and examined the next day using a Nikon Eclipse Ti-E Inverted fluorescence microscope, using Nikon NIS Elements software. The images were taken

with a 60X oil immersion objective lens. Multiple images were acquired and representative images are presented. A total of 100 cells from randomly selected fields were imaged.

Alamar Blue assay

Beas-2B cells were plated onto 96-well microplates (10,000 cells/well) for 24 hours. Cells were treated with different concentrations of AG1478 (Cayman Chemical), and 0.1% of DMSO was used as a control. Cells were incubated with AG1478 for 24 hours. Then, 10 µL (10% of total volume) of Alamar Blue Reagent (Bio-Rad) was added to each well of the 96-well microplate. After cell incubation for 2 hours at 37°C, the fluorescence of each well was measured in plate reader with Gen5 BioTek software at 530nm of excitation wavelength and 590nm emission wavelength. The data were plotted as the percentages of the cell viability vs. the increasing concentrations of AG1478.

RT-qPCR

RNA was isolated from Beas-2B cells by using RNAqueous-Micro Total RNA Isolation Kit (Thermo Fisher, AM1931). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher, 4368814). qPCR was performed using TaqMan Gene Expression Master Mix (Thermo Fisher, 4369016), and the primers were commercially designed by Thermo Fisher. The following primers were used for qPCR analysis: EGFR (Catalog# 4331182, Assay ID Hs01076089), ErbB2 (Catalog# 4331182, Assay ID Hs01001580_m1), AREG (Catalog# 4331182, Assay ID Hs00950669_m1), BTC (Catalog# 4331182, Assay ID Hs00156140_m1), and TGFα (Catalog#4331182, Assay ID Hs00608187_m1). All primers have been validated either by the company itself or in the literature. The 96-well plate (Thermo Fisher, 4346906) was read in StepOnePlus System (Thermo Fisher, 4376600). GAPDH was used as a housekeeping gene. For each experiment, gene expressions (average of the technical replicates) were

normalized to the average GAPDH levels. Data were plotted as the fold difference compared to the untreated Beas-2B cells at week 0.

CHAPTER 3

RESULTS

The overarching goal of these experiments was to understand the molecular mechanisms of arsenite-induced overexpression of EGFR and to identify novel roles of chronic arsenite exposure in the EGFR endocytic trafficking. We hypothesized carcinogenicity of chronic arsenite exposure induces improper endocytic trafficking of EGFR, resulting EGFR overexpression.

Chronic arsenite exposure does not alter the morphology of Beas-2B cells.

As a typical epithelial morphology, Beas-2B cells maintain cell-to-cell adhesion, cell polarity, and have projectile morphology (Fig. 6). The cell morphology can change in response to heavy metals, such as chromium, by altering cell-cell contact, and losing the formal shapes [262]. To test whether low levels of arsenite alter the morphology of the cells, Beas-2B cells were grown in the presence of 100 nM arsenite for 24 weeks. Interestingly, we did not observe any morphological changes, as compared to the passage-matched untreated cells and 0 week Beas-2B cells (Fig. 6).

Chronic arsenite exposure induces overexpression of EGFR.

Overexpression of EGFR in response to acute micromolar range of arsenite exposure is now well-established [120, 160] (Fig. 3). To determine whether chronic exposure to “a physiologically relevant” level of arsenite (100 nM) stimulate the EGFR levels in a similar way, the EGFR and phosphotyrosine (pY1068) levels were measured in cells that were grown in arsenite for 24 weeks. As a measure of EGFR phosphorylation (activity), pY1068 levels were monitored. The passage-matched untreated Beas-2B cells were used as a negative control to make sure the cells’

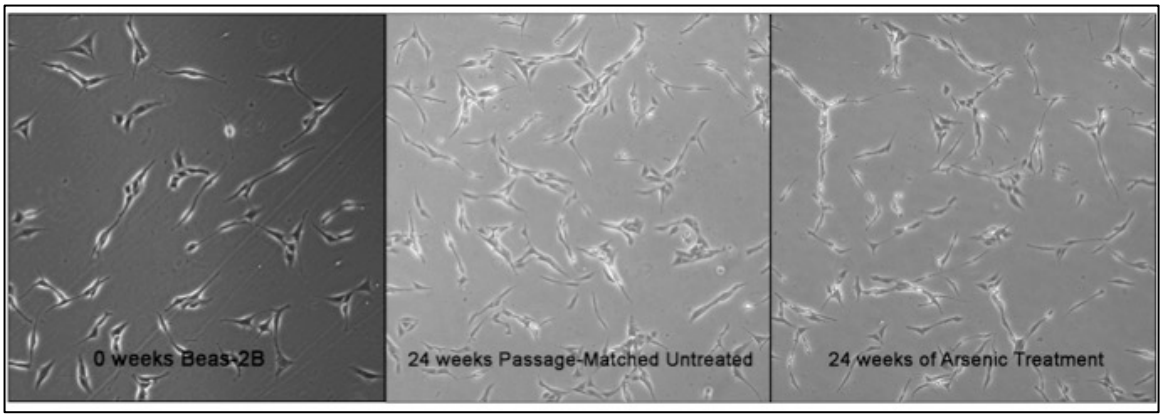

Figure 6. Chronic arsenic treatment does not alter the morphology of Beas-2B cells. The Beas-2B cells were grown in the presence of 100nM arsenic for 24 weeks, and the morphology was compared to the passage-matched untreated Beas-2B cells and 0 week Beas-2B cells.

characteristics did not change over time. We did not observe any differences in the expression levels of both EGFR and pY1068 among the passage-matched untreated cells (Fig. 7). Similarly to acute exposure of arsenite, chronic low levels of arsenite, too, induced the overexpression of EGFR (Fig. 7). When 24-weeks arsenite treated cells were co-treated with AG1478, a small molecule inhibitor of the kinase domain, the expression level of pY1068 significantly decreased, which suggests arsenite acts directly on the tyrosine kinase domain, rather than via another protein (i.e. a non-receptor tyrosine kinase) to induce autophosphorylation of the EGFR (Fig. 8). The expression level of pY1068 of the untreated cells establishes the basal level of EGFR phosphorylation (Fig. 8, lane 1).

Chronic arsenite exposure increases the mRNA levels of EGFR ligand, TGFα.

To understand the underlying mechanism of arsenite-induced overexpression of EGFR, we investigated mRNA levels of the ErbB family receptors, as well as their ligands. RT-qPCR data showed chronic exposure of "a physiologically relevant" level of arsenite increases TGFα mRNA levels in an exposure time-dependent manner (Fig. 9).

Chronic arsenite exposure alters the route of the EGFR endocytic trafficking pathway.

Previous studies have observed increase in protein and mRNA levels of proteins that are highly involved in the recycling of EGFR in response to acute arsenite exposure [248, 249, 252]. However, whether arsenite alters the EGFR endocytic trafficking pathway still remains elusive, thus we wanted to determine any alterations in the route of the EGFR endocytic trafficking pathway when the cells are chronically exposed to "a physiologically relevant" level of arsenite. The EGFR in the untreated Beas-2B cells were punctate and randomly scattered, but when the cells were exposed to 100 nM of arsenite for 24 weeks, the route of EGFR trafficking was altered; the EGFRs were aggregated (Fig. 10). However, due to obscurity in the EGFR localization, further studies

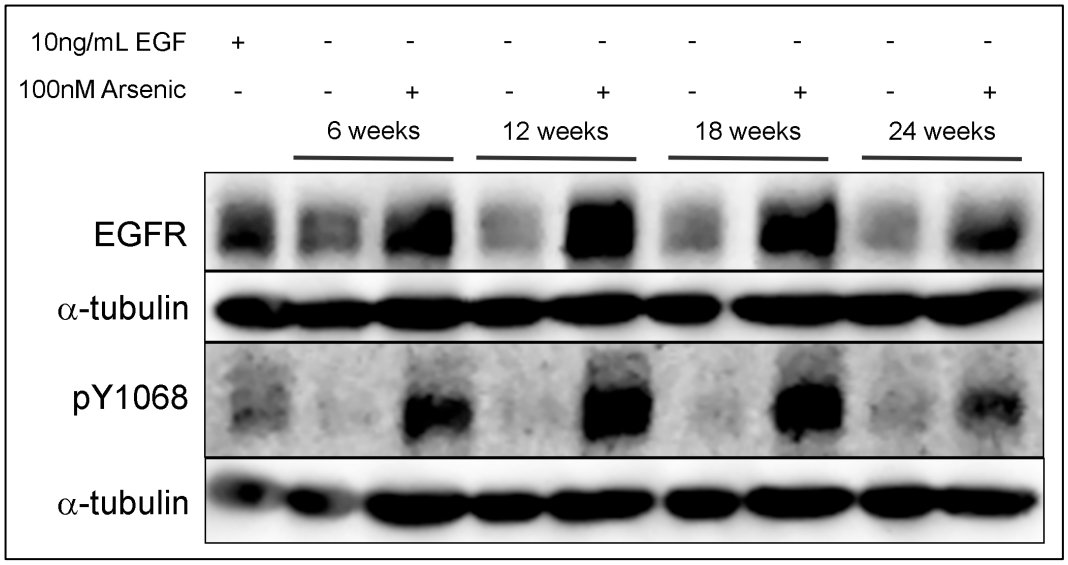

Figure 7. Chronic exposure to low levels of arsenite induces EGFR overexpression and activity. 10 ng/mL of EGF was stimulated after 2 hours of serum-starvation, and it was stimulated for 7 minutes. The samples were resolved on 7.5% SDS-PAGE. The EGFR and pY1068 expression levels of Beas-2B cells that were treated with arsenite were compared with the passage-matched untreated cells.

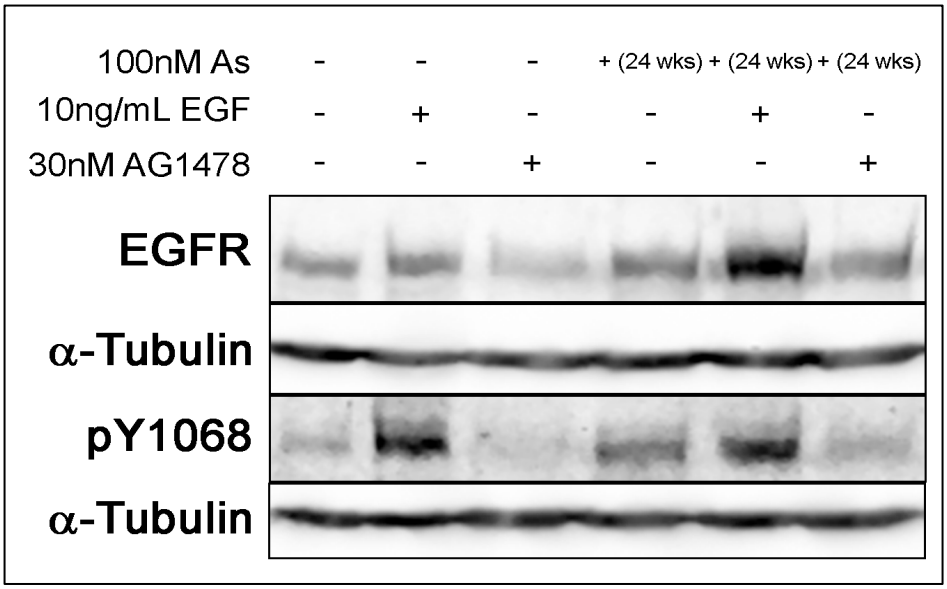

Figure 8. AG1478 prevents chronic-arsenite induced EGFR activity. The EGFR and pY1068 expression levels were measured in the cells that were grown in arsenite, EGF, or AG1478. 10 ng/mL of EGF was stimulated after 2 hours of serum-starvation, and it was stimulated for 7 minutes. The cells were exposed to 30 nM of AG1478 for 24 hours. The samples were resolved on 7.5% SDS-PAGE.

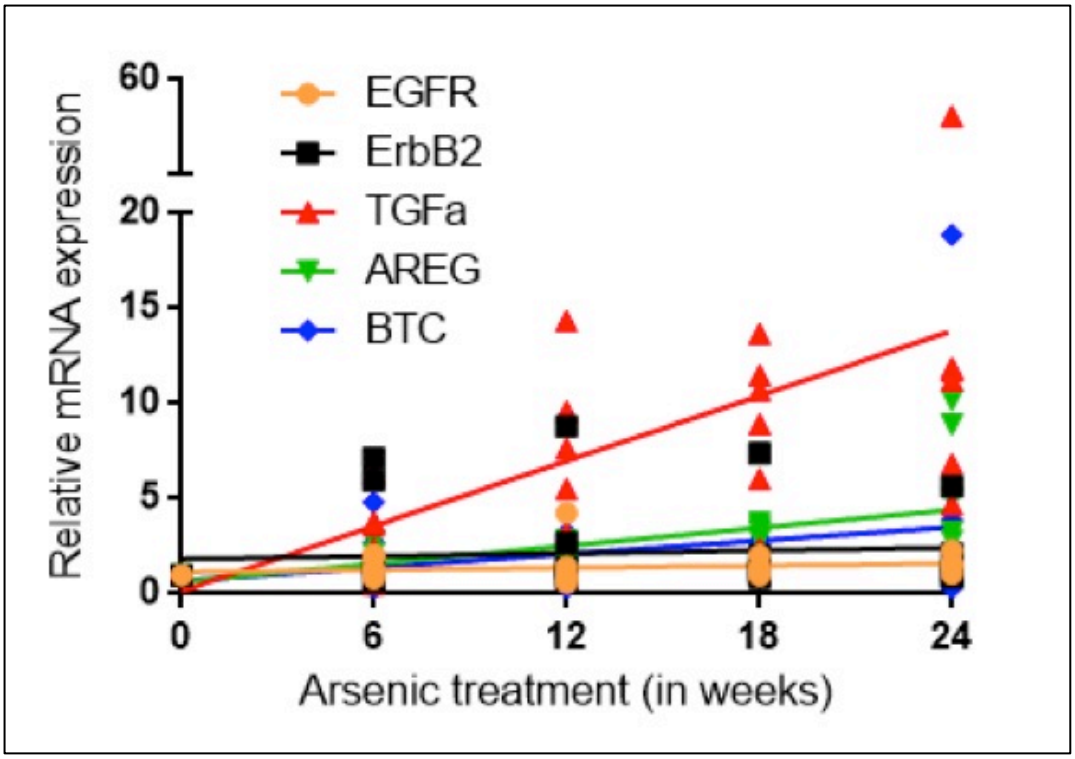

Figure 9. Chronic arsenite increases mRNA levels of EGFR ligand, TGFα. RNA was isolated from Beas-2B cells (untreated, 6 weeks, 12 weeks, 18 weeks, and 24 weeks) and cDNA was synthesized. qPCR was performed using the primers that were commercially designed. For each experiment, gene expressions (average of the technical replicates) were normalized to the average GAPDH levels. Data were plotted as the fold difference compared to the untreated Beas-2B cells at week 0. (N=2)

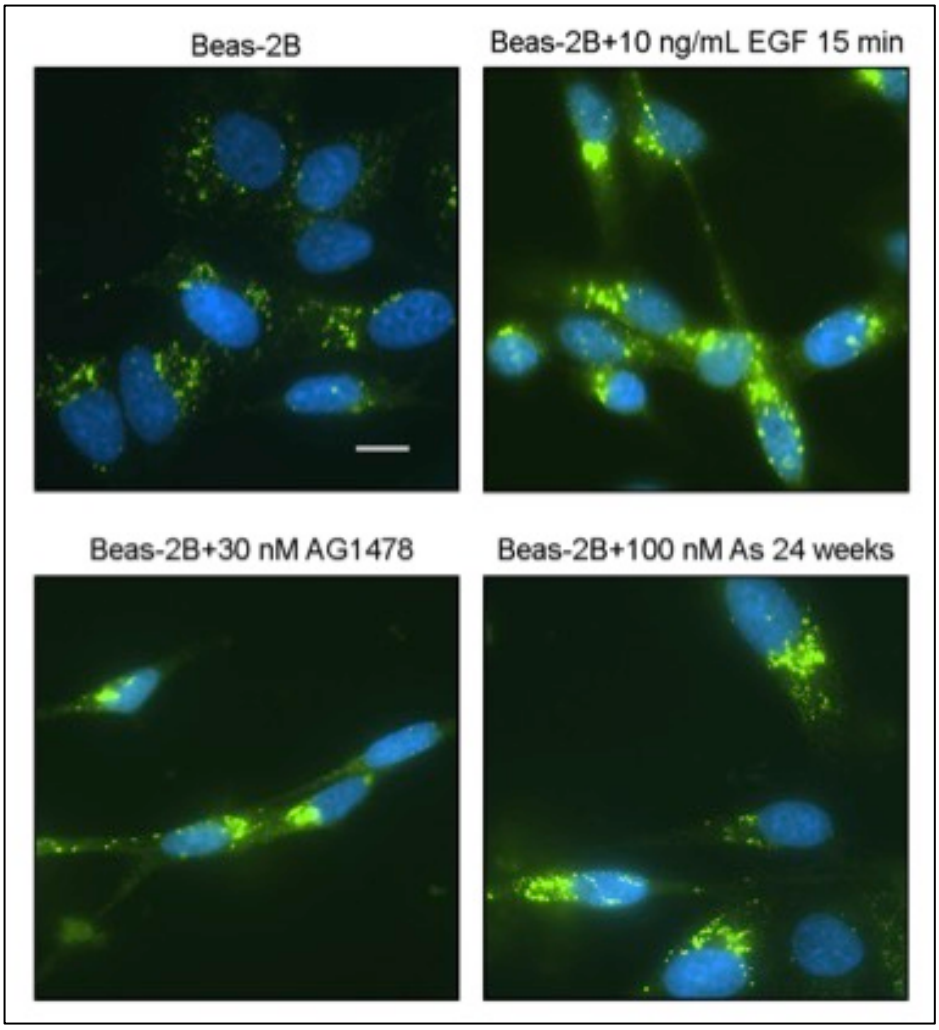

Figure 10. Chronic arsenite exposure alters the route of the EGFR endocytic trafficking pathway. Immunofluorescence was performed to analyze the EGFR trafficking in response to chronic arsenic exposure. Cells were fixed with 4% paraformaldehyde and stained for EGFR (Alexafluor-488 (Green)). Scale bar=10 μm

are needed to draw conclusions.

Chronic arsenite does not sensitize Beas-2B cells to tyrosine kinase inhibitor.

Because we observed chronic arsenite-induced EGFR overexpression, we next wanted to determine how chronic exposure to "a physiologically relevant" level of arsenite treatment affects cell sensitivity to tyrosine kinase inhibitor. Tyrosine kinase inhibitor is a well-established EGFR-targeted chemotherapy. A previous study has shown overexpression of EGFR in thyroid cancer cells increases its sensitivity to a well-known tyrosine kinase inhibitor, getfitnib [149]. Because arsenite stimulates overexpression of EGFR, we performed Alamar Blue assay to determine whether chronic arsenite alters the sensitivity of Beas-2B cells to AG1478. AG1478 is known to share the same structural backbone with getfitnib [263], and it is known to have the same biological function as getfitnib [264]. We performed the assay by using different concentrations of AG1478 (10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM) and measured cell viability. The overall trend of dose-response between the untreated and the 24-weeks arsenite treated cells was comparable (Fig. 11). Thus, there were no changes in sensitivity to AG1478 in the chronic arsenic-treated Beas-2B cells.

Chronic arsenite may prevent EGFR lysosomal degradation.

A previous study observed prevention of the EGFR degradation in response to acute arsenite exposure, and this phenomenon persisted with cyclohexamide treatment [265]. To determine whether the overexpression of EGFR is mediated by arsenite-induced prevention of the EGFR lysosomal degradation, we performed immunoblot on EGF-treated cells to measure the EGFR and pY1068 expression levels in the absence and presence of 24-weeks of arsenite exposure, and stimulated with 50 ng/mL of EGF with selected time points (0, 5, 10, 20, 40, 60 minutes) to analyze the EGFR protein degradation efficiency. We predicted, with time, we would observe prevention of EGFR degradation in arsenite-treated cells, as compared to the untreated cells. Although the

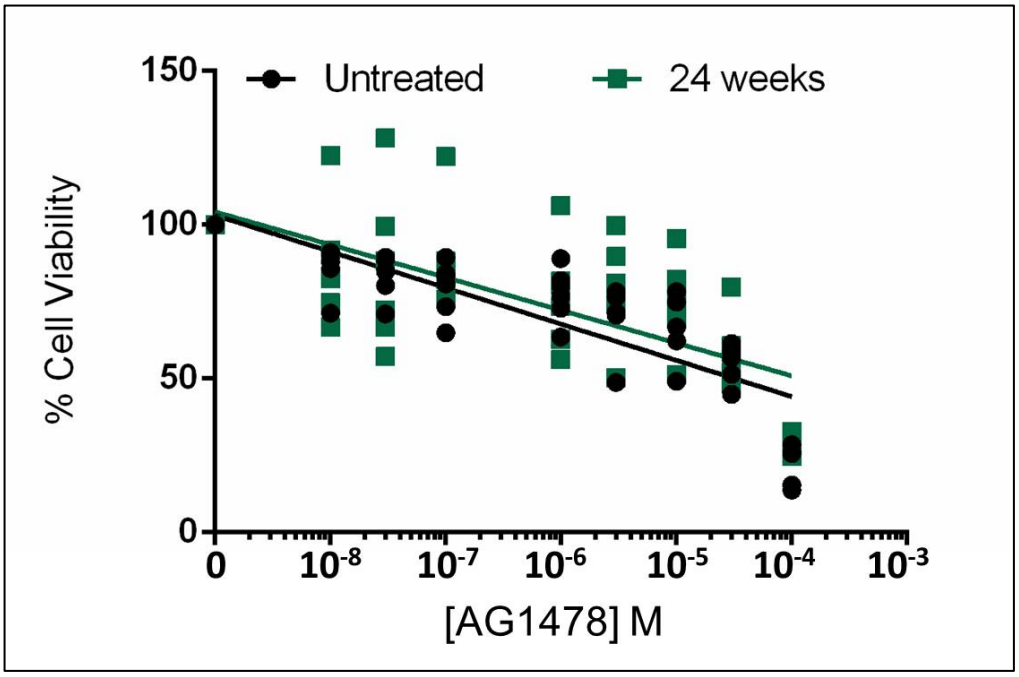

Figure 11. Chronic arsenite does not sensitize Beas-2B cells to tyrosine kinase inhibitor. Alamar Blue assay was performed to measure and compare the cell viability of 24 weeks arsenite-treated cells and the passage-matched untreated cells in response to different concentrations of AG1478 for 24 hours. (N=2)

kinetics of EGFR degradation were not altered with arsenite treatment, the overall protein expression levels of the EGFR and its activity were stronger in the arsenite-treated cells than in the untreated cells (Fig. 12).

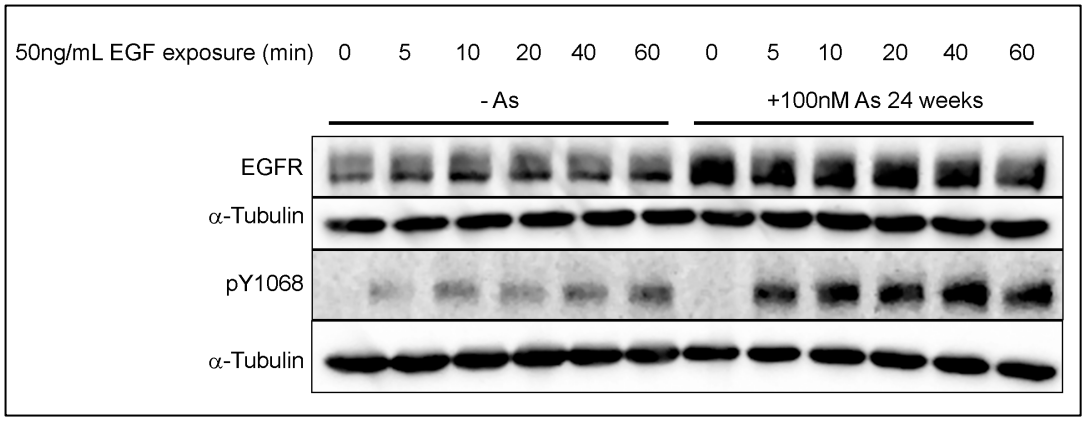

Figure 12. Chronic arsenite may prevent degradation of EGFR. Immunoblot was performed to measure any prevention in the EGFR degradation in response to 24-weeks of arsenic treatment. The EGFR and pY1068 expression levels were measured in cells that were grown with or without 100 nM of arsenite. Beas-2B cells were treated with 50 ng/mL of EGF for selected time points, after 2 hours of serum-starvation. The samples were resolved on 7.5% SDS-PAGE.

CHAPTER 4

DISCUSSION

The overall purpose of this work was to identify novel roles of chronic arsenite exposure in cancer development to mitigate arsenite toxicity. Acute high levels of arsenite-induced EGFR overexpression have been well-established based on previous studies and our own preliminary data [120, 160] (Fig. 3). In this thesis, we examined the effect of “a physiologically relevant” level of arsenite on the EGFR signaling. This study was achieved by first, performing immunoblot to support chronic arsenite exposure does increase EGFR expression levels and activity (Fig. 7A, B). Our results were consistent with previous studies that observed the activation of EGFR in response to chronic arsenite exposure [93, 119]. We also measured the expression levels and the activity of EGFR in response to AG1478 exposure to determine whether arsenite acts directly on the kinase domain to induce autophosphorylation of the EGFR. We observed decrease in EGFR expression and activity in both of the untreated and the arsenite-treated cells when the cells were exposed to AG1478 (Fig. 8). With 30 nM AG1478 treatment, a nearly complete inhibition of the EGFR activity (pY1068) was observed (Fig. 8), however, there is no clear explanation to decreased protein expression level of the EGFR in both untreated and arsenic-treated cells. One possible explanation is that AG1478 prevents activation of EGFR downstream signaling, resulting prevention of further EGFR synthesis.

Previous studies support a role of arsenite in inducing expression levels of protein and mRNA that are highly involved in the trafficking, such as TGFα [248] and Rab4 [249], respectively. As mentioned earlier, TGFα is an EGFR ligand that is involved

in the constitutive recycling of EGFR, which can delay the EGFR lysosomal degradation [181], resulting the overexpression of the receptors. Rab4, too, is a protein that is involved in the recycling of EGFR [249]. Additionally, acute high levels of arsenite exposure is known to prevent microtubule disassembly by losing the integrity of the tubulins and mircotubules [252], and microtubules are critical component in movement of proteins between the organelles [253]. As a result, the EGFR endocytic trafficking pathway could be a potential target site of chronic arsenite exposure to induce the overexpression of EGFR in the cells. We performed immunofluorescence to analyze any changes in the trafficking of EGFR in response to chronic arsenite exposure. Based on our immunofluorescence results, we observed alterations in the trafficking of EGFR, but it is unclear whether the receptors are localized on the cell surface or inside the cell. Further studies, such as confocal analysis and co-localization immunofluorescence, are needed to draw conclusions on their altered trafficking phenotype in response to chronic arsenic exposure. There are several possibilities; 1) chronic arsenite exposure may prevent the EGFR to transport from the ER-Golgi intermediate compartment (ERGIC) to the plasma membrane, 2) chronic arsenite exposure may prevent lysosomal degradation of EGFR by inducing accumulation of the EGFR in the early endosomes and allow constitutive downstream signaling, or 3) chronic arsenite may allow continuous recycling of the EGFR from the early endosomes to the plasma membrane by overexpressing the proteins that are involved in the recycling, such as Rab4. The possibility of EGFR accumulation in the ERGIC is likely, because chronic arsenic exposure may prevent EGFR endocytic transport by hindering microtubule disassembly, as a previous study has shown acute high levels of arsenic exposure prevent microtubule disassembly [252]. The second possibility is also likely, because a recent study from our laboratory observed signaling from the early endosomes [266]. Even when the EGFR are accumulated in the early endosomes, the receptors are able to emit signals and activate

the downstream effectors. This study also supports our observation of chronic arsenite exposure-induced overexpression of EGFR and strong EGFR activation at 60 minutes post-stimulation by EGF (Fig. 7, Fig. 12). Because previous studies have observed increased levels of proteins that are involved in recycling, the third possibility is highly likely as well, as we observed a similar results in our RT-qPCR data (Fig. 9). Overall, in response to chronic arsenite exposure, the route of EGFR trafficking was altered, and they were aggregated, which was significantly different from the untreated Beas-2B cells. However, further studies need to be performed to draw conclusions.

Another approach to identifying alterations in the endocytic trafficking of EGFR was to measure the mRNA levels of the ErbB family receptors as well as their ligands. We performed RT-qPCR and observed a significant increase in mRNA level of TGFα. The increase in TGFα was observed in an exposure time-dependent manner (Fig. 9). Though the actual protein level of TGFα is still yet to be measured, our data suggest that in response to chronic arsenite exposure, the cells try to make more of the ligand that is associated with the recycling of the EGFR, due to possible decreased cell surface expression of the EGFR. Though it may not be the predominant mechanism to chronic arsenite carcinogenicity, it is likely that an increase in TGFα mRNA levels will contribute to arsenite carcinogenicity, as increase in TGFα mRNA levels have shown to accelerate cell proliferation [248, 267].

To determine if there are any alterations in sensitivity to AG1478 in arsenite-treated Beas-2B cells, we performed Alamar Blue assay and measured the cell viability in response to different concentrations of AG1478. Because 24-weeks arsenite treated cells stimulated overexpression of EGFR, we expected the cells to have higher sensitivity to AG1478, as Schiff et al. have shown increased sensitivity to getfitnib in thyroid cancer cells that overexpress EGFR [149]. However, we observed a comparable trend of cell viability between 24-weeks arsenite treated cells and the untreated cells

(Fig. 11). Further studies are needed to make a concrete conclusion on their sensitivity to tyrosine kinase inhibitor.

Because Andrew et al. observed prevention of EGFR degradation in response to acute high levels of arsenite treatment [119], we investigated whether chronic exposure of “a physiologically relevant” level of arsenite treatment prevents the EGFR degradation in a similar manner. Under normal physiological conditions, EGF-induced EGFR activation increases significantly within 5 minutes, and diminishes within an hour. Although we did not observe a clear decrease in the EGFR degradation in response to chronic arsenite treatment, the overall protein expression levels of the EGFR and activity in the arsenite-treated cells were stronger than the untreated cells (Fig. 12). This incomplete EGFR degradation suggests we will need to select longer time points for EGF treatment to observe a nearly complete degradation of EGFR. The present work is summarized in a schematic diagram in Figure 13.

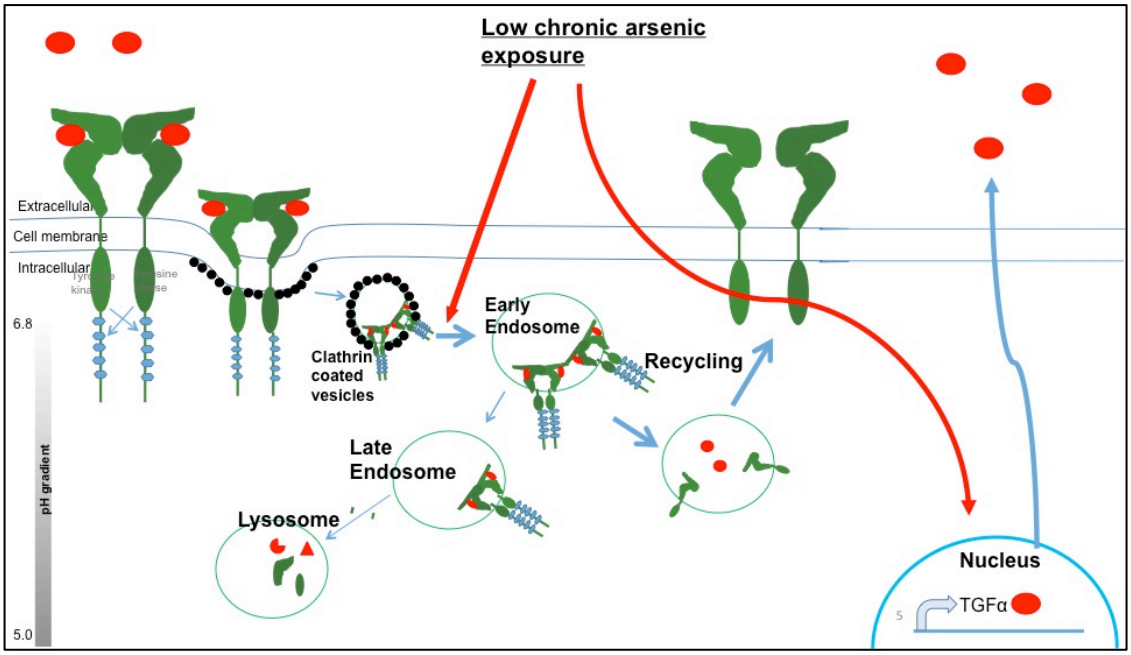

Figure 13. The schematic diagram of the possible mechanisms of chronic arsenite-induced carcinogenesis. Chronic exposure of "a physiologically relevant" level of arsenite alters the endocytic trafficking of EGFR via constitutive recycling of the internalized EGFR, leading to overexpression of EGFR on the surface of the cell. Another possible mechanism of chronic arsenite-induced overexpression of EGFR is chronic arsenite-induced increase in induction of TGFα synthesis.

CHAPTER 5

FUTURE STUDIES

This thesis contributes in identifying novel roles and targets of chronic arsenite exposure. To determine whether chronic exposure to “a physiologically relevant” level of arsenite alter the EGFR endocytic trafficking pathway, we performed immunofluorescence. We observed altered EGFR distribution in cells that were grown in arsenite for 24 weeks, as compared to the untreated cells, but we were not able to conclude the exact location of the EGFR. To understand the underlying mechanism behind this phenomenon, the EGFR co-localization with other organelles, such as the ER and Golgi apparatus, or with other endocytic compartment, such as the early endosomes, will be needed to identify which step(s) of the EGFR endocytic trafficking are being inhibited by chronic arsenite exposure.

In order to draw conclusions from RT-qPCR analysis, we would need to analyze other ErbB family receptors and their ligands, and repeat the experiments with other human bronchial epithelial cell lines, such as HBEC. We observed a significant increase in TGFα mRNA levels in an exposure time-dependent manner. After we determine a consistent increase in the protein expression level of TGFα and cell surface EGFR localization, we will knockout TGFα by transfection of its shRNA to the cells and observe its phenotypes, such as proliferation efficiency and anchorage-independent growth, to determine contribution of increased level of TGFα in arsenite-induced carcinogenicity. If we do not observe a consistent increase in TGFα protein expression level and observe less cell surface EGFR localization, it is possible that chronic arsenite-treated cells may be upregulating TGFα mRNA level to bring the EGFR to the plasma membrane due to

chronic arsenite-induced accumulation of EGFR in the endocytic compartments. If this were the case, we will introduce exogenous TGFα to the chronic arsenite-treated cells and determine if TGFα mRNA level is still being induced. Once this has been completed, we will then be able to determine the significance of TGFα role in an arsenite-induced carcinogenesis. An inconsistent changes in the protein levels of TGFα can be explained by chronic arsenite exposure affecting the expression of proteins that control ligand processing and ligand:receptor down-regulation. By analyzing expression levels of proteins involved in ligand processing, such as ADAMs (A Disintegrin And Metalloproteinase) [268], we will be able to refine our model and identify appropriate molecular mechanisms. Also, it is possible that TGFα mRNA level is not consistent with the protein level due to non-coding RNA, such as miRNA, thus we will investigate miRNA that are involved in TGFα regulation. Partial complementarity between miRNA and the target mRNA can decrease protein expression level without decreasing the mRNA level. It should be noted that there was no observed change in EGFR mRNA level in response to chronic arsenite exposure. This phenomenon can be due to 1) arsenite-induced increased translation of EGFR, 2) arsenite-induced decreased EGFR degradation, or 3) combination of both mechanisms. To elucidate whether arsenite-induced overexpression of EGFR is due to prevention of EGFR degradation, we will perform immunoblot analysis using cyclohexamide to measure EGFR degradation efficiency in response to chronic arsenic exposure.

Furthermore, as mentioned earlier, the interplay between clathrin-mediated endocytosis and caveolin-mediated endocytosis controls the expression of EGFR in the cells to prevent overstimulation, as caveolin-mediated endocytosis preferentially couples to EGFR degradation [251]. In our study, we observe overexpression of EGFR in response to chronic arsenite exposure, this suggests a possible role of arsenite in dysregulating the interplay between the two distinct endocytic trafficking pathways,

contributing to arsenite-induced carcinogenesis by allowing overstimulation of the EGFR signaling. Ironically, however, an earlier study showed sub-chronic high levels of arsenite increases caveolin protein expression level in liver sinusoidal endothelial cells [269]. If we observe increase in caveolin levels in cells treated with low levels of chronic arsenite exposure, we will, then, use chloroquine (CHQ), lysosomal inhibitor, to assess relationship between caveolin expression and chronic arsenite-induced EGFR overexpression. If we observe a greater EGFR overexpression in cells that are co-treated with arsenite and CHQ, as compared to cells with arsenite treatment only, this will suggest chronic arsenite-induced caveolin-mediated endocytosed EGFR are being degraded via lysosomes, suggesting chronic arsenite does not cause accumulation of the EGFR in the cells by disrupting the interplay between the two distinct endocytic trafficking pathways. Alternatively, if we observe comparable levels of the EGFR expression with CHQ, as compared to the cells with arsenite treatment only, this suggests caveolin-mediated endocytosed EGFR are not being degraded via lysosomes, and this could further lead to a possible perturbed degradation pathway.

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HONORS & AWARDS

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PRESENTATIONS

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RESEARCH EXPERIENCE

University of Louisville, Louisville, KY — August 2016-present
Graduate Fellow
• EGFR dependency in arsenic-induced transformation of non-malignant human bronchial epithelial cells.
• Arsenic regulation the synthesis of ErbB receptors and their ligands.
• EGFR trafficking in arsenic-transformed cells.

University of Kentucky, Lexington, KY — August 2012-June 2015
Graduate Fellow
• Histone modification of H3K36 by cigarette smoking condensate.
• The impact of cigarette smoking condensate on chromatin assembly by histone modification.

Purdue University, West Lafayette, IN — Summer 2009-May 2011
Laboratory Technician
• Investigate activated Cdc42 kinase regulation on Dock localization in male germ cells during Drosophila spermatogenesis.

GRANTS/FELLOWSHIPS

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Title: Assessing the Role of Arsenic in EGFR Signaling Axis.
Goal: To determine the impact of chronic arsenic in EGFR signaling axis.

PUBLICATIONS

• Tong, D., Ortega, J., **Kim, C.,** Huang, J., Gu, L., Li, GM. (2015) Arsenic inhibits DNA mismatch repair by promoting EGFR expression and PCNA phosphorylation. *J.Biol.Chem*. PMID: 25907674

• Abdallah, A.M., Zhou, X., **Kim, C**., Shah, K.K., Hogden, C., Schoenherr, J.A., Clemen, J.C., Chang, H.C. (2013) Activated Cdc42 kinase regulates Dock localization in male germ cells during Drosophila spermatogenesis. *Dev. Biol.* 378, 141-153. PMID: 23562806