METABOLISM REPROGRAMMING IN HEXAVALENT CHROMIUM-INDUCED HUMAN LUNG CARCINOGENESIS

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METABOLISM REPROGRAMMING IN HEXAVALENT CHROMIUM-INDUCED HUMAN LUNG CARCINOGENESIS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

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2019

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ABSTRACT OF DISSERTATION

METABOLISM REPROGRAMMING IN HEXAVALENT CHROMIUM-INDUCED HUMAN LUNG CARCINOGENESIS

Hexavalent chromium, Cr(VI), is an established human carcinogen that is a worldwide environmental health concern. It is well understood that reactive oxygen species, genomic instability, and DNA damage repair deficiency are important contributors to Cr(VI)-induced carcinogenesis. After decades of research some cancer hallmarks remain understudied for the mechanism of Cr(VI) carcinogenesis. Dysregulated cellular energetics have been established as a hallmark of cancer. Energy pathways that become dysregulated in cancer include mitochondrial respiration, lipogenesis, pentose phosphate pathway, one carbon metabolism, and increased anaerobic glycolysis in the presence of oxygen or ‘Warburg effect’.

To investigate metabolism changes in Cr(VI) carcinogenesis, we exposed human lung epithelial cells (BEAS-2B cells) to Cr(VI) for six months and isolated a colony from soft agar. To confirm the results in the BEAS-2B cells, we used two other sets of Cr(VI)-transformed cells, human lung epithelial cells (BEP2D cells) and human lung fibroblasts (WTHBF-6 cells).

We found increased lipogenesis related protein expressions including: ATP citrate lyase (ACLY), acetyl-CoA carboxylase 1 (ACC1), and fatty acid synthase (FASN) in Cr(VI)-transformed cells as compared to passage-matched control cells. We also observed increased palmitic acid levels, confirming that Cr(VI)-transformed cells were making more lipids. Cr(VI)-transformed BEAS-2B cells had decreased colony formation in soft agar and decreased cell growth when treated with a FASN inhibitor (C75). ACLY, ACC1, and FASN protein expressions were also increased in chromate-induced lung tumors in human tissue samples.

We also observed that Cr(VI)-transformed human lung cells (BEAS-2B, BEP2D, and WTHBF-6 cells) had no major changes in their mitochondrial respiration as measured by the Seahorse Analyzer when compared to their passage-matched control cells. Conversely, xenograft tumor-derived cells had mitochondrial respiratory dysfunction.

Interestingly, we also found that Cr(VI)-transformed human lung cells (BEAS-2B, BEP2D, and WTHBF-6 cells) had no major changes in their glycolytic function as measured by the Seahorse Analyzer when compared to their passage-matched control cells. Similarly, these cells did not have changes in glycolytic enzymes or extracellular L-lactate.
levels. Moreover, xenograft tumor-derived cells showed no changes in glycolytic endpoints or L-lactate levels. This indicates these cells did not undergo the ‘Warburg effect’.

These data demonstrate that increased lipogenesis is important to Cr(VI)-induced lung carcinogenesis and are consistent with the cancer literature which reports that increased lipogenesis proteins occur during carcinogenesis. Additionally, our results indicate mitochondrial respiratory dysfunction is likely a result of the tumor microenvironment and a later step during Cr(VI) carcinogenesis. Lastly, we observed the ‘Warburg effect’ is not required for Cr(VI)-induced carcinogenesis in vitro. However, it remains to be shown if the ‘Warburg effect’ is still a consequence or contributing factor for tumorigenesis. Future studies are needed to investigate other metabolic pathways in Cr(VI)-induced carcinogenesis. In conclusion, some metabolism pathways are important to Cr(VI)-induced carcinogenesis, while others appear not to be.

KEYWORDS: Cellular Energetics, Hexavalent Chromium, Lung Cancer, Lipogenesis, “Warburg effect”.

James Tate Fortin Wise

02/22/2019
Date
METABOLISM REPROGRAMMING IN HEXAVALENT CHROMIUM-INDUCED HUMAN LUNG CARCINOGENESIS

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02/22/2019
Date
I would like to dedicate this to my grandparents, Jane M. Fortin, Roger T. Fortin, Kathleen Harrigan, Steve Seekins, and Francis Wise, Ph.D. Each has had their own battle with cancer. I would also like to dedicate this to two of my uncles, Franklin D. Wise and Mark C. Wise, both who passed during my time as a Ph.D. Student.
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Chapter 1 Introduction

1.1. Overview

Chromium is a naturally occurring element found ubiquitously worldwide. It was first discovered by Louis-Nicholas Vauquelin in Siberian red lead (crocoite) in 1797. Chromium is a transition metal found in group 6 of the periodic table. It has a ground state electron configuration of \([\text{Ar}]3d^54s^1\) and its abbreviation is \(\text{Cr}\), and has an atomic number of 24 with an atomic mass of 51.9961 (IARC, 1990). Chromium occurs in various valence states, with the most common and stable being \(\text{Cr}(0)\), \(\text{Cr}(\text{III})\), and \(\text{Cr}(\text{VI})\). \(\text{Cr}(0)\), or metallic chromium, is a steel-gray solid that has a high melting point and is used to make steel and other metal alloys. There are other unstable valance states that occur in biological systems [e.g., \(\text{Cr}(\text{IV})\) and \(\text{Cr}(\text{V})\)]. \(\text{Cr}(\text{III})\) was considered for a long time as an essential element but has recently been deemed by the European Union and experts in the field as non-essential and subsequently has been removed from the United States Dietary Associations’ 2015-2020 dietary guidelines (USDA, 2017; Vincent, 2010;). Trivalent chromium is generally considered safe at pharmacological and dietary exposure levels (Vincent, 201; Wise et al., 2018b). Meanwhile, \(\text{Cr}(\text{VI})\) is an established human and animal carcinogen and is the most toxic form of \(\text{Cr}\).

The first suspected documented case of \(\text{Cr}(\text{VI})\)-induced cancer was reported in 1890, a \(\text{Cr}(\text{VI})\)-pigment worker was diagnosed with adenocarcinoma of the nasal turbinate bone (Newman, 1891). Following this finding, there have been countless studies demonstrating a correlation between occupational exposures to \(\text{Cr}(\text{VI})\) and cancers. Most of these reported cancers being lung and nasopharyngeal in origin, suggesting that inhalation is the primary route of exposure (Wise et al., 2008). Furthermore, epidemiologic studies of chromate-exposed workers have estimated a 2 to 80-fold increased risk in
respiratory system cancers (Langard, 1990; Leonard and Lauwerys, 1980; OSHA, 2006). In conjunction with these studies, animal studies have supported the epidemiologic data, and further implicated Cr(VI) in respiratory cancers (IARC, 2012; Levy 1986a, 1986b).

Cr(VI) found in the environment is largely due to industrial activity and it is considered stable when there are no available reducing agents. Most of the serious adverse health effects documented for chromium are attributed to its hexavalent form as other valence states, namely Cr(III), have not been shown to be carcinogenic; however, the latter has shown some negative health effects, including contact dermatitis and kidney and liver damage (EPA, 1984; NIOSH, 2006).

Cr(VI) is the most potent form of chromium due to its ability to be transported through the cell membrane, Cr(III) is unable to penetrate the cell membrane and has poor passive absorption. Once inside the cell Cr(VI) is rapidly reduced to Cr(III) while the extracellular Cr(VI) is slowly reduced and contributes to a chronic exposure to Cr(VI). Prolonged exposure to Cr(VI) is key to its toxic and carcinogenic effects (Wise et al., 2008). It has been established that either chronic or repeated prolonged exposures to Cr(VI) leads to neoplastic transformation (Kim et al., 2015; Kim et al., 2016; Wise J et al., 2018a; Wise S et al., 2018). Additional changes include: redox imbalances, imbalance of numerous proteins, growth signal changes, and DNA repair inhibition that results in chromosome instability (Wise S et al., 2018). We hypothesize that Cr(VI)-transformed cells will have cancer cell properties (i.e., dysregulated energy metabolism).

Here we summarize the significance of chromium exposure and the exposed populations. We also discuss the known physico-chemical mechanisms of Cr(VI) exposure which contribute to its carcinogenesis. Next, we discuss the current models of Cr(VI)-
induced carcinogenesis. Then we discuss dysregulated energy metabolism in cancer. Lastly, we discuss how dysregulated energy metabolism may have a role in Cr(VI)-induced lung carcinogenesis.

1.2. Chromium Exposure

Chromium has several unique properties that make it a unique metal and thus when addressing the exposure of Cr(VI) it is important to understand its complexity. It is also important to understand the relevance of this complexity for exposed human populations.

1.2.1 Chromium is an Important Element

Chromium is a common abundant element found in the Earth’s crust. Elemental chromium is a steel grey, hard metal with a high melting point. Since chromium is highly resistant to corrosion and heat, it is widely used in the metallurgic industry. The name chromium is derived from the Greek word “chroma”, meaning color. When chromium is combined with other chemicals it can produce compounds of a variety of colors, making these compounds highly attractive for producing pigments and dyes.

Chromium can exist in oxidation states from -2 to +6 (ATSDR, 2012). The most commonly occurring stable oxidation states are 0, 3, and 6. Other states are occasionally found but, are considered highly unstable and rapidly oxidized or reduced to more stable states. In the natural environment, pH and oxidative states of the environment (soil and water) will greatly influence which species of chromium will predominate. The bulk of Cr(VI) found readily available in the environment is from anthropogenic causes (ATSDR, 2012).
The major uses for Cr(VI) include chemical industries, metallurgic, refractory, and the leading use is in stainless steel. Stainless steel consists of 12 to 30 percent chromium (the higher quality of stainless correlates with more chromium). Other major uses include anticorrosive applications, chrome plating, leather tanning, paints, and dyes (including tattoo ink), as well as a wood preservative. Table 1.1 provides a list of some of the chromium compounds and their major uses.
Table 1.1 Uses of Chromium Compounds

<table>
<thead>
<tr>
<th>Activity/Use</th>
<th>Type of Chromium Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromate production and smelting</td>
<td>Sodium, potassium, calcium, and ammonium chromates and dichromates; alkaline chromates, chromic sulfate, sodium chromate, sodium dichromate</td>
</tr>
<tr>
<td>Chrome plating</td>
<td>Chromium trioxide, chromic chloride, chromic sulfate</td>
</tr>
<tr>
<td>Pigment production and use</td>
<td>Zinc chromate, lead chromate, chromic hydroxide, chromic acid, chromic oxide, chromic phosphate, calcium chromate, chromium trioxide, potassium dichromate, sodium chromate, barium chromate, strontium chromate</td>
</tr>
<tr>
<td>Corrosion inhibition</td>
<td>Calcium chromate, chromic oxide, chromic nitrate, chromic chloride, chromium trioxide, barium chromate, strontium chromate, zinc chromate</td>
</tr>
<tr>
<td>Stainless steel and alloy production</td>
<td>Metallic chromium, cobalt chromium, ferrochromium, chromic oxide, strontium chromate</td>
</tr>
<tr>
<td>Welding</td>
<td>Alkaline chromates, chromic oxide</td>
</tr>
<tr>
<td>Tanning</td>
<td>Chromic acetate, chromic hydroxide, chromic sulfate, potassium chromic sulfate, ammonium dichromate, sodium chromate, sodium dichromate</td>
</tr>
<tr>
<td>Dyeing</td>
<td>Chromic acid, potassium chromic sulfate, ammonium dichromate, potassium chromate, potassium dichromate, sodium chromate, sodium dichromate</td>
</tr>
</tbody>
</table>

Adapted from IARC, 1990
1.2.2. Chromium Exposure: Who is Exposed?

1.2.2.1. Occupational Exposure

Chromate ore is used to produce hexavalent chromium for industrial purposes. Around 14 million tons of chromate ore are mined yearly, with the majority of chrome ore mined in India, Kazakhstan, Russia South Africa, and Turkey (IARC, 1990; Wise et al., 2008). The production method affects the final form for Cr(VI). For example, the final product can be primarily insoluble Cr(VI) when using lime in production or soluble Cr(VI) when using soda ash in production.

Occupational exposures to Cr(VI) is of major health concern and is the top source of high dosage human exposure. The main routes of exposure are dermal contact and inhalation. Recently ingestion has become an exposure concern, given the prevalence in drinking water of Cr(VI). It is estimated that workers in industries that involved Cr(VI) typically have exposure rates that are two orders of magnitude higher than the general population (ATSDR, 2012). OSHA (2006) estimates that approximately 558,000 U.S. workers are exposed to Cr(VI). Table 1.2 is a list of occupations in which workers may be exposed to Cr(VI).
<table>
<thead>
<tr>
<th>Occupation</th>
<th>Potential Chromium Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abrasives manufacturers</td>
<td></td>
</tr>
<tr>
<td>Acetylene purifiers</td>
<td></td>
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<tr>
<td>Adhesives workers</td>
<td></td>
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<tr>
<td>Aircraft sprayers</td>
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<tr>
<td>Alizarin manufacturers</td>
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<tr>
<td>Alloy manufacturers</td>
<td></td>
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<tr>
<td>Aluminum anodizers</td>
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<tr>
<td>Anodizers</td>
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<tr>
<td>Battery manufacturers</td>
<td></td>
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<tr>
<td>Biologists</td>
<td></td>
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<td>Blueprint manufacturers</td>
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<tr>
<td>Boiler scalers</td>
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<tr>
<td>Candle manufacturers</td>
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<tr>
<td>Cement workers</td>
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<tr>
<td>Ceramic workers</td>
<td></td>
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<tr>
<td>Chemical workers</td>
<td></td>
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<tr>
<td>Chromate workers</td>
<td></td>
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<tr>
<td>Chromium-alloy workers</td>
<td></td>
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<tr>
<td>Chromium-alum workers</td>
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<tr>
<td>Chromium platers</td>
<td></td>
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<tr>
<td>Copper etchers</td>
<td></td>
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<tr>
<td>Copper-plate strippers</td>
<td></td>
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<tr>
<td>Corrosion-inhibitor workers</td>
<td></td>
</tr>
<tr>
<td>Crayon manufacturers</td>
<td></td>
</tr>
<tr>
<td>Diesel locomotive repairmen</td>
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<tr>
<td>Drug manufacturers</td>
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<tr>
<td>Dye manufacturers</td>
<td></td>
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<tr>
<td>Dyers</td>
<td></td>
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<tr>
<td>Electroplaters</td>
<td></td>
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<tr>
<td>Enamel workers</td>
<td></td>
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<tr>
<td>Explosives manufacturers</td>
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<tr>
<td>Fat purifiers</td>
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<td>Fireworks manufacturers</td>
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<tr>
<td>Flypaper manufacturers</td>
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<tr>
<td>Furniture polishers</td>
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<tr>
<td>Fur processors</td>
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<tr>
<td>Glass-fiber manufacturers</td>
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<td>Glass frosters</td>
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<td>Glass manufacturers</td>
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<tr>
<td>Glue manufacturers</td>
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<tr>
<td>Histology technicians</td>
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</tbody>
</table>

Adapted from IARC, 1990
In occupational settings, airborne Cr(VI) concentrations can range from 5 µg/m³ to 600 µg/m³ depending on the industry (Stern, 1982). Occupational setting levels are significantly higher than the Cr concentrations in ambient air in industrial cities which generally ranges from 0.01 to 0.03 µg/m³ (Fishbein, 1981). Blood levels of chromium in occupationally exposed workers are also increased and chromium lung burdens are higher (Gerhardsson et al., 1988; McAughey et al., 1988). It has been reported that workers at a British pigment factory exposed to strontium and lead chromates had chromium levels in their blood from 3-216 µg/L, while in non-occupationally exposed workers chromium levels were less than 1 µg/L (McAughey et al. 1988). The chromium lung burden was higher in occupationally exposed workers was 2.3 to 4 times higher than both urban and rural residents (Gerhardsson et al., 1988). Given the high level of health effects (including lung cancer), in 2006, the National Institute for Occupational Safety and Health (NIOSH) lowered the permissible exposure limit in ambient air for an 8 hour period from 52 µg/m³ to 5 µg/m³ Cr(VI). Still, studies report that industrial air Cr(VI) concentrations frequently exceed the occupational exposure limits (Blade et al., 2007). Therefore, despite permissible limits being set, workers in industries using Cr(VI) compounds are often exposed to high levels of Cr(VI) and are still at a higher risk for developing adverse health effects.

1.2.2.2. Exposure in the General Public

Cr(VI) mainly enters the environment from anthropogenic sources. The anthropogenic sources of Cr(VI) include industrial waste streams from electroplating, leather tanning, and textile industries, and factories making dyes and pigments. Typically, Cr(VI) is in soils as a result of the disposal of commercial products containing chromium, coal ash from electric utilities, and chromium waste from industry. Cr(VI)
generally enters the air largely as particulate from the manufacturing and use of metal chromates. Cr(VI) can also enter the environment in lesser amounts from cement production, the combustion of fossil fuels, industrial cooling towers, and municipal incinerators. Chromium has been found in at least 1,127 of the 1,699 hazardous waste sites listed on the EPA’s National Priorities List (ATSDR, 2012).

The majority of high-dose chromium exposures occurs in the industrial settings, however, there is still a risk to the general populations. The general population is exposed to chromium via inhalation or ingestion of contaminated food and water. Levels of Cr(VI) in food are generally lower (IARC 2012). Interestingly, in salt water environments, chromium predominates as Cr(VI). Thus, given this and that chromium bioaccumulates in marine organisms, it is possible that chromium exposure may also occur due to consumption of seafood containing a high dose of bioaccumulated chromium. Air quality values of Cr(VI) for U.S. urban and nonurban areas were reported to be 5-525 ng/m³ (ATSDR 2012). More precise levels for the different regions of the United States can be found from the Environmental Protection Agency (EPA).

1.2.2.2.1 Water

Chromium predominates as Cr(VI) in the public drinking water systems (Moffat et al., 2018; Zhitkovich 2011). Members of the general population that reside near industrial facilities and waste sites will have a higher risk of exposure. A well-known example of chromium exposure to a general human population, is Hinkley, California, where the legal case against Pacific Gas and Electric Company’s contamination of the drinking water was documented in the movie Erin Brockovich. Another example is Hudson County, New Jersey, where it is estimated that 2 million tons of industrial waste were produced by local chromium industries (ATSDR, 2012).
has been documented as a contributor to occasional blooms of particulate Cr(VI) on the ground’s surface. Given that Cr(III) can be oxidized by chlorine to Cr(VI), it is possible that Cr(III) contamination of drinking water sources can result in Cr(VI) in the water supply (Zhitkovich, 2011). The level of chromium in U.S. freshwaters ranges from <1-30 μg/L and U.S. drinking water contains total chromium levels of 0.2–35 μg/L (ATSDR 2012). In California (CA) drinking water supplies, it was reported that 86% of the sources tested reported levels of Cr(VI) below 10 μg/L (Moffat et al., 2018). A recent epidemiological report of individuals living near industrial waste sites in CA has shown lasting reproductive and developmental impacts on the non-occupationally exposed population (Remy et al., 2017).

1.2.2.2 Air

Roughly 60-70% of the atmospheric chromium emissions are from human activities (ATSDR, 2012). Sources of chromium contamination in the air can be from industrial processes and waster, municipal water incineration, cement dusts, cigarette smoke, and e-cigarettes, and brake lining dust (ATSDR, 2012; Farsalions and Rodu, 2018). Cigarette smoke can significantly increase chromium levels in indoor air. More data are still needed to determine the public significance of environmental Cr(VI) exposure as the likelihood of adverse health effects are not limited to occupational exposures.

Roughly 2,700 to 2,900 tons of chromium is released into the atmosphere in the U.S. and about a third can be hexavalent (ATSDR, 2012). Total chromium in ambient air in the U.S. is estimated to be 0.005 to 0.525 μg/m³ (ATSDR, 2012) while the concentrations in ambient air within the vicinity of ferrochrome industries can be 10 to 100-fold higher. Cr(VI) particles half-life in the atmosphere varies on the particle size
and density. The smaller, more aerodynamic particles (less than 10 µm) remain longer in the air (ATSDR, 2012). These smaller particles are also potentially more likely to negatively impact human health due to this smaller size enabling them to pass through the larger areas of the lung and nose. The particles can then deposit and possibly accumulate in the bifurcation sites within the lung rodents (Speer and Wise, 2018; Wise et al., 2008).

Some urban cities are known to have high levels of fine particulate matter (PM$_{2.5}$), which may contain chromium, specifically in Mexico City. Residents in this city are exposed to year-round air pollution concentrations above the United States’ National Air Ambient Air Quality Standards. (1) In a study by Calderón-Garcidueñas et al. (2013), the lungs of polluted air-exposed young adults of Mexico City contained chromium equivalent to 2524 ± 581 µg/g dry weight whereas in the control reference lungs it was 368 ± 179 µg/g dry weight. In the same study, the frontal lobe of the brain had chromium levels of 910 ± 63 µg/g dry in adults and 527 ± 85 µg/g dry weight in the reference individuals. The chromium levels in the lungs of the Mexico City adults was higher than the study’s control group was not statistically significant, but the levels in the brains were. (2) However, chromium levels were higher in both the control and high exposed young adults than those reported in chromate workers (21.3 µg/g wet weight) (Tsuneta et al., 1980). (3) Adjusting for wet weight versus dry weight (assuming a 75% moisture), the adjusted mean values for the Mexico City residents is 3367 µg/g wet weight and the reference residents is 1824 µg/g wet weight and the chromate workers levels would be 15.98 µg/g dry weight. Thus, these values show us that the Mexico City residents had a 150 to 160-fold increase in chromium levels compared to workers that died of chromate
induced lung tumors, while the fold increase for the Mexico City resident paper was 85-115 times higher than in chromate workers. While the authors did not measure any known health effects from Cr(VI), these data are important as it shows that urbanites exposed to particulate matter in the city can accumulate chromium in their tissues and environmental exposures may be of health concerns.

1.3. Chromium is a Human Carcinogen

Multiple regulatory agencies have classified Cr(VI) as a human carcinogen. The International Agency for Research on Cancer (IARC) lists Cr(VI) as a human carcinogen. IARC listing is based on sufficient evidence from humans and animals studies on the carcinogenicity of Cr(VI) compounds (IARC, 2012). More specifically, for humans, the risk of respiratory cancers is high for workers in chromate production, chromate pigment production, and chromium plating industries. The EPA lists airborne Cr(VI) as a Group A or a human carcinogen via inhalation exposure (EPA, 1984). Furthermore, the Agency for Toxic Substances and Disease Registry (ATSDR) states that occupational exposure to Cr(VI) compounds are associated with increased incidences of lung cancers (ATSDR, 2000). Lastly, the NIOSH considers that all Cr(VI) compounds are potential occupational carcinogens (NIOSH, 2006).

Most of the epidemiological studies have implicated Cr(VI) as a respiratory tract carcinogen and some studies provide evidence to suggest that Cr(VI) may also play a role in other cancers. Chromium-exposed workers have been reported to have higher incidences of digestive tract cancer, but these results were not statically significant (Enterline, 1974; Machle and Gregorius, 1948; Royle, 1975; Sheffet et al., 1982). Recent data have indicated that Cr(VI) exposure in drinking water can cause
gastrointestinal carcinomas in mice and rats (Moffat et al., 2018; NTP, 2008; Stout et al., 2009). Although there is no data on these cancer types in Cr(VI) contaminated drinking water human exposed population, studies indicate Cr(VI) may cause gastrointestinal tract cancers. Interestingly, oral exposure to Cr(VI) in combination with UV exposure increases the levels of chromium in the skin and increases skin carcinogenesis (Davidson et al., 2004; Uddin et al., 2007). Yet, Cr(VI) effects on the respiratory system remain the primary health concern and thus studies focus on Cr(VI) role as a human lung carcinogen. While, chromium has been found above permissible limits in tattoo inks, it remains unclear if there is a higher incidence in skin cancer rates of individuals with tattoos (Bocca et al., 2018).

1.3.1. Evidence in Chromium Exposed Workers

The first reported epidemiologic studies in Cr(VI)-exposed workers was in 1948. Table 1.3 highlights some early epidemiologic studies in Cr(VI)-exposed workers in the U.S. The 1948 study reported that 22 percent of the chromate production workers died of respiratory cancers compared to less than 2 percent of an unexposed reference population. These chromate workers had an 18 to 50-fold increased risk of death due to lung cancer (Machle and Gregorius, 1948). A similar study reported lung cancer death rates among chromate workers were 15 times higher than the general population (Mancuso, 1951). Subsequent studies have provided supporting data showing the insoluble Cr(VI) compounds produced from the high lime production process are more carcinogenic than the soluble Cr(VI) forms (Alderson et al., 1981; Baetjer, 1950; Hayes et al, 1979; NIOSH, 2006).

The chromate pigment production industry also has a high rate of occupational
exposures to chromium. These industries rely heavily on the use of insoluble forms of Cr(VI), for example, lead chromate and zinc chromate. Epidemiological studies of chromate pigment workers have showed similarly high lung cancer risk as those works in the chromate production industry (Langard, 1990; NIOSH, 2006). The first chromate pigment epidemiologic study calculated a risk ratio of 38 in exposed workers (Langard and Norseth, 1975). Epidemiological studies from countries other than the U.S. have also shown a two- to three-fold increased risk of developing respiratory cancer in chromate workers (Davies, 1979, 1984; Frentzel-Beyme, 1983; Sheffet et al., 1982). Additionally, chronic exposure in the pigment industry is associated with a higher risk of lung cancers (Hayes et al., 1989).

Recent epidemiological studies reported an improvement in the risks posed to chromate workers. This is due to the increased hazard awareness and improved protection procedures for those at highest risk; however, the risk remains elevated compared to control populations (Luippold et al., 2003; Mancuso, 1997; Proctor et al., 2016). A key factor to the observed decline of lung cancers in chromate workers is due to the elimination or reduction of lime usage during the production process. In 2006, the United States’ Occupational Safety and Health Admiration (OSHA) reduced their 8 h permissible exposure limits (PEL) from 52 µg/cm³ to 5.0 µg/cm³ (OSHA, 2009) and thus future studies may show an additional reduction in respiratory cancers in chromate-related employments.
Table 1.3 List of Chromate Epidemiology Studies in the United States

<table>
<thead>
<tr>
<th>Study Population</th>
<th>Years of Exposure</th>
<th>Reference Population</th>
<th>Major Endpoints</th>
<th>No. of Cases</th>
<th>Estimated Relative Risk</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 chromate plants; 156 deaths</td>
<td>4-17 years before 1948</td>
<td>Oil refining company</td>
<td>bronchial and lung cancers</td>
<td>32</td>
<td>26</td>
<td>Machle and Gregorius, 1948</td>
</tr>
<tr>
<td>7 United States chromate factories</td>
<td>1940-1948</td>
<td>US male; white and black</td>
<td>Lung cancer</td>
<td>10</td>
<td>14.3</td>
<td>Galafer, 1953</td>
</tr>
</tbody>
</table>
1.3.2. Evidence from Animal Studies

Many animal studies have assessed the carcinogenicity of chromium and these studies support the epidemiological data. These studies indicate that Cr(VI) is the cancer-causing form of chromium. Furthermore, these studies support that the more potent carcinogenic forms of Cr(VI) are the insoluble forms (IARC, 1990). Inhalation, intrabronchial instillation or implantation exposures of calcium chromate in mice and rats induced lung tumors (Nettesheim et al., 1971; Steinhoff et al., 1986; Levy 1986a, 1986b). Chromium trioxide as a mist induced tumors in mice (Adachi et al., 1986); the same result was seen with inhalation and intratracheal administrations (Adachi et al., 1986; Levy 1986a, 1986b). Intrabronchial implantation of either lead chromate or strontium chromate led to lung tumors in rats (Levy 1986b). Other insoluble forms of chromium, for example, sodium dichromate, have been shown to induce lung tumors in animals (Glaser et al., 1986; Steinhoff et al., 1986).

1.3.3 Evidence in Cell Culture

Numerous cell culture studies have provided invaluable data for the mechanisms of Cr(VI)-induced carcinogenesis. Cellular transformation studies also support the epidemiologic and animal studies and further demonstrate that the particulate forms of Cr(VI) are the more potent carcinogenic form of Cr(VI) (Patierno et al., 1988; Elias et al., 1989; IARC, 1990). Cell culture studies demonstrated that extracellular dissolution of Cr(VI) particles is important for the genotoxic effects of chromium (Wise et al., 2004a). Cell culture studies in human cell lines also showed that acute/prolonged (less than 120 h) high dose exposures or chronic (6 months) environmentally relevant dose
exposures can induce neoplastic transformation (Kim et al., 2015; Pratheeshkumar et al., 2016; Wise J et al., 2018a; Wise S et al., 2018; Xie et al., 2007).

1.4. Physico-Chemical Factors in Cr(VI) Carcinogenesis

1.4.1. The Role of Valence

The carcinogenic potency differences between Cr(III) and Cr(VI) are due to their cellular uptake differences, being a result of their valence states. Cr(III) is poorly absorbed by cells as it forms large bulk molecules with ligands such as water, cysteine, glutathione, sulfates or organic acids (Costa and Klein 2006; EPA 1990; Wise J et al., 2018b). Whereas Cr(VI) forms a chromate (CrO$_4^{2-}$) anion, which structurally resembles phosphate (PO$_4^{3-}$) and sulfate (SO$_4^{2-}$), that readily allow cell internalization via facilitated diffusion (De Flora and Wetterhahn, 1989). Intracellular Cr(VI) does not directly interact with DNA or proteins (evidenced in cell-free systems) (Fornace et al., 1981). Cr(VI) is rapidly reduced intracellularly to Cr(III) by a variety of mechanisms that are dependent on the reducing agents, like glutathione and ascorbate (De Flora and Wetterhahn, 1989). Major results of this intracellular reduction of Cr(VI) to Cr(III) include highly reactive intermediates such as Cr(IV), Cr(V) (De Flora and Wetterhahn, 1989; Wise J et al., 2018b). Cr(III) is not readily excreted from the cells and can lead to high intracellular chromium concentrations. It is still unknown if Cr(III) or its reactive intermediates are the ultimate carcinogen, as all three have the ability to react with and damage DNA and cellular proteins (De Flora and Wetterhahn, 1989).

1.4.2. The Role of Reactive Oxygen Species

In regards to Cr(VI)-induced carcinogenesis, the reactive oxygen species produced from the reduction of Cr(VI) to Cr(III) is considered important to the mechanism underlying the carcinogenesis of Cr(VI) (Shi et al., 1998, 1999). Cr(III) from this reduction
can interact with various cellular components (discussed in section 1.5). Reactive oxygen species (ROS) are generated by Cr(VI) and Cr(III). These ROS occur from a Haber-Weiss reaction (Figure 1.1). In the classical reactions (Haber-Weiss and Fenton) involving iron, \( \text{O}_2^- \) mediates \( \cdot \text{OH} \) generation from \( \text{H}_2\text{O}_2 \) and also participates in the reduction of Fe(III) leading to the Fenton reaction. In the case of Cr(VI) and Cr(III), both species can replace Fe(II) to produce \( \cdot \text{OH} \). The Haber–Weiss reaction may also involve the different valence states of chromium, such as Cr(IV) and (V). The Haber–Weiss-type mechanism of \( \cdot \text{OH} \) generation is likely to be more predominant \textit{in vivo} than other reaction (Shi \textit{et al.}, 1998, 1999).
Figure 1.1. Cr-mediated Haber-Weiss Reactions.
This figure shows a schematic of the reactive oxygen species produced by Cr(III) and Cr(VI), the intermediate chromium species produced, and the various reactive oxygen species produced during these processes.
1.4.3. The Role of Reducing Agents

Multiple intracellular components, like ascorbate, cysteine, glutathione, NADPH, ribose, fructose, and cytochrome P450, as well as certain cellular organelles, can act as cellular reductants for Cr(VI) (Figure 1.2) (Shi et al., 1999; Wise J et al., 2018b). Studies suggest that ascorbate may be the principal reductant in vivo (Standeven and Wetterhahn 1991, 1992; Zhitkovich 2011); however, these studies were carried out by exposing extracted cytosolic fractions to Cr(VI) and did not use whole animals (Wise et al., 2008). Unlike rodents, humans are not able to synthesize ascorbate like rodents. Rodents may have evolved a system that overemphasizes ascorbate as a primary mechanism for pro-oxidant reductions. Subsequent studies have relied on these rat studies and presume that ascorbate is the principal reductant and ascorbate is supplemented to the media in cell culture studies. Interestingly, addition of ascorbate to cell culture media resulted in higher mutation frequencies (base substitutions and deletion mutations) (Quievryn et al., 2003, 2006; Reynolds et al., 2007a). The physiological levels of ascorbate in human lung tissues are relatively low and thus the pro-oxidant activity of ascorbate may not be the primary contributing mechanism for Cr(VI) reduction in human lung tissue and cells (Slade et al., 1985; Yavorsky et al., 1934). This possibility is further supported by the low levels of mutations seen in Cr-induced human lung cancers. Further, whole animal studies utilizing electron paramagnetic resonance (EPR) analysis revealed that Cr(VI) is preferentially reduced by NADPH, producing high valent chromium species, that are believed to be responsible for reactions with DNA (Liu et al., 1995). Unfortunately, the principal reductant for Cr(VI) in human lung tissue still remains unknown at this time.
Figure 1.2. Mechanisms of Intracellular Cr(VI) Reduction.
This figure shows the mechanisms by which Cr(VI) is reduced to Cr(III) inside the cell. Glutathione can reduce Cr(VI) in a one- or two-electron reaction whereas cysteine is almost exclusively considered to reduce Cr(VI) in a one-electron reaction with NADPH providing electrons. Ascorbate reduces Cr(VI) in a two-electron reaction skipping the Cr(V) intermediate and immediately forming Cr(IV). As a result of all of these reductions. ROS are formed which can lead to oxidative damage to nucleic acids, proteins, and lipids. Reprinted from Reference Module in Chemistry, Molecular Sciences and Chemical Engineering, 2018, Speer RM and Wise, JP Sr. Current Status on Chromium Research and Its Implications for Health and Risk Assessment, Pages No. 3, Copyright (2018), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also, Lancet special credit - "Reprinted from The Lancet, 2018, Speer RM and Wise, JP Sr. Current Status on Chromium Research and Its Implications for Health and Risk Assessment, Pages No. 3, Copyright (2018), with permission from Elsevier."
1.4.4. The Role of Solubility

Solubility plays a key role in the carcinogenic potential of chromium compounds. Animal studies and human epidemiologic studies demonstrate particulate (or water-insoluble forms) of Cr(VI) are potent carcinogens. Studies of soluble chromium compounds showed relatively small effects in the increases of lung tumors and these results are linked to the fact that soluble forms of Cr(VI) are rapidly reduced (De Flora et al., 1997; IARC, 1990). Additionally, these results in rodent studies were attributed to the much higher level of ascorbate in rodents (Speer and Wise, 2018; Wise et al., 2008).

Many studies have established that the more insoluble forms of Cr(VI), e.g. calcium chromate, lead chromate, strontium chromate, and zinc chromate, cause significant increases in lung tumor formation (IARC, 1990). Similarly, workers who died of Cr(VI)-induced lung cancer had Cr(VI) deposits at their lung bifurcations sites, where the particles are likely to impact pathology and cancer (Ishikawa et al., 1994a, 1994b). Given the slow rate of clearance for particulate Cr(VI), these deposits can efficiently produce a prolonged exposure of Cr(VI) to lung tissue. Prolonged exposure to particulate Cr(VI) in human lung cell culture studies further support this conclusion resulting in increased levels of aneuploidy, chromosome damage, DNA damage repair deficiency, and neoplastic transformation (Holmes et al., 2006a, 2006b, 2010; Wise S et al., 2018; Xie et al., 2007; Xie et al., 2015). Interestingly, recent studies of chronic exposure to soluble Cr(VI) showed that chronic exposure results in neoplastic transformation (Kim et al., 2015, 2016; Pratheeshkumar et al., 2016, 2017; Wise J et al., 2018). Even through prolonged exposure to soluble Cr(VI) can induce
transformation in cell culture, animal, cell culture and human studies still show that the particulate form is a more potent carcinogen.

1.4.5. A Physico-Chemical Mechanism for Cr(VI)

Extracellularly, particulate Cr(VI) dissolves releasing the chromate oxyanion, which then enters the cell by facilitated diffusion (Wise et al., 1994; Wise S et al., 2004a) (Figure 1.3). While the cation and the particle can be taken into the cell, studies report that these internalized particles do not dissolve intracellularly and thus both the particle and cation have no reported cellular effects (Wise S et al., 2004a; Xie et al., 2004; Holmes et al., 2005). On the other hand, soluble Cr(VI) compounds make the chromate oxyanion directly available to enter the cell through facilitated diffusion. After the Cr(VI) ion is in the cell it is rapidly reduced by intracellular reductants to Cr(III) (De Flora and Wetterhahn, 1989). Cr(III) or the reactive Cr intermediates then interact with intracellular molecules and induce the observed damaging effects. Studies have demonstrated that Cr can bind to DNA directly altering its conformation or in the event of DNA replication, leading to a DNA break (Cupo and Wetterhahn, 1985; Madhusudanan et al., 1999; Standeven and Wetterhahn, 1992; Tsapakos et al., 1983; Zhitkovich et al., 1996). Interestingly, studies on the binding of Cr with DNA reveal there are inconsistencies in our understanding of the binding mechanism and exactly where Cr binds DNA remains elusive. Recently, it was observed that Cr(III) binds weakly to the DNA phosphate backbone in a reversible interaction likely through electrostatic forces (Zhou et al., 2016). Cr(III) also interacted with nucleobases and formed stable cross-links. When neutral or high pH was restored in these experiments, Cr(III) gradually lost its DNA binding ability which was likely due to hydrolysis. While
this study reveals some information about Cr-DNA binding kinetics, the physiological relevance remains elusive. There are limited other studies regarding the direct interaction of Cr with cellular molecules. This part of the Cr(VI) carcinogenesis mechanism still remains unclear.
Figure 1.3. Cr(VI) Cellular Uptake.
This figure shows a model for the cellular uptake of chromate (VI) anion. Following exposure to Cr(VI) (insoluble or soluble), Cr(VI) anion is the key species and not the cation. The chromate anion is taken into the cell (via facilitated diffusion mimicking sulfate and phosphate ions) and reduced to reactive intermediates, Cr(IV) and Cr(V), and lastly to Cr(III). While, the particulate Cr(VI) enters the cell typically through phagocytes and has no contribution to Cr(VI) carcinogenic mechanism.
1.5. Cr(VI) Genotoxicity

It is well accepted that Cr(VI) can induce DNA damage. Specifically, Cr(VI) can cause DNA single and double strand breaks; DNA adducts and crosslinks; oxidative lesions of DNA bases; and chromosome instability. Cr(VI) itself does not directly react with DNA but Cr(III) and the same intermediates (produced during intracellular reduction) have a high level of reactivity toward DNA (O’Brien et al., 2002; Speer and Wise, 2018). Chromium can also form covalent and electrostatic bonds with intracellular molecules (e.g., amino acids, ascorbate, cysteine, histidine, and glutathione) and then can lead to DNA adducts (O’Brien et al., 2002; Speer and Wise, 2018). In support of this concept, lymphocytes from Cr(VI)-exposed workers had increased levels of DNA-protein crosslinks (Medeiros et al., 2003). The Cr-DNA adducts and crosslinks are considered important in Cr(VI)-induced carcinogenesis, by causing cell cycle arrest, mutations, gene expression changes, and polymerase arrest (Patierno and Landolph, 1989; Manning et al., 1992; Yang et al., 1992; Bridgewater et al., 1994; Chen and Thilly 1994; Speer and Wise, 2018).

1.5.1. Oxidative DNA Damage

The ROS from the reduction of Cr(VI) leads to oxidative DNA damage and produces alterations in DNA including abasic sites, base and sugar lesions, DNA-protein cross-links, and strand breaks. If these damages are left unrepaired, they can lead to biological consequences that are detrimental to an organism, including cell death, mutations and ultimately malignant transformation (Wise et al., 2008). Studies have shown Cr(VI) damages both purines and pyrimidines (Blasiak and Kowalik, 2000; Qi et al., 2000; Slade et al., 2005; Sugden and Martin, 2002). Studies have provided evidence
that the main lesion of nucleobases is on the guanine residue and forms 7,8-dihydro-8-oxoguanine (8-oxoG) (Slade et al., 2005). Oxidative DNA damage from Cr is not limited to the guanine residues, but, can also occur at adenine bases in Cr(VI)-treated lung cells (Arakawa et al., 2012). Another study suggested that the 8-oxoG lesion is susceptible to additional oxidation by Cr and forms the lesion spiroiminodihydantoin (Sp) and may be capable of carcinogenic initiation following Cr(VI) exposure and toxicologically more important than the 8-oxoG lesion (Slade et al., 2005). However, these lesions have not been explored after particulate Cr(VI) exposure and additional investigations are needed.

1.5.2. DNA Adducts and Crosslinks

Cr(VI) is able to cause Cr-DNA adducts, Cr-DNA crosslinks and DNA-protein crosslinks, which can lead to additional cellular injuries (e.g. DNA polymerase arrest, gene expression changes, and mutagenesis) (Singh et al., 1998). Macfie et al., 2010 proposed a stepwise mechanism for the formation of protein-Cr-DNA crosslinks. Cr(VI) is reduced to Cr(III) and Cr(III) then binds to DNA. Following the slow capture of proteins by the binary Cr-DNA adduct, a ternary protein-Cr-DNA crosslink forms. Additionally, the repair of these lesions may cause DNA single and double strand breaks. The isolation and detection of a specific Cr-DNA adduct have proven difficult despite a robust effort from many laboratories and the isolation of the exact lesion remains elusive. It remains unclear if Cr is binding directly to DNA bases or is electrostatically associating with the phosphate backbone. The Cr interactions with DNA are important because these interactions lead to the various consequences of DNA strand breaks and ultimately chromosomal aberrations.
1.5.3. DNA Single Strand Breaks

Insoluble and soluble forms of Cr(VI) have been reported to induce DNA single strand breaks (Hodges *et al*., 2001; Sugiyama *et al*., 1986; Yang *et al*., 2005). Animal Cr(VI) exposure studies have shown single strand breaks occurred in lymphocytes and somatic cells of the brain, kidney, liver, lung, and spleen (Kleinsasser *et al*., 2001; Ueno *et al*., 2001). It remains unclear if the single strand breaks are formed by direct DNA damage or are formed indirectly as a byproduct of excision repair. Cell-free system studies suggest that they are indirect (Messer *et al*., 2006), but more studies are warranted. It is possible a DNA double strand break may occur from the conversion of single strand break to a double strand break (Speer and Wise, 2018).

1.5.4. DNA Double Strand Breaks

In addition to single strand breaks, both particulate and soluble forms of Cr(VI) have been shown induce DNA double strand breaks in animals and cultured human cells (Ha *et al*., 2004; Reynolds *et al*., 2007a; Wakeman *et al*., 2004; Wang *et al*., 2006; Wise *et al*., 2010; Xie *et al*., 2005, 2009;). These double strand breaks were detected by increased migration of broken DNA in an agarose gel electrophoresis (comet assay) and gamma-H2A.X (g-H2A.X) foci. DNA double strand breaks can arise from the collapse of a replication for, the conversion of a single strand break to a double strand break during replication, and a stalled replication fork (as their formation is limited to the late S- and G2-phases of the cell cycle) (Ha *et al*., 2004; Reynolds *et al*., 2007a; Speer and Wise, 2018).

1.5.5. Microsatellite Instability

Cr(VI)-induced tumors have shown microsatellite instability (MIN) at two or more loci and longer Cr(VI) exposures have a greater frequency of MIN (Hirose *et al*., 2002;
Takahashi et al., 2005b). MIN and Cr(VI) has been investigated in only one cell culture study. Human lung epithelial (BEAS-2B) cells were transformed by a chronic low dose of soluble Cr(VI). One Cr(VI)-transformed cell line was isolated which had an increased expression of a mismatch repair gene but had no evidence of microsatellite instability (Rodrigues et al., 2009). This Cr(VI)-transformed cell line did display a high degree of chromosomal instability. The results from these suggest that MIN may not be a driving factor in Cr(VI)-induced carcinogenesis but may be a result of later stages or a potential consequence from other genomic changes.

1.5.6. Mechanism of DNA Repair of Cr(VI)-Induced Lesions DNA Repair

Cr(VI) is a well-known genotoxicant agent and can produce several types of DNA damage and unchecked these damage can lead to apoptosis, cytotoxicity, growth arrest, and mutations. In normal basal conditions, cells are able to swiftly and proficiently repair damage. If damages are left unrepaired or misrepaired, it can lead to neoplastic transformation and ultimately tumorigenesis. Therefore, scientists have been investigating how Cr(VI) interferes with different DNA repair mechanisms for decades.

Base excision repair (BER) is the primary pathway used to repair DNA alkylation, oxidation lesions, and single strand breaks. Data indicate that BER and single strand break repair occur after Cr(VI) exposure (Speer and Wise, 2018; Wise and Wise, 2018; Wise et al., 2018). Further, the specific mechanisms involved in repairing these lesions are still poorly understood. Currently, it is too difficult to formulate a repair pathway for Cr-induced lesions and more investigations are required to identify these specific repair mechanisms after Cr(VI) exposures (Speer and Wise, 2018; Wise and Wise, 2018; Wise et al., 2018).

Single-strand breaks are generally detected by poly [ADPribose] polymerase 1
(PARP1), and this detection is followed by processing of the DNA ends. These steps are coordinated by the enzyme X-ray repair cross-complementing protein 1(XRCC1). XRCC1 acts as recruiter and scaffold for other proteins required for repair (Caldecott, 2008). Cell culture studies have shown that XRCC1 appears to be involved in the repair of Cr(VI)-induced lesions and these data are supported from an epidemiological study in Cr(VI) workers (Zhang et al., 2012). Additionally, failed single-strand break repair after Cr(VI) exposure leads to more complex forms of damage (Bryant et al., 2006; Grlíková-Duževík et al., 2006a, 2006b; Zhang et al., 2006).

Nucleotide excision repair is used to repair chemically modified bases and helical distortions in the DNA (Gillet and Scharer, 2006). Data indicate that nucleotide excision repair occurs after Cr(VI) exposure, which is consistent with the observations that these lesions are produced by Cr(VI). To date, the specific mechanism for repairing these lesions after Cr(VI) exposure is still uncertain. More studies are needed to formulate a repair pathway for these lesions. Interestingly, studies have shown evidence that nucleotide excision repair may be pro-mutagenic or anti-mutagenic after Cr exposures, but more studies are necessary (Speer and Wise, 2018; Wise and Wise, 2018; Wise et al., 2018).

Mismatch repair functions to recognize and repair mispaired bases and insertion/deletion loops generated during DNA replication. Cr-induced lung cancer patients have shown higher rates of replication errors than lung cancer patients lacking Cr exposures and some mismatch repair proteins are repressed at a higher rate (Takahashi et al., 2005b). Likewise, Cr(VI)-exposed cancer patients with alterations in mismatch repair proteins (MLH1) were at a higher risk for lung cancer development (Halasova et al.,
In 2016). Additionally, these results are supported by cell culture studies, which have provided further evidence that mismatch repair (MMR) proteins may increase the genotoxicity of Cr-DNA adducts by causing replication fork collapse thus leading to DNA double strand breaks (Peterson-Roth et al., 2009; Reynolds et al., 2007a; Reynolds and Zhitkovich, 2007) Mismatch repair proteins were able to recognize ternary Cr-DNA adducts, but did not recognize binary Cr-DNA adducts. The generation of Cr-induced chromosomal breaks and double strand breaks was dependent on the ternary Cr-DNA adducts, as they were mistakenly processed by MMR proteins as mismatches and consequently processed into these DNA breaks (Reynolds et al., 2009). These studies show that mismatch repair is active after Cr(VI) exposure but the specific repair mechanism remains unclear. Studies provide evidence that both MLH1 and MSH2 are candidate genes involved. Remarkably, as was observed for nucleotide excision repair; proficient mismatch repair seems to be more genotoxic than deficient repair, this is additionally interesting as the Cr-induced human tumors appear MMR-deficient. This further suggests that obtaining a state of mismatch repair-deficiency is likely a later event in Cr(VI)-carcinogenic process and that Cr(VI)-induced mismatch damage is likely acquired after other carcinogenic changes have occurred.

The two primary DNA double strand break repair pathways are homologous recombination (HR) and non-homologous end joining (NHEJ). Non-homologous end joining is considered a low fidelity pathway due to the loss of genetic material during its repair process. A study reported NHEJ is not critical in protecting cells against Cr(VI) exposure (Camyre et al., 2007). Unlike NHEJ, homologous recombination is considered to be a high-fidelity repair mechanism and is critical in preventing chromosome
instability. Multiple groups have found homologous recombination is impaired following Cr(VI) exposure and recently observed a loss of RAD51 protein expression and function (Browning et al., 2016; DeLoughery et al., 2015; Qin et al., 2014; Reynolds et al., 2007).

DNA crosslink repair is utilized to repair adducts that tether both strands of DNA together. Interstrand crosslinks cause a stalled DNA replication fork. Studies report that Cr does induce DNA crosslink repair but the components of the crosslinks remain uncertain but are likely to involve amino acids, DNA, and/or proteins (Savery et al., 2007; Vilcheck et al., 2002, 2006). The specific mechanisms involved in repairing these crosslink regions remain elusive and inadequate to formulate a repair pathway for these Cr-induced lesions. Future studies are still required to elucidate the specific crosslink repair mechanisms.

1.5.7. Chromosome Instability

Particulate and soluble Cr(VI) have also been reported to damage chromosome structure, which can manifest as chromosomal aberrations. Numerous studies have extended these findings to human lung epithelial cells and fibroblasts and demonstrate that damage occurs in both a concentration- and time-dependent manner (Holmes et al., 2006a, 2006b, 2010; Kost et al., 2012; Wise et al., 2002, 2004a, 2004b, 2004c, 2004d, 2006b, 2018; Wise and Wise, 2012; Xie 2004, 2008, 2009, 2015). Cr(VI) has also been shown to affect mismatch repair which is used to repair Cr-DNA adducts (discussed above) (Zecevic et al., 2009). Additionally, failure of this process can lead to a stalled replication fork and ultimately double strand breaks (Barbour and Xiao, 2003). These double strand breaks occur during late S and G2 (DeLoughery et al., 2015; Reynolds et al., 2004; Xie et al., 2009). If DNA double strand breaks are unattended to, they can lead to chromosomal instability (CIN) (Masuda A and Takahashi, 2002). CIN is a common occurrence in lung
cancers and has been observed in chromate-induced tumors (Maeng et al., 2004). CIN can occur in multiple forms, including in the form of numerical CIN (change in chromosome number), structural CIN (form of translocations), or DNA breaks contributing to Cr(VI) carcinogenesis (Figure 1.4) (Albertson et al., 2003; Speer and Wise, 2018). Improper chromosomal segregation after Cr(VI) exposures appears to due to supernumerary centromeres. Centromeres are important for the chromosomal separation during cell division (Holmes et al., 2010; Martino et al., 2015).

These studies provide significant evidence that both structural and numerical CIN play a key role in the mechanism of Cr(VI)-induced carcinogenesis. The abnormal chromosome numbers or deletions/insertions in whole chromosomes or genes can result in aberrant gene expression. These changes can then lead to more carcinogenic outcomes. The complete pathways involved in maintaining fidelity in these mechanisms are still being examined. There is significant evidence that genomic instability is a key driver of Cr(VI)-induced carcinogenesis.

1.6. Models for Cr(VI) Carcinogenesis

Although, various mechanisms for Cr(VI)-induced carcinogenesis have been established, the ultimate mechanism of Cr(VI)-induced carcinogenesis remains elusive. Currently, there are three well-recognized paradigms for chemical carcinogenesis that can be applied to a probable model of Cr(VI)-induced carcinogenesis. These models are epigenetic modification, multistage carcinogenesis, and genomic instability.

1.6.1. Characteristics of Cr(VI)-Induced Lung Tumors

Chromium accumulates high concentrations at lung bifurcation sites in chromate-exposed workers (Ishikawa et al., 1994a). Another study showed multiple tumors at these bifurcation sites, mainly squamous cell carcinomas (Ishikawa et al., 1994b). Additionally,
in these workers with lung cancer, the levels of chromium were higher than workers who did not have lung cancer (Ishikawa et al., 1994b). It has been reported that there is no correlation between chromium accumulation in the lungs, but there was a correlation between chromium accumulation and the malignancy stage of the lung cancer (Kondo et al., 2003). This finding was further supported in animal studies (Takahashi et al., 2005a).

Studies have reported there are very few mutations in key oncogenes or tumor suppressor genes in chromium-induced tumors. There were fewer mutations found in p53 in Cr(VI)-induced lung tumors when compared to lung tumors with no Cr(VI) exposure and those tumors with mutations in p53 had no association with the length of chromium exposure (Kondo et al., 1997). Additionally, Katabami et al. (2000) reported there was no difference in p53 protein expression in Cr(VI)-induced lung tumors. Likewise, there were no point mutations found in the ras oncogene (Ewis et al., 2001).

Chromate-induced lung tumors have been characterized by features that are associated with genomic instability. Microsatellite instability markers were found in 79% of Cr(VI) induced tumors compared to only 15% of non-Cr(VI) exposed tumors (Hirose et al., 2002). Microsatellite instability markers were increased proportionally with chromium exposure (Hirose et al., 2002) and further correlated with a decrease in the hMLH1 (mismatch repair gene) protein expression but microsatellite instability was not associated with the hMSH2 (mismatch repair gene) repression (Takahashi et al., 2005b). Furthermore, Hirose et al. (2002) also reported a higher level of methylation in CpG islands in the MLH1 gene was present in the tumors of Cr(VI) exposed tumors when compared to non-Cr(VI) tumors. However, this change in CpG islands in the gene was not correlated with the
observed decreased in protein expression. These studies suggest a mechanism for MIN in Cr(VI)-induced lung cancers that does not involve a loss of mismatch repair proteins.

Studies have also revealed that Cr(VI)-induced lung tumors exhibit chromosome instability. Loss of heterozygosity was reported at 6 different loci in 50-75% of Cr(VI)-induced lung tumors but this result was not significantly different from non-Cr(VI) lung tumors (Hirose et al., 2002). These findings could implicate loss heterozygosity may be a general mechanism for lung carcinogenesis given that most lung cancers exhibit significant chromosome instability (Masuda and Takahashi, 2002). No further studies have investigated chromosome instability in Cr(VI)-induced lung tumors and no examinations of chromosome complement have carried out on.

Other characteristics reported of Cr(VI)-induced lung tumors include alterations in cyclin D1 and p16 protein expressions (Katabami et al., 2000; Kondo et al., 2006), methylation of the APC (adenomatosis polyposis coli) gene (Ali et al., 2011), alterations in the surfactant protein B (SP-B) gene (Ewis et al., 2006), and changes to EGFR and Nrf2 (Kim et al., 2015, 2016). However, the EGFR and Nrf2 were only examined in one chromate worker lung tumor. APC is the gene that has been reported to be mutated in patients with familial adenomatous polyposis coli (colon cancer). Further, the silencing of this gene activates the Wnt signaling pathway which could lead to excessive cell proliferation and eventual tumor development (Nusse, 2005). Alterations of the SP-B gene may potentially lead to abnormal clearance ability in these individuals and enhance susceptibility to particulates in Cr(VI)-exposed workers. Both cyclin D1 and p16 are important cell cycle regulators (Katabami et al., 2000; Kondo et al., 2006) and changes of these protein expressions could allow for otherwise damaged cells to escape the normal
cell cycle arrest and continue to proliferate. Increased activate EGFR protein expression could lead to further cancer cell growth and tumor proliferation and increased Nrf2 and downstream antioxidant proteins could protect the cell against the harsh pro-oxidant environment (Kim et al., 2015, 2016).

1.6.2. Characteristics of Cr(VI)-Transformed Cells

There are no cell lines available from chromate lung tumors. Chronic exposure to low doses of Cr(VI) has been shown to induce neoplastic transformation of human lung cells (Kim et al., 2015; 2016, Pratheeshkumar et al., 2016; 2017). Additionally, acute exposure to high doses of Cr(VI) can also induce neoplastic transformation (Xie et al., 2007). Over the past decade these Cr(VI)-transformed cells have been investigated for different cancer endpoints. These Cr(VI)-transformed cells have distinct properties when compared to passage-matched control cells. EGFR overexpression and constitutive activation have been shown to be important in these transformed cells (Kim et al., 2016). Additionally, constitutive Nrf2, HIF-1α, and p62 expression have been shown to be important in the cancer properties of these transformed cells (Kim et al., 2015, 2016; Pratheeshkumar et al., 2016; 2017; Roy et al., 2016). Epigenetic changes have also been reported in Cr(VI)-transformed cells (Pratheeshkumar et al., 2016; 2017; Wang Z et al., 2018) Cr(VI)-transformed cells also have the ability to evade apoptosis ((Kim et al., 2015, 2016; Pratheeshkumar et al., 2016; 2017). Lastly, Cr(VI)-transformed cells have genomic instability and altered DNA repair responses (Wise S et al., 2018; Xie et al., 2007). Given the difficulty in assessing all endpoints in chromate tumors, these data from Cr(VI)-transformed cells can be used to understand changes during Cr(VI)-induced transformation and can be used to further understand the mechanism underlying Cr(VI) carcinogenesis.
1.6.3. Cr(VI) and Epigenetic Modification

Epigenetics are modifications that are heritable changes that can alter cellular function but do not require changes to genetic material. (Herman and Baylin, 2003). Epigenetic modifications or changes to the epigenome suggested as an important carcinogenic mechanism. The two main epigenetic modifications are DNA methylation and histone modifications. Other reported epigenetic changes include histone variants, microRNA (miRNA) expression, and nucleosome repositioning (Sharma et al., 2010). These modifications can alter gene expression and last for several generations or the lifetime of the cell. Changes can down- or upregulate pathways. Cr(VI) has been shown to induce epigenetic changes in methylation of DNA and histone modifications and cause gene expression changes and signaling pathway changes in cell culture studies. However, studies in Cr(VI)-induced tumors and epigenetic alterations are limited.

Takahashi et al. (2005a) reported hMLH1 and hMLH2 expression was decreased in chromate tumors due to hypermethylation of the promoter region. Additionally, these tumors also were reported to have significant repression of the tumor suppressor p16INK4a. This repression was correlated with DNA hypermethylation of the promoter in patients with exposure to chromium for more than 15 years (Kondo et al., 2006). It was also reported that tumor suppressor genes, APC and hMLH1 were hypermethylated in Cr(VI)-exposed lung cancers (Ali et al., 2011). Wang et al. (2012) found that in individuals actively exposed to Cr(VI) with Cr accumulation in their blood showed global DNA hypomethylation which correlated with low levels of folate, DNA strand breaks, and urinary 8-hydroxy-2'-deoxyguanosine. Similarly, other changes in gene expression in chromate tumors have been observed, e.g. increased cyclin D1 and decreased survivin
(Halasova et al., 2010; Katabami et al., 2000). Interestingly, 61.3% of chromate tumors had surfactant B gene variants (deletion or insertion) (Ewis et al., 2006). Another study found hypermethylation of CpG sites in DNA repair genes, including RAD51, increased in Cr(VI)-exposed workers compared to non-exposed workers. Furthermore, these data were correlated to blood Cr levels of the workers and this study confirmed their results in human bronchial epithelial cells (16HBE cells) treated with Cr(VI) (Hu et al., 2018). A study trying to develop a biomarker for Cr(VI) reported that mitochondrial DNA isolated from the blood of chromate workers was hypomethylated (Yang et al., 2016). No studies have investigated miRNAs in chromate tumors. Li et al. (2014) reported decreased miR-3940-5p levels in the blood with Cr exposure from chromate workers.

Cell culture studies have addressed different epigenetic changes as a result of chromium exposures. Some cell culture studies have shown significant changes in DNA methylations (Klein et al., 2002; Lou et al., 2015). Other epigenetic modifications observed in cell culture studies include histone acetylation and methylation (Hu et al., 2016; Schnekenburger et al., 2007; Sun et al., 2009; Zhou et al., 2009). Acetylation of histone tails is commonly associated with increased gene expression, while methylation is associated with both increased and decreased repression (depending on where the methylation site is). Sun et al. (2009) found changes in histone methylation leading to tumor suppressor gene silencing events. Interestingly, Wei et al., 2004 found Cr(VI) could crosslink a histone deacetylase to inducible promoters, which resulted in increased gene expression. Similarly, acetylation of histone H4 of the stressor protein Nupr1 was downregulated after Cr(VI) exposure in BEAS-2B cells which led to increased Nupr1
protein expression. The overexpression of Nupr1 protein is associated with cancers (Chen et al., 2016).

Another histone modification is biotinylation, the covalent binding of biotin to other molecules. The biological role of histone biotinylation remains unclear but is believed to be involved with histone acetylation and DNA damage response (Xia et al., 2014). Cr(VI) treated 16HBE cells had differential histone biotinylation and differential distribution of biotinidase depending on the Cr(VI) concentration (Xia et al., 2014). Furthermore, in Cr(VI) treatments the authors reported histone deacetylation could play a role in histone biotinylation further adding to the complexity of these histone modification mechanisms.

Human bronchial epithelial cells (BEAS-2B cells) were evaluated for gene expression changes following a 2-month exposure to chromium. The authors utilized single-cell RNA sequencing (Park et al., 2017). This study reported that Cr(VI) with or without a CRISPR/cas9 deletion of Gene 33 (a protein involved in transformation) induced differential expression of more than 80 genes. Some of these genes were included proteins for epithelial-mesenchymal transition/metastasis, cell adhesion, oxidative stresses, ubiquitination, and WNT signaling. Gene expression changes can be widely regulated by epigenetic mechanisms. However, investigations on epigenetic changes in tumors are limited and have been focused on methylation changes.

A limited number of cell culture studies have investigated the involvement of miRNAs in Cr(VI) carcinogenesis. Li et al., 2016 reported that in 16HBE cells miR-3940-5p enhanced the HR response following treatment with Cr(VI). Similarly, He et al., 2013 found changes in miRNA expression associated with Cr(VI)-transformed BEAS-2B cells. This reduction was associated with the up-regulation of proteins involved in angiogenesis
and proliferation (He et al., 2013). Likewise, a study reported after Cr(VI)-induced malignant cell transformation of BEAS-2B cells, transformed cells had increased miR-21 expression and inhibition of a tumor suppressor. Further blocking miR-21 suppressed Cr(VI)-induced transformation (Pratheeshkumar et al., 2017).

These epigenetic investigations highlight the complexities and connectedness between different mechanisms of carcinogenesis. Still, collectively these data provide evidence that Cr(VI) has the ability to induce epigenetic changes. Nonetheless, it is unclear precisely when some of these epigenetic changes happen and if they occur early or late during Cr(VI) carcinogenesis. Additional studies are still warranted to determine if epigenetic changes alone can induce carcinogenesis or occur in conjunction with the potent DNA damaging effects of chromium. While the current studies provide strong evidence that epigenetic changes are a key piece to the Cr(VI) carcinogenesis, the complete epigenetic mechanisms are still unknown.

1.6.4. Cr(VI) and Multistage Carcinogenesis

The Multistage carcinogenesis mechanism is a stepwise progression and an accumulation of mutations in tumor suppressor genes and oncogenes that leads to cancer development (Volgelstein and Kinzler, 1993). The first step in multistage carcinogenesis paradigm is initiation. Initiation involves some form of permanent genetic change or mutation in an oncogene or a tumor suppressor gene. Next, this genetic change or mutation is then promoted by selective clonal expansion of the initiated cell or cells. Further, this promotion is then followed by more genetic changes which then lead to malignant transformation and ultimately tumor progression (Volgelstein and Kinzler, 1993). Therefore, for a chemical or compound to follow this paradigm for its carcinogenesis
mechanism it must be able to induce significant mutations in oncogenes or tumor suppressor genes.

Both animal studies and cell based studies suggest that Cr(VI) can be mutagenic. One study examined Cr(VI)-induced mutation frequency in rodents using the Big Blue transgenic mouse model (Cheng et al., 2000). These mice were treated with soluble potassium dichromate via intratracheal instillation and the authors found a dose- and time-dependent increase in mutation frequencies. Mutations observed were largely G:C base substitution and minor deletions (Cheng et al., 2000). Inflammation alone has been reported to induce mutations (Federico et al., 2007). Cr(VI) causes inflammation (Ding et al., 2000; Roy et al., 2016). Since there were no measures in this aforementioned mutagenesis study to account for inflammation, it is unclear if the mutations reported were a direct result of the chromium exposure or secondary from the inflammatory response to Cr(VI).

Cell culture studies applying a shuttle vector mutagenesis assay reported mutations increase with Cr(VI) treatments (Zhitkovich et al., 2001, 2002; Quievryn et al., 2003, 2006). Additionally, these studies found that Cr(VI)-induced mutations resulted from Cr(III)-DNA adducts and, importantly, were not from the reactive oxygen species or reactive intermediates produced (Zhitkovich et al., 2001, Quievryn et al., 2006). More in-depth studies found that Cr(III) forms binary and ternary adducts with DNA and these ternary Cr(III) DNA-adducts were the most mutagenic (Zhitkovich et al., 2001, Quievryn et al., 2003). Consistent with the Big Blue transgenic mouse study data, the predominant mutations observed were single base substitutions of G:C (Zhitkovich et al., 2001) and
interestingly, an additional study suggested that similar levels of base substitutions and deletion mutations can occur from Cr(VI) exposure (Quievryn et al., 2003).

Additional cell culture studies used the method of focusing on an existing chromosomal locus. One reported a 3 to 3.5-fold increase in mutation frequency with 30-50% of these mutations being deletions when using a bacterial promoter in lung cells (Klein et al., 2002). Another study found an increase in mutation frequency at the *hp*rt locus in Chinese hamster ovary cells, though this was only observed when cells were preloaded with high (mM level) concentrations of ascorbate (reducing agent) and, notably, there no increases in mutations were observed without ascorbate (Reynolds et al., 2004). Brooks et al. (2008) reported an increase in Cr(VI)-induced mutations, but proficient nucleotide excision repair was necessary for the induction of these mutations.

These studies suggest that Cr(VI) may induce G:C base substitution mutations. The evidence is conflicting on the ability of Cr(VI) to induce deletion mutations and this discrepancy may be due to the reducing agent present in the studies. For example, with cysteine used as a major reducing agent, limited deletion mutations were observed (Zhitkovich et al., 2001). Conversely, when ascorbate is the reducing agent similar levels of both base substitutions and deletion mutations were found (Quievryn et al., 2003). These reducing agent comparisons and the results from the *in vivo* Big Blue transgenic mouse study, which showed a predominance of base substitutions with few deletions, further support the observations by Liu et al. (1995) that ascorbate is not the principle *in vivo* reductant for Cr(VI). Therefore it is reasonable to conclude that cysteine may be the more important reducing agent *in vivo*. Thus, the increases in mutations observed in the ascorbate
cell culture study may be experimentally derived and not occur in tissues and this conclusion is consistent with the lower ascorbate levels in the lungs.

Cell culture studies and the animal study suggest that Cr(VI) is mutagenic. Yet, these data suggest that these mutagenic events may only occur under non-exposure relevant conditions, specifically may require a high dose, high toxicity, or experimentally contrived systems. Furthermore, data from Cr(VI)-induced tumors from chromate workers show very limited or no mutations in oncogenes such as p53 and ras. These results further support the argument that Cr(VI) is either weakly or indirectly mutagenic. Thus there are insufficient mutations to support Cr(VI) carcinogenic mechanism is through the multistage carcinogenesis paradigm.

1.6.5. Cr(VI) and Genomic Instability

Genomic instability is a common event in lung cancers with both chromosome instability and microsatellite instability and happening concurrently, and has been extensively studied in Cr(VI)-induced tumors, which have microsatellite instability (Holmes et al., 2008; Speer and Wise, 2018; Wise and Wise, 2018). Chromosome instability comprises of both numerical and structural chromosome abnormalities. Chromosome instability occurs in Cr(VI)-induced tumors evidenced by an increase in the loss of heterozygosity, which is consistent with many cell culture studies that report a significant effect on chromosome structure in cultured cells treated with Cr(VI) (Holmes et al., 2008; Wise et al., 2010; Wise S et al., 2018). Numerical chromosomal abnormalities, have not been measured in Cr(VI)-induced tumors, however, cell based studies report that Cr(VI) dramatically alters chromosome number in cultured cells treated with Cr(VI) (Speer and Wise, 2018; Wise and Wise, 2010; Wise and Wise, 2018).
Epidemiology, animal, and cell culture studies demonstrate that the particulate form of Cr(VI) is most potent with regards to carcinogenicity (IARC, 1990; Leonard and Lauwerys, 1980). An evergrowing mechanism for particulate Cr(VI)-induced chromosome instability has been proposed by the Wise Laboratory of Environmental and Genetic Toxicology and supported by the literature (Speer and Wise, 2018) (Figure 1.4). Once the particle dissolves, the Cr(VI) oxyanion crosses the cell membrane using sulfate/phosphate transfer proteins aids and is rapidly transported into the cell (Leonard and Lauwerys, 1980; Levy et al., 1986b; Patierno et al., 1988; Langard, 1990). Once Cr(VI) enters the cell, Cr(VI) is rapidly reduced to Cr(III) which forms stable complexes with intracellular structures (e.g., DNA, proteins, and other components) and will accumulate within the cell (De Flora and Wetterhahn, 1989). This can lead to DNA adduct-based lesions and then lead to stalled replication forks and ultimately DNA double strand breaks (Zhitkovich et al., 2001, 2002; Quievryn et al., 2003, 2006; Reynolds and Zhitkovich, 2007; Brooks et al., 2008). These double strand breaks will overwhelm the repair systems of the cells and then go unrepaired or are mis-repaired and ultimately leading to translocations and other structural chromosomal aberrations (Xie et al., 2007, 2008). The accumulation of double strand breaks also causes a G2 arrest leading to centrosome amplification, spindle assembly checkpoint bypass, premature centriole disengagement, centrosome amplification, and eventually numerical chromosome instability (Holmes et al., 2006b; Martino et al., 2015; Wise S et al., 2006). Epigenetic modifications may also contribute to structural and numerical chromosome instability further contributing to neoplastic transformation (Wise and Wise, 2018). Epigenetic modifications may change the DNA packaging and protein and gene signaling and affect the genomic instability observed, however, more
investigations are warranted. Reactive oxygen species produced during the reduction of Cr(VI) to Cr(III) also contribute to oxidative damage in the cell, and if left unrepaired will cause other problems and contribute to the neoplastic transformation (Xu et al., 2018).

Reports of patients with cobalt-chromium metal on metal hip implants have shown increases in chromosome instability (measured in their peripheral blood) and increases in chromosomal aberrations and translocations (was not overall statistically significant) (Briggs et al., 2015; Dunstan et al., 2008). Interestingly, the first time point was at 1 year follow up and patients had increased translocations (statistically significant), while the aberrations had a strong linear relationship with Cr levels (not statistically significant) (Briggs et al., 2015). However, the lasting consequences of these outcomes are uncertain. To elucidate the impact that chromium may have on these results, Figgett et al. (2010) examined the effects of physiologically relevant metal (cobalt and chromium) levels on human primary skin fibroblasts. They reported that cells treated with these physiological concentrations of Cr(VI), had both numerical and structural chromosome aberrations, and complex aneuploidy.

Cr(VI)-induced DNA damage can be repaired by several mechanisms depending on the type of damage. Base excision repair, crosslink repair, homologous recombination, mismatch repair, nucleotide excision repair, and single strand break repair have all been investigated in the repair of Cr(VI)-induced DNA damage as reviewed by Speer and Wise (2018). All of the lesions types caused by Cr(VI) if left unrepaired or mis-repaired, can have a direct negative impact on chromosome structure and overall genomic integrity of the cell and ultimately lead to carcinogenesis (Figure 1.4).
**Figure 1.4. Mechanisms of Numerical and Structural CIN in Cr(VI) Carcinogenesis.** This figure shows some of the major mechanisms underlying CIN in our proposed mechanism of Cr(VI) carcinogenesis. Particulate Cr(VI) dissolves extracellularly and if the cation enters the cell there is no contribution to the effect. Similarly, if the Cr(VI) particle enters the cell by phagocytosis there is also no contribution to the effect. When the Cr(VI) oxyanion enters the cell it is reduced to Cr(III) and ROS are produced. The ROS can induce oxidative damage which base excision repair (BER) attempts to repair. However, if it fails double strand breaks form. Positively charged Cr intermediates and Cr(III) form as result of the intracellular reduction process potentially binding to cellular elements including the formation of DNA-Cr adducts or crosslinks and consequentially stalled replication forks and the formation of a DNA double strand break. Crosslink repair or MMR can attempt to resolve these effects, but if they fail will result in double strand breaks. The formation of double strand breaks results in a G2 arrest as the attempts to repair the damage. Cr(VI)-impaired HR repair leads to the use of a low fidelity repair mechanism and structural CIN. At the same time, Cr(VI) induces spindle assembly checkpoint bypass and premature centriole disengagement leading to centrosome amplification and numerical CIN. Underlying structural and numerical CIN are Cr(VI)-induced epigenetic alterations have yet to be elucidated. Finally, taken together structural and numerical CIN contribute to the neoplastic transformation of Cr(VI) exposed and cancer. Reprinted from Reference Module in Chemistry, Molecular Sciences and Chemical Engineering, 2018, Speer RM and Wise, JP Sr. Current Status on Chromium Research and Its Implications for Health and Risk Assessment, Pages No. 10, Copyright (2018), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also, Lancet special credit - "Reprinted from The Lancet, 2018, Speer RM and Wise, JP Sr. Current Status on Chromium Research and Its Implications for Health and Risk Assessment, Pages No. 10, Copyright (2018), with permission from Elsevier."
1.7 Dysregulated Cellular Energetics and Cancer

The seminal peer-reviewed article by Hanahan and Weinburg (2010), currently list the hallmarks of cancer as self-sufficiency in growth signals, insensitivity to anti-growth signals, evading programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion, and metastasis, deregulated metabolism, evading the immune system, genome instability, and inflammation. Altered energy metabolism was first purposed as an important part of carcinogenesis back in the 1920s by Otto Warburg (Warburg et al., 1927). Typically, the major pathways affected are glycolysis, lactate metabolism, lipid metabolism, mitochondrial respiration, pentose phosphate pathway, and one carbon and nucleotide synthesis. It has also been reported that cancer cells and tumors have increased glutamine and glucose uptake compared to normal cells or adjacent non-cancerous tissues.

1.7.1 Increased Anaerobic Glycolysis or the “Warburg Effect”

Energy metabolism was first suggested as a vital to carcinogenesis back in the 1920s by Otto Warburg. In his initial publications and book Warburg noticed that cells from cancer tissue slices had increased fermentation (we would later call this anaerobic glycolysis or lactate fermentation) and some had increased respiration, but typically he reported an increased in fermentation, similar to the Pasteur effect, while normal cells and tissues had high levels of respiration and low fermentation levels. Subsequently, as our understanding of normal cellular energetics evolved, so did Warburg’s interpretation of his results grew. He would later in the 1950s refer to what he previously called fermentation as anaerobic respiration or conversion of glucose to pyruvate and then pyruvate to lactic
acid (lactate and H\(^+\)) and refer to the conversion of pyruvate into the mitochondrial for use oxidative phosphorylation as aerobic glycolysis or aerobic respiration (Warburg, 1956a, 1956b). Additionally, Warburg noted that most cancer cells or tumor tissues studied (when compared to normal cells and tissues) had increases in their anaerobic respiration, he noted this phenomenon stayed even in the presence on ambient oxygen and seemed to be a permanent change of the cancer cells. Whereas normal cells will switch to an increase of aerobic glycolysis or aerobic respiration in the presence of ambient or physiological oxygen respiration (Warburg, 1956a, 1956b). Researchers in the 1970s would revisit Warburg’s finding only briefly and it wasn’t until the technology evolved with our understanding of cellular energetic at the turn of the century did cancer researchers really go back and understand the impact of Warburg’s work (Weinhouse, 1976). Since his time, the paradox Warburg described, of an increased of anaerobic glycolysis in the presence of ambient and physiological oxygen, with a sometimes lower level of the oxidation of pyruvate, has been termed the “Warburg effect” and is permanent energy metabolism shift (Otto, 2016). Aerobic glycolysis with oxidative phosphorylation produces more ATP than anaerobic glycolysis and it remains paradoxical that the cells do not revert back after oxygen levels are increased. Glycolysis is 10 step process that converts glucose to pyruvate. In the presence of oxygen, pyruvate then enters the mitochondria and is converted to Acetyl-CoA by pyruvate dehydrogenase (PDH) and enters the Citric Acid Cycle (TCA)/Kreb cycle, this is aerobic respiration. The process of glucose to acetyl-CoA is called aerobic glycolysis. Conversely, in the absence of oxygen pyruvate is converted to lactate, named anaerobic respiration. The process of glucose to lactate is termed anaerobic glycolysis (Figure 1.5).
There is now a growing body of literature in many cancer cells and tumor systems, which supports, the increase of anaerobic glycolysis of cancer cells and tumors. This change to an increase of anaerobic glycolysis over aerobic glycolysis is thought to provide the cancer cells with a survival advantage in the harsh tumor microenvironment. However, it remains unclear whether if the “Warburg effect” is a cause or consequence of tumor development or a contributing of both (Otto, 2016).
Figure 1.5. Glycolysis Overview. Glycolysis is a 10 step process that converts glucose to pyruvate. In the presence of oxygen, pyruvate then enters the mitochondria and is converted to Acetyl-CoA by pyruvate dehydrogenase (PDH) and enters the Citric Acid Cycle (TCA)/Kreb cycle, this is aerobic respiration. Conversely, in the absence of oxygen pyruvate is converted to lactate, this is anaerobic respiration.
**1.7.2 Increased *de novo* Lipogenesis in Cancer**

Remarkably, Warburg also noticed that tumors were very fatty compared to adjacent normal tissue and appeared to be making more lipids. Most adult cells acquire necessary lipids via exogenous sources; these lipids originate from dietary sources or are synthesized in the liver and adipose (Otto, 2016; Santos and Schulze, 2012). *De novo* lipogenesis occurs in the liver, adipose tissue, lactating breast, and at very low basal levels in other tissues. Increased *de novo* lipogenesis and its proteins [ATP-citrate lyase (ACLY), acetyl-CoA carboxylase alpha/1 (ACC1), and fatty acid synthase (FASN)] expressions have been reported as key to carcinogenesis processes (Otto, 2016; Santos and Schulze, 2012).

In human cells, ACLY converts citrate into acetyl-CoA. The source of citrate can be from acetate or glutamine metabolism, citrate uptake or citrate from the citric acid cycle. Acetyl-CoA then has four ultimate destinations: (1) TCA/Kreb cycle; (2) cholesterol synthesis; (3) acetylation of proteins or histones; (4) lipogenesis. In the case of lipogenesis, Acetyl-CoA is then carboxylated by ACC1 to make malonyl-CoA. Finally, through a series of steps involving NAPDH and more acetyl-CoA, malonyl-CoA is converted by FASN to palmitate (the first free fatty acid produced by the cell) (Figure 1.6). Phosphorylation of ACLY can increase its activity, while phosphorylation of ACC1 decreases its activity. This palmitate can then be used to make triglycerides and phospholipids or for palmitoylation of proteins.

Specifically, increases in lipogenesis and these proteins have been observed in breast, colon, and lung, ovarian, pancreatic, and prostate cancers. Lipid increases in cancer
cells are important for different aspects of cancer development; (1) they may be stimulated by oncogenic signaling (e.g. EGFR, HER2, HIF-1α, etc.); and (2) they directly contribute to the growth and proliferation of cancer cells, oxidative stress resistance, and survival under energy stress, redox balance, and invasive properties of cancer (Menendez and Lupu, 2007; Santos and Schulze, 2012). Furthermore, overexpression of ACC1 induced neoplastic transformation in murine skin epidermal cells (Li et al., 2016). Overexpression of FASN drove neoplastic transformation of human mammary epithelial cells (Vazquez-Martin et al., 2008; Yang et al., 2002). These studies show that these proteins when overexpressed can induce neoplastic transformation and may be oncogenes when overexpressed.

In human lung patients, these proteins increased expression is associated with decreased patient survival. ACLY overexpression had a hazard ratio of 6.242 in lung cancer patients (Osugi et al., 2015). In the case of ACC1, its phosphorylation (inactivity) is associated with increased lung adenocarcinomas and squamous cell carcinomas patient survival and risk ratio of mortality was 0.5 (95% CI, 0.06-0.7; P = .01) (Conde et al., 2007). FASN tumor expression was linked to a decreased lung squamous cell carcinoma patient survival (Visca et al., 2004).

Inhibition of ACLY, ACC1, and FASN causes a loss of many cancer cell properties, e.g. decreased tumor growth and loss of growth in soft agar, programmed cell death and decreased cancer cell proliferation, and increased drug sensitivity to chemotherapies (Migita et al., 2008; Menendez and Lupu, 2007; Orita et al., 2007; Santos and Schulze, 2012).
Figure 1.6. *De novo* Lipogenesis. The source of citrate can be from acetate or glutamine metabolism, citrate uptake or citrate from the citric acid cycle. ACLY converts citrate into acetyl-CoA. Acetyl-CoA then has four ultimate destinations: (1) TCA/Kreb cycle; (2) cholesterol synthesis; (3) acetylation of proteins or histones; (4) lipogenesis. In the case of lipogenesis, Acetyl-CoA is then carboxylated by ACC1 to make malonyl-CoA. Finally, through a series of steps involving NAPDH and more acetyl-CoA, malonyl-CoA is converted by FASN to palmitate (the first free fatty acid produced by the cell) (Figure 1.4). Phosphorylation of ACLY can increase its activity, while phosphorylation of ACC1 decreases its activity.
1.7.3 Pentose Phosphate Pathway in Cancer

An elevation of the pentose phosphate pathway in cancer cells has been postulated as a way to distinguish cancer cells from normal cells. Intermediates from pentose phosphate pathway feed into other metabolism pathways (i.e., lipogenesis) and intermediates from other energy pathways (i.e., glycolysis) can be used in the pentose phosphate pathway. Increasing the pentose phosphate pathway leads to: (1) high NADPH levels which can reduce ROS, high levels of nucleotides for DNA synthesis and repair; (2) may promote resistance to therapies for increasing oxidative stress and DNA damage; (3) drug resistance; (4) sustained high levels of glucose-6-phosphate dehydrogenase and glutathione after drug resistance (Ferretti et al., 1993; Friesen et al., 2004; Gessner et al., 1990; Patra and Nissim, 2015; Yeh et al., 1987). Multiple cancer cell lines studies have been reported to have the acquisition of drug resistance accompanied by elevation of the oxidative pentose phosphate pathway. Drug resistance could be reversed by treatment from inhibition of early steps in the pentose phosphate pathway (Patra and Nissim, 2015). Therefore, targeting the pentose phosphate pathway has also been proposed as a therapeutic target for cancer (Patra and Nissim, 2015).

1.7.4. One-Carbon Metabolism and Nucleotide Synthesis in Cancer

One-carbon metabolism and nucleotide biosynthesis are important in one line with such cancer-related metabolic alteration (Seyfried and Shelton, 2010; Shuvalov et al., 2017). Increased one-carbon metabolism and nucleotide biosynthesis are found in many different cancers (Locassale et al., 2013; Vazquez et al., 2013). One carbon metabolism
can provide additional “building blocks” (e.g., nucleotides, some amino acids) and contributes to epigenetic (e.g., DNA methyltransferases and protein methylation) and redox homeostasis. There have been limited studies on developing drugs for the inhibition of one-carbon metabolism enzymes Shuvalov et al., 2017). It is proposed due to the necessity of some one-carbon metabolism pathways for normal cell functions, the targeting of enzymes in these pathways can be difficult and may need to be used in combination with other treatments.

1.7.5. Mitochondria and Cancer

Mitochondria changes in cancer have demonstrated as part of the dysregulated energy metabolism reported in cancer. There are multiple endpoints of mitochondria to investigate, this includes mitoDNA, mitochondrial respiratory function, mitophagy (mitochondria autophagy), mitochondria membrane potential, fusion, and fission. There are reports investigating mitochondrial respiration during transformation of human cell lines. For example, one study found that mitochondrial dysfunction plays a pivotal role in pancreatic carcinogenesis (Hardie et al., 2017). It was reported that mitochondrial dysfunction is important in prostate carcinogenesis (Stueckle et al., 2012). However, most studies are done in cancer cell lines and there still is needed for more studies for the role of mitochondria dysfunction during the transformation process.

1.8. Chromium and Cellular Energetics

There remains limited reports on Cr(VI)-induced carcinogenesis and dysregulated cellular energetics. One group reported that acute exposure to Cr(VI) in human skin fibroblasts could upregulate cholesterol metabolism and SREBP-1 (Guo et al., 2013). SREBP-1 is the main transcription factor for ACLY, ACC1, and FASN (Santos and
Schulze, 2012). Another study, acute exposures to high concentrations of Cr(VI) to human lung epithelial cells exhibited increased glycolytic function and decreased mitochondrial respiration (Cerveira et al., 2014). Conversely, human skin cells that survived the toxicity from exposure to high concentrations of Cr(VI) has elevated spare respiratory capacity of their mitochondria (Nickens et al., 2012). These studies indicate that acute exposures to Cr(VI) can induce dysregulated cellular energetics, but it is unclear from these data whether these changes are due to a response to the toxicity of Cr(VI) or a permanent change.

Rationale for this study is supported by evidence from previous reports. First, as mentioned above, some preliminary reports show that Cr(VI) exposure can alter cellular energetics. Second, previous reports indicate that Cr(VI) can activate the PI3K/AKT pathway and EGFR and dthat ownstream signaling of EGFR (Kim et al., 2015, 2016) increases of both pathways have been implicated in upregulation of lipogenesis in cancer cells (Bia et al., 2015; Lei et al., 2016; Migita et al., 2008; Orita et al., 2007; Otto, 2016; Santos and Schulze, 2012) Next, HIF-1α is constitutively activated in Cr(VI)-transformed lung cells (Kim et al., 2015, 2016; Wang et al., 2011) and HIF-1α is able to induce FASN expression (Menendez et al., 2005) Lastly, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) has been reported to be constitutively activated in Cr(VI)-transformed lung cells, but has altered function (Kim et al., 2015, 2016; Pratheeshkumar et al., 2016, 2017; Wang et al., 2011) and it was previously reported that Nrf2 may help control metabolic enzymes and control ACLY expression in murine liver, however how Nrf2 is involved with ACLY regulation remains unclear (Kitteringham et al., 2008; Liu et al., 2018). These studies suggest that in Cr(VI)-transformed cells, given the dysregulation of upstream factors, lipogenesis may become activated and be a key to Cr(VI)-transformed cell survival and
cancer properties and be a potential therapeutic target. Additionally, Cr(VI) causes acutely to glycolysis and mitochondria, and thus Cr(VI)-transformed cells may have dysregulated mitochondria. Further because most cancer cells exhibit a permanent shift to anaerobic glycolysis or the “Warburg effect”, and increases in anaerobic glycolysis, which are associated with increased lipogenesis (Santos and Schulze, 2012), it is plausible this pathway will be upregulated in Cr(VI)-transformed cells.

1.9. Study Overview

Given, the lack of literature on energy metabolism in Cr(VI) carcinogenesis, we chose to investigate some of these energy pathways in Cr(VI)-induced carcinogenesis. Using the model Cr(VI)-transformed human lung cells, we chose to examine lipogenesis, the “Warburg effect”, and mitochondrial respiratory dysfunction. We hypothesize: Cr(VI)-induced transformation of human lung cells will cause altered cellular energetics, specifically resulting in increased de novo lipogenesis, the “Warburg effect”, and mitochondrial respiratory dysfunction.

For this study, we induced neoplastic transformation of human bronchial epithelial airway cells (BEAS-2B cells) by exposing them to sodium chromate for 180 days and we then isolated an individual colony from soft agar [BEAS-2B Cr(VI)-transformed cells]. Parallel untreated control BEAS-2B cells were passaged alongside the treatment dishes and agar dish and used as a passage-matched control cell line. We compared the passage-matched BEAS-2B cells and Cr(VI)-transformed BEAS-2B cells results to two other sets of Cr(VI)-transformed lung cell line and their passage-matched, the two cell line sets used were human bronchial epithelial airway cells (BEP2D cells) and human lung fibroblasts (WTHBF-6 cells). We also performed a tumor growth assay using the xenograft nude
mouse model for all sets of cells used. We isolated some xenograft tumor-derived cells from the Cr(VI)-transformed fibroblasts. We then investigated three aims:

Specific Aim #1: \textit{Cr(VI)-transformed cells exhibit increased de novo lipogenesis, which is important to their carcinogenic properties.}

Specific Aim #2: \textit{Mitochondrial respiratory dysfunction does not occur during \textit{Cr(VI)-transformation but rather during tumorigenesis.}}

Specific Aim #3: \textit{Cr(VI)-transformed cells do not exhibit increased anaerobic glycolysis or the 'Warburg effect'.}

In brief, we observe increased lipogenesis enzymes and activity in the \textit{Cr(VI)}-transformed cells as compared to their passage-matched control cells. This pathway was important for the \textit{Cr(VI)}-transformed BEAS-2B cells. No mitochondrial respiratory dysfunction in the \textit{Cr(VI)}-transformed cells as compared to their passage-matched control cells was observed. We did see mitochondrial respiratory dysfunction in xenograft tumor-derived cells. We observed no increased anaerobic glycolysis or “Warburg effect” in the \textit{Cr(VI)}-transformed cells or the xenograft-tumor derived cells. Lastly, we show that human chromate-induced lung cancer tissues had increased pACLY, ACLY, ACC1, and FASN expressions and lung tumor tissues had no changes in LDHA expression.

\textbf{1.10. Summary}

The known mechanism of \textit{Cr(VI)}-induced carcinogenesis include: (1) \textit{Cr(VI)} is a potent genotoxic agent that acts as a carcinogen by inducing DNA double strand breaks, chromosome instability, altered DNA repair, and genomic alterations, epigenetics plays a role with the genomic endpoints; (2) \textit{Cr(VI)} induces reactive oxygen species that cause
downstream changes in protein signaling and redox imbalance; and (3) Cr(VI) is able to increase cell death evasion and growth signal expressions. (4) Lastly, growing evidence suggests that Cr(VI) is able to induce altered cellular energetics.
Chapter 2 Materials and Methods

2.1. Materials

2.1.1. Cell Culture

Phosphate buffered saline (PBS), Trypsin/EDTA, sodium pyruvate, penicillin/streptomycin, LHC, LHC-8, LHC-9, and L-Glutamine were purchased from Invitrogen (Carlsbad, CA). Dulbecco’s minimal essential medium and Ham’s F-12 (DMEM/F-12) were purchased from Mediatech (Herndon, VA). Cosmic calf serum was purchased from Hyclone (Logan, UT). Tissue culture dishes, flasks, and plasticware were purchased from Corning (Acton, MA). Moxi GO II™ and cassettes were purchased from ORFLO Technologies (Ketchum, ID). Ethanol was purchased from VWR Life Science (Radnor, PA).

2.1.2. Metals and Metal Preparation

Sodium chromate (NaCrO₄), anhydrous 98%, was purchased from Alfa Aesar (Haverhill, MA). 0.22 µM filters and syringes were purchased from VWR Life Science (Radnor, PA).

2.1.3. Drug Preparation and Treatment

4-Methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75) dimethyl Sulfoxide (DMSO) were purchased from Millipore-Sigma (St. Louis, MO).

2.1.4. Antibodies

Primary antibodies for acetyl-CoA carboxylase 1 (ACC1), phosphorylated acetyl-CoA carboxylase (Ser79) (pACC), ATP-citrate lyase (ACLY) and phosphorylated ATP-citrate lyase (Ser455) (pACLY), fatty acid synthase (FASN), histone 3 (H3), hexokinase I, hexokinase II, lactate dehydrogenase A (LDHA), phosphofructokinase (PFKP), pyruvate
dehydrogenase (PDH), pyruvate kinase isozymes M1/M2 (PKM 1/2), and pyruvate kinase
isozymes M2 (PKM 2) were purchased from Cell signaling (Danvers, MA). Primary
antibodies for sterol regulatory binding protein 1 was purchased from BD Biosciences
(Franklin Lakes, NJ). Primary antibodies for acetyl-CoA carboxylase alpha, ATP-citrate
lyase, fatty acid synthase, and lactate dehydrogenase B (LDHB) were purchased from
Abcam (Cambridge, United Kingdom). Primary antibodies for phosphorylated acetyl-CoA
carboxylase alpha (Ser79) and phosphorylated ATP-citrate lyase (Ser455) were purchased
from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibody for β-actin were
purchased from Sigma-Aldrich (St. Louis, MO). Secondary antibodies for anti-mouse and
anti-rabbit HRP were purchased from Cell Signaling (Danvers, MA).

2.1.5. Protein Preparation and Quantification

PBS was purchased from Invitrogen (Carlsbad, CA). Cell lifters were purchased
from VWR Life Science (Radnor, PA). 10x RIPA buffer was purchased from Cell
Signaling (Danvers, MA). Protease inhibitor cocktail and phosphatase inhibitor were
purchased from Millipore-Sigma (St. Louis, MO). Phenylmethylsulfonyl fluoride (PMSF)
was purchased from RPI (Mt. Prospect, IL). Bradford assay reagents were purchased from
Bio-Rad (Hercules, CA). Albumin standards were purchased from ThermoFisher Scientific
(Waltham, MA).

2.1.6. Immunoblotting

3-8% tris-acetate gels, tris-acetate buffer, and gel cassettes were purchased from
Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) was purchased from Gemini
Bio-Products (West Sacramento, CA). 2-beta mercaptoethanol, bromophenol blue,
ponceau, and sodium azide was purchased from (St. Louis, MO). Acrylamide/bis-
Acrylamide (29:1 Ratio Solution), ammonium persulfate (APS), glycine, glycerol, sodium
chloride, sodium dodecylsulfate (SDS) tris, and Tween 20 were purchased from RPI (Mt. Prospect, IL). Precision Plus Protein Dual Color Standards was purchased from Bio-Rad (Hercules, CA). Acetic Acid, hydrochloric acid, methanol and N,N,N',N'-tetramethylethylenediamine (TEMED) was purchased from VWR Life Science (Radnor, PA).

2.1.7. Immunofluorescence

Xylene and ethanol were purchased from VWR Life Science (Radnor, PA). Antigen unmasking solution and Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) were purchased from Vector laboratories Heat-Inactivated horse serum was purchased from Invitrogen (Burlingame, CA). Secondary antibodies against mouse and rabbit for Alexa Fluor 488 and Alex Fluor 568 were purchased from Invitrogen (Carlsbad, CA).

2.1.8. Oil Red O Staining

Oil red O stain was purchased from Millipore-Sigma (St. Louis, MO). Ethanol, 0.22 μM filters and syringes were purchased from VWR Life Science (Radnor, PA). Formaldehyde was purchased from Ricca Chemical (Batesville, Indiana).

2.1.9. Growth Curve

Moxi GO II™ and cassettes were purchased from ORFLO Technologies (Ketchum, ID).

2.1.10. Metabolism Kits

The glycolysis cell based kit was purchased from Cayman Chemical Company (Ann Arbor, MI). Free Fatty Acid Quantification Kit from BioVision (Milpitas, CA). Chloroform and TRITON X-100 were purchased from VWR Life Science (Radnor, PA).

2.1.11. Seahorse Analyzer
Antimycin A, L-carnitine, 2-deoxyglucose, glucose, glutamine, Etomoxir, oligomycin, palmitate, rotenone, sodium pyruvate, ultra fatty acid free BSA were purchased from Millipore-Sigma (St. Louis, MO). XF base medium, 96 well seahorse plates, and seahorse cartridges were purchased from Agilent Technologies (Santa Clara, CA).

2.1.12. Soft Agar Transformation Assay and Colony Isolation

Agar was purchased from Fisher Scientific (Hampton, NH). 4-Nitro blue tetrazolium chloride (NBT) was purchased from Roche Holding AG (Basel, Switzerland).

2.1.13. Xenograft Tumor Studies

Athymic nude mice, NU/NU, 6–8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME). 22 gauge needles and syringes were purchased from VWR Life Science (Radnor, PA). Matrigel™ GFR Membrane Matrix was purchased from Corning Inc. (Corning, NY).

2.1.14. Cell Line Development

Collagenase type II was purchased from Stemcell Technologies (Vancouver, CA). 0.22 µM cell filters were purchased from BD Biosciences (San Jose, CA).

2.1.15. Statistics

Graphpad Prism 7 was purchased from GraphPad Software (La Jolla, CA).

2.2. Methods
2.2.1. Cells and Cell Culture

BEAS-2B, BEP2D and WTHBF-6 were used as model human lung cells, respectively. BEAS-2B are SV40 immortalized human bronchial airway cells purchased from ATCC (Ke et al., 1988). BEP2D cells are HPV E6 and E7 immortalized human bronchial airway cells, which were gifted by Curtis Harris at the National Institute of
Health (Wiley et al., 1991). WTHBF-6 cells are hTERT-expressing human lung fibroblasts and were gifted by the Wise Laboratory of Environmental Health and Toxicology at the University of Louisville (Wise et al., 2004b). These cells exhibit a diploid karyotype, normal growth parameters and an extended lifespan. WHBF-6 and transformed WHTBF-6 cell lines were cultured in a 50:50 mix of Dulbecco’s minimal essential medium and Ham’s F12 medium plus 15% cosmic calf serum, 1% L-glutamine and 1% penicillin/streptomycin. BEAS-2B and transformed BEAS-2B cell lines were cultured in LHC-9 media. BEP2D and transformed BEP2D cell lines were cultured in LHC-8 media. All cells were maintained in a 37°C, humidified incubator with 5 % CO₂. At least once a week, cells were subcultured using 0.25% trypsin/1mM EDTA solution and all experiments were performed on logarithmically growing cells.

2.2.2. Metals and Metal Preparation

Sodium chromate is a soluble form of Cr(VI) and was dissolved as a solution in ddH₂O as 5 mM and filtered through a 0.22 μM filter. Then diluted further in water to prepare final treatment concentrations in media as 0, 0.25, 0.5, 1, and 2.5 μM Cr(VI).

2.2.3. Inhibition Compound Preparation and Treatment

C75 was prepared aseptically in DMSO. DMSO was also used as a vehicle treatment. 0, 1, 5, and 10 μM C75, and 0.1% DMSO treatments were used in fresh media.

2.2.4. Chronic Chromium Treatment

BEAS-2B cells were exposed to sodium chromate (0.25 and 0.5 μM) for 180 d (6 months) and routinely cultured with sodium chromate re-added during sub-culturing and when media was refreshed (48-72 h). Passaged match control BEAS-2B were cultured alongside the two treatment groups. Following 6 months of exposure, cells were seeded into agar and a colony was isolated from the 0.5μM treatment group. Passage match control
BEAS-2B cells were cultured alongside the agar dishes. Additionally, BEAS-2B cells, BEP2D cells, and WHTBF-6 cells were seeded at 650,000 cells (BEAS-2B and BEP2D cells) and 500,000 cells (WHTBF-6 cells) in 100 mm dishes 48 h prior to treatment. Then cells were treated for 24 h with the 0, 0.25, 0.5, 1 or 2.5 µM sodium chromate. Dishes were then harvested for protein for immunoblotting.

2.2.5. Protein Preparation and Quantification

Cells were washed with 4°C cold PBS 2x and scraped with a cell scraper on ice in cold PBS. Pellets were collected in a microcentrifuge tube and centrifuged at 5,000 at 4°C for 5 min. PBS was then aspirated. Pellets were homogenized in 60-100 µL of RIPA buffer (RIPA buffer with protease cocktail inhibitors and phosphatase inhibitor). Pellets were incubated on ice for 10 min and sonicated at 1 amplitude for 1 sec. Then pellets were centrifuged at 16g for 10 min at 4°C. A Bradford assay was performed to measure the protein concentrations against protein standards. Samples were then prepared at 30 µg/15 µL in RIPA buffer and 1x sample buffer.

2.2.6. Immunoblotting

Western blot analysis was carried out using standard laboratory procedures. Protein samples were heated at 95°C for 10 min and then centrifuged at 16g for 10 min at 4°C. For all glycolytic-related enzymes: 30 µg of protein samples were loaded into mini-prep tris-glycine gels and run in a tris-glycine buffer. Running time was about 2 hours at -100 v. For all lipogenesis related enzymes: 30 µg of protein samples were loaded into mini-prep 3-8% tris-acetate gels and run in a tris-acetate buffer. Running time was about 90 min at -125 v. Gels were transformed to nitrocellulose membranes overnight at 20 mAmps. To visualize and confirm transfer, membranes were incubated in Ponceau S for 5 min. Membranes were then blocked in 5% blocking buffer (BSA) for 1 h. Membranes were incubated in primary
antibodies up to 72 h at 4°C. Primary antibodies were diluted at 1:1,000 for all except β-actin (1:5,000) in 1% BSA in TBST + 200 μL sodium azide. Following primary antibody incubation, membranes were washed 6 times with 1x TBST buffer over 1 h. Membranes were then incubated with secondary antibodies (HRP antibodies) for 1 h in 2.5% blocking buffer (BSA in TBST) at 1:5,000 (β-actin was 1:10,000). Following incubation with secondary antibodies, membranes were washed 6 times with 1x TBST buffer over 1 h. Membranes were then exposed to ECL reagent for up to 5 min and the developed on the Azure c600 and bands were excised using Photoshop.

2.2.7. Patient Lung Sample Collection

Lung tumor tissue and adjacent normal lung tissue from three chromate workers were obtained from the Tokushima University hospital, Tokushima, Japan. Worker 1 was a male non-smoking chromate worker (Age 62) that had been exposed to a chromate (Na2Cr2O7, K2Cr2O7, CrO3, and Cr2O3) in Hokkaido, Japan for 19 years. This worker was diagnosed with stage I (T1N0M0) squamous lung carcinoma. Worker 2 was a male non-smoking chromate worker (Age 61) that had been exposed to chromate (Na2Cr2O7, K2Cr2O7, CrO3, and Cr2O3) in Hokkaido, Japan for 38 years. This worker was diagnosed with stage II (T2N1M0) squamous lung carcinoma.

2.2.8. Immunofluorescence

Formalin-fixed tissue slices from the chromate workers were incubated at 60°C overnight to dissolve the paraffin. Tissues then went through a series of xylene and ethanol washes to remove the paraffin and rehydrate the tissue. Tissues were incubated with unmasking solution for 30 minutes at 95°C then washed twice with TBS (with 0.025% Triton). Tissues were then blocked with 10% horse serum with 1% BSA in TBS (with
0.025% Triton) and washed twice with TBS (with 0.025% Triton). Tissues were then incubated with primary antibodies overnight at 4°C. Primary antibodies were diluted 1:50 in TBS with 1% BSA. Primary antibodies were removed and tissues were washed twice with TBS (with 0.025% Triton) and re-probed with secondary antibody at room temperature for 1 h. Secondary antibodies were removed and tissues were washed three times with TBS. Slides were mounted with a cover slip and mounting solution containing DAPI. Finally, the cells were visualized using Olympus BX53 fluorescence microscope (Pittsburgh, PA).

**2.2.9. Oil Red O Staining**

Cells were seeded into 6 well plates and when cells reached 80-90% confluence were processed for lipid droplet formation. Briefly, dishes were washed with PBS three times and fixed with 4% formaldehyde for 40 min. Cells were then incubated in Oil Red O solution (3 parts Oil Red O to 2 parts water) for 40 min. Following staining with Oil Red O the dishes were washed 3 times with water and imaged on the Zeiss Observer A1 Microscope (Carl Zeiss AG, Germany). Following imaging cells were dissolved and quantified. Quantification was carried out at 490nm on the BioTek EL800 plate reader (Winooski, Vermont).

**2.2.10. Growth Curve**

BEAS-2B Cr(VI)-transformed cells (B2B-CrT cells) were treated with the FASN inhibitor C75 for 24 h in a 100 mm dish. Cells were washed with PBS once and trypsinized then centrifuged at 1000 rpm at 4°C for 5 min and resuspended in media after collecting the cell pellet. Cells were counted with the Orflo cell counter. Each concentration was seeded into 3 wells of a 6 well plate at 12,500 cells (this was considered day 0). One well per
concentration was harvested and counted per concentration every two days for up to six days.

2.2.11. Metabolism Kits

2.2.11.1. L-Lactate Acid Levels

Cells were seeded in 96 well plates at the density to achieve 90% confluence after 24 h in appropriate growth medium. A blank well without cells was included as the control. After 24 h incubation, L-Lactate concentrations in the culture medium were measured using the glycolysis cell based kit purchased from Cayman Chemical Company.

2.12.2. Palmitic Acid Levels

Cells (1 x 10^6) were harvested for free fatty acids. Cells were homogenized by pipetting in 200 μL of chloroform with 1% triton-x. Samples were centrifuged at 16g for 5 min at 4°C. Then the lower phase (non-polar) was transferred to a new tube and dried at 50°C, vacuum centrifuged for 30min and resuspended in 200 μL of sample buffer and 50uL of sample was used per well. Then the manufactures’ instructions for the Free Fatty Acid Quantification Kit from BioVision were followed and quantification was carried out at 560nm on the BioTek plate reader. Using a standard curve and correcting for the ¼ dilution factor, palmitic acid levels were determined for all samples. Three independent experiments were performed for each cell line.

2.2.12. Seahorse Analyzer

2.2.12.1. Fatty Acid Oxidation

The Seahorse XF96 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA) was used to measure fatty acid oxidation in all cells. 24 h prior to the assays BEAS-2B, BEP2D, and WTHBF-6 cells were seeded at a density of 4.0 x 10^4, 4.5 x 10^4, and 3.5 x 10^4 cells per well in a XF96 plate, respectively. The seeding densities used for
the Cr(VI)-transformed cells were the same as their passage-matched control cells. To measure the rate of fatty acid oxidation, cells were incubated overnight in their normal cell culture media with 0.5mM L-carnitine supplementation in XF96 cell culture plates. Forty-five minutes prior to the beginning of OCR measurement, the cells were switched to the Assay Medium (DMEM with 25 mM glucose, 0.5 mM carnitine, 2 mM glutamine, and 1 mM pyruvate). Etomoxir (40 µM) was added to one set of cells to reveal the amount of FAO-associated OCR. The endogenous FAO-respiration was calculated by subtracting OCR levels in ETO-treated cells receiving BSA from those in untreated cells receiving BSA. The exogenous FAO-respiration was calculated by subtracting OCR levels in ETO-treated cells receiving BSA-palmitate from those in untreated cells receiving BSA-palmitate. All Seahorse experiments were performed in at least triplicate by the Redox Metabolism Shared Resource Facility at the University of Kentucky.

2.2.12.2. Glycolytic Rate Assay

The Seahorse XF96 Extracellular Flux Analyzer was used to measure glycolysis activity in all the cells. 24 h prior to the assays BEAS-2B, BEP2D, and WTHBF-6 cells were seeded at the density of 4.0x10^4, 4.5x10^4, and 3.5x10^4 cells per well in a XF96 plate respectively. The glycolytic rate tests were performed per manufacturer’s protocol. For injections 10mM glucose, 1uM oligomycin, and 50 mM 2-deoxyglucose were used. The relative levels of non-glycolytic acidification, glycolysis, glycolytic capacity and glycolytic reserve were calculated based on ECAR data obtained in the glycolysis stress using Seahorse Wave software (Agilent Technologies) for XF analyzers. All Seahorse experiments were performed by the Redox Metabolism Shared Resource Facility at the University of Kentucky.

2.2.12.3. Glycolytic Stress Test
The Seahorse XF96 Extracellular Flux Analyzer was used to measure glycolysis activity in all the cells. 24 h prior to the assays BEAS-2B, BEP2D, and WTHBF-6 cells were seeded at the density of 4.0x10^4, 4.5x10^4, and 3.5x10^4 cells per well in a XF96 plate respectively. The glycolytic rate tests were performed per manufacturer’s protocol. For injections 0.5 mM antimycin A, 0.5 µM rotenone, and 50 µM 2-deoxyglucose were used. The basal glycolysis and compensatory glycolysis were calculated based on the Proton Efflux Rates using the Seahorse Wave software for XF analyzer. All Seahorse experiments were performed by the Redox Metabolism Shared Resource Facility at the University of Kentucky.

2.2.12.4. Mitochondrial Stress Test

The Seahorse XF96 Extracellular Flux Analyzer was used to measure mitochondrial respiratory activity in all cells. 24 h prior to the assays BEAS-2B, BEP2D, and WTHBF-6 cells were seeded at a density of 4.0 x 10^4, 4.5 x 10^4, and 3.5 x 10^4 cells per well in a XF96 plate, respectively. The seeding densities used for the Cr(VI)-transformed cells were the same as their passage-matched control cells. The mitochondrial stress tests were performed per manufacturer’s protocol. For basal media for the experiments we used XF base medium with 25 mM glucose, 2.0 mM glutamine, and 1.0 mM pyruvate. During the assay, the values used for injections were 100 µM oligomycin and 50 mM 2-deoxyglucose. For the injections of FCCP BEAS-2B control and Cr(VI)-transformed cell lines received 0.3 µM FCCP and BEP2D and WTHBF-6 control, Cr(VI)-transformed cell lines, and xenograft tumor-derived cells received 0.6 µM FCCP. Data were normalized to baseline read for the third oxygen consumption read and is presented as a percentage (Wise J et al., 2018). Coupling efficiency was not normalized to the baseline as it has an internal normalization in its calculation. The basal respiration, coupled respiration, coupling
efficiency, maximal respiration, proton leak, non-mitochondrial oxygen consumption and spare respiratory capacity were calculated using the Seahorse Wave software for XF analyzers. All Seahorse experiments were performed in at least triplicate by the Redox Metabolism Shared Resource Facility at the University of Kentucky.

2.2.13. Soft Agar Transformation Assay and Colony Isolation

To confirm malignant transformation occurred of the Cr(VI) treated BEAS-2B cells, an anchorage-independent cell growth assay was used. Soft agar colony formation assays were performed as described (Wise J et al., 2018). Briefly, 2 mL of 0.67% agar in LHC-9 media was placed into each well of a 6-well culture plate for the bottom layer. A suspension (2 mL) containing 5 x 10⁴ cells was mixed with 2 mL of 0.33% agar-LHC-9 and placed on the bottom layer of the agar and grown for 8 weeks (24 h after seeding 1 mL of media was put into each well to keep the agar from drying out). 24 h after plating, cultures were examined microscopically to confirm an absence of large clumps of cells. Colonies were stained with 5% 4-Nitro blue tetrazolium chloride and stained colonies were counted following 24 h in stain. To establish cultures from anchorage-independent colonies, one soft-agar colony was plucked under sterile conditions and then dispersed in trypsin and re-plated into culture dishes.

2.2.14. Xenograft Tumors

Animal studies were conducted in accordance with NIH animal use guidelines and the animal usage protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky (Lexington, KY). Athymic nude mice were housed in a pathogen-free room within the animal facilities at the Chandler Medical Center, University of Kentucky (Lexington, KY). Cells (1 x 10⁶ cells per mouse) from each cell line were re-suspended in serum-free medium containing basement membrane matrix at a
1:1 ratio (total volume of 100 μl) and subcutaneously (s.c.) injected into the flanks of nude mice up and mice were maintained for up to 6 months post injection. A total of 8 injection sites per cell line used. Each mouse received one s.c injection per side (8-12 mice were used per experiment). Mice were checked weekly for tumor appearance. At the termination of the experiment, mice were sacrificed; tumors were excised into pieces for cell line development. Tumor weight and volume were also recorded. Tumor volume was determined by Vernier caliper, using the formula of A x B² x 0.52, where A is the longest diameter of tumor and B is the shortest diameter.

2.2.15. Cell Line Development

Tumors were rinsed briefly with ethanol and then PBS, minced into small pieces and then digested for 2 h with collagenase type II in PBS. Cells were then run through a 40 μM mesh cell filter and plated into T-25 flasks, media was replaced after 72 h and routine culture was carried out once cells reached confluence.

2.2.16. Statistics

Graphpad Prism 7 was used to determine statistical relevance. The Student’s t-test was used to calculate p-values to determine the statistical significance of the difference in means. For tumor incidence, a chi-squared test was performed.
Chapter 3  De novo Lipogenesis is Important for Hexavalent Chromium-Induced Lung Carcinogenesis

3.1. Overview

The first aim of this project is the investigation of whether de novo lipogenesis is important in Cr(VI)-induced lung carcinogenesis. The results in this chapter show Cr(VI) treatment can induce lipogenesis-related cells in human lung cell lines. Further the results report that three different sets of Cr(VI)-transformed lung cells have increased lipogenesis related protein expression and increased lipogenesis as compared to their passage-matched control cells. Furthermore, inhibit of a key lipogenesis protein provided evidence that this energy metabolism pathway is important to Cr(VI)-transformed cells. Lastly, lipogenesis protein expressions were higher in chromate-induced lung tumors than adjacent normal lung tissue.

3.2. Prolonged Cr(VI) Exposure Induces Lipogenesis-Related Protein Expressions in Human Lung Cell Lines

As mentioned in the introduction (chapter 1), a previous report shows that acute exposures to Cr(VI) can induce SREBP-1 expression in human skin cells. Here, we exposed human lung cell lines to sodium chromate for 120 h (sodium chromate concentrations were chosen due to observed toxicity with higher concentrations). We saw that pACLY, ACLY, and FASN expressions were increased and pACC expression was decreased in BEAS-2B cells treated with Cr(VI) (Figure 3.1A). We observed that pACLY, ACLY, ACC1, and FASN expressions were increased in BEP2D cells treated with Cr(VI) (Figure 3.1B). Lastly, we reported that pACLY, ACLY, ACC1, and FASN expressions were increased in WTHBF-6 cells treated with Cr(VI) (Figure 3.1C).
Figure 3.1. Prolong Exposure to Cr(VI) Induces Lipogenesis-related Protein Expressions in Human Lung Cells. This figure shows three preliminary western blots. (A) BEP2D cells exposed to Cr(VI) for 120 h had increased ACLY, pACLY, ACC1, and FASN expression compared to untreated control. While pACC expression was decreased. (B) BEAS-2B cells exposed to Cr(VI) for 120 h had increased ACLY, pACLY, ACC1, and FASN expression compared to untreated control. (C) WHTBF-6 cells exposed to Cr(VI) for 120 h had increased ACLY, pACLY, ACC1, and FASN expression compared to untreated control. β-actin and α-tubulin were used as loading controls.
3.3. Chromium(VI)-Transformed BEAS-2B Cells Have Increased *de novo* Lipogenesis

Using BEAS-B cells and Cr(VI)-transformed BEAS-2B Cells (B2B-CrT), we investigated lipogenesis endpoints in these cells (protein and functional palmitic acid levels). B2B-CrT cells had increased ACLY, pACLY, ACC1, and FASN protein expressions compared to passage-matched control BEAS-2B cells. SREBP-1 protein expression levels were not increased, indicating that the increase in protein expression is not due to more transcription (Figure 3.2). Phosphorylation of ACC1 indicates decreased activity. We observed that pACC protein expression was lower in the B2B-CrT cells compared to the passage-matched BEAS-2B cells. B2B-CrT cells had increased palmitic acid levels compared to passage-matched control cells (Figure 3.3), confirming a functional change in the lipogenesis pathway. Typically, cancer cells increase lipid production for various different endpoints (e.g. membrane synthesis, changing lipid profiles, protection against oxidation, energy usage, and lipid droplet storage). We measured energy usage and lipid droplet storage. We saw no major changes in endogenous or exogenous fatty acid oxidation of the B2B-CrT cells compared to passage-matched control BEAS-2B cells (Figure 3.4). Lastly, to measure lipid droplet formation, we employed Oil Red O staining. No changes in lipid droplets were observed in the Cr(VI)-transformed cells compared to passage-matched control cells (Figure 3.5).
Figure 3.2. Chromium(VI)-Transformed BEAS-2B Cells Have Increased de novo Lipogenesis Enzymes. Cr(VI)-transformed BEAS-2B cells (B2B-CrT cells) had increased ACLY, pACLY, ACC1, and FASN protein expressions compared to passage-matched control BEAS-2B cells. pACC expression was decreased in the B2B-CrT cells compared to their passage-matched control BEAS-2B cells. β-actin was used as a loading control.
Figure 3.3. Chromium(VI)-Transformed BEAS-2B Cells Have Increased Palmitic Acid Levels. Cr(VI)-transformed BEAS-2B cells (B2B-CrT cells) had increased palmitic acid levels compared to passage-matched control BEAS-2B cells (Data presented as percent increase of passage-matched control BEAS-2B). Data represent 3 experiments, errors bars represent the SEM, *p<0.05 compared to passage-matched control cells.
Figure 3.4. Chromium(VI)-Transformed BEAS-2B Cells Do Not Have Increased Fatty Acid Oxidation. Endogenous and exogenous fatty acid oxidation were measured on the Seahorse Analyzer. Cr(VI)-transformed BEAS-2B cells (B2B-CrT cells) did not have increased fatty acid oxidation as compared to their passage-matched control BEAS-2B cells. Data represent 3 experiments, errors bars represent the SEM, *p<0.05 compared to passage-matched control cells.
Figure 3.5. Chromium(VI)-Transformed BEAS-2B Cells Do Not Have Increased Lipid Droplets. Cr(VI)-transformed BEAS-2B cells (B2B-CrT cells) had no increases in lipid droplet formation (Oil Red O staining) when compared to passage-matched control BEAS-2B cells. Data represent 3 experiments, errors bars represent the SEM, *p<0.05 compared to passage-matched control cells.
3.4. Chromium(VI)-Transformed BEP2D Cells Have Increased *de novo* Lipogenesis

Next we used BEP2D cells and Cr(VI)-transformed BEP2D Cells (BPD-CrT) from a previous study. We investigated lipogenesis endpoints in these cells (protein and functional palmitic acid levels). We aimed to confirm that our results with the BEAS-2B cells were not cell line specific. BPD-CrT cells had increased ACLY, pACLY, ACC1, and FASN protein expressions compared to passage-matched controls. SREBP-1 protein expression levels were not increased, indicating that the increase in protein expression is not due to more transcription (Figure 3.6). We also observed pACC expression was lower in BPD-CrT cells (Figure 3.6). BPD-CrT cells had increased palmitic acid levels compared to passage-matched control cells (Figure 3.8). We saw no changes in endogenous or exogenous fatty acid oxidation of the BPD-CrT cells compared to passage-matched control BEP2D cells (Figure 3.8) Lastly, no changes in lipid droplets were observed in the Cr(VI)-transformed cells compared to passage-matched control cells (Figure 3.9).
Figure 3.6. Chromium(VI)-Transformed BEP2D Cells Have Increased De Novo Lipogenesis Enzymes. Cr(VI)-transformed BEP2D cells (BPD-CrT cells) had increased ACLY, pACLY, ACC1, and FASN protein expressions compared to passage-matched control BEP2D cells. pACC expression was decreased in the BPD-CrT cells compared to their passage-matched control BEP2D cells. β-actin was used as a loading control.
Figure 3.7. Chromium(VI)-Transformed BEP2D Cells Have Increased Palmitic Acid Levels. Cr(VI)-transformed BEP2D cells (BPD-CrT cells) had increased palmitic acid levels compared to passage-matched control BEP2D cells (Data presented as percent increase of passage-matched control BEP2D). Data represent 3 experiments, errors bars represent the SEM, *p<0.05 compared to passage-matched control cells.
Figure 3.8. Chromium(VI)-Transformed BEP2D Cells Do Not Have Increased Fatty Acid Oxidation. Endogenous and exogenous fatty acid oxidation were measured on the Seahorse Analyzer. Cr(VI)-transformed BEP2D cells (BPD-CrT cells) did not have increased fatty acid oxidation as compared to their passage-matched control BEP2D cells. Data represent 3 experiments, errors bars represent the SEM, *p<0.05 compared to passage-matched control cells.
Figure 3.9. Chromium(VI)-Transformed BEP2D Cells Do Not Have Increased Lipid Droplets. Cr(VI)-transformed BEP2D cells (BPD-CrT cells) had no increases in lipid droplet formation (Oil Red O staining) when compared to passage-matched control BEP2D cells. Data represent 3 experiments, errors bars represent the SEM, *p<0.05 compared to passage-matched control cells.
3.5. Chromium(VI)-Transformed WTHBF-6 Cells Have Increased de novo Lipogenesis

Next, using WTHBF-6 cells (C52-2) and Cr(VI)-transformed WTHBF-6 Cells (T23-3 and T73-3) from a previous study, we investigated lipogenesis endpoints in these cells. T23-3 and T73-3 cells had increased ACLY, pACLY, ACC1, and FASN protein expressions compared to passage-matched controls. SREBP-1 protein expression levels were not increased, indicating that the increase in protein expression is not due to more transcription (Figure 3.10). We also found pACC expression was lower in T23-3 and T73-3 cells (Figure 3.10). T23-3 and T73-3 had increased palmitic acid levels compared to passage-matched control cells (Figure 3.11). We observed no changes in endogenous or exogenous fatty acid oxidation of the T23-3 and T73-3 cells compared to passage-matched control cells (Figure 3.12). Similarly, we did not observed no changes in lipid droplets in the Cr(VI)-transformed cells compared to passage-matched control cells (Figure 3.13).
Figure 3.10. Chromium(VI)-Transformed WTHBF-6 Cells Have Increased De Novo Lipogenesis Enzymes. Cr(VI)-transformed WTHBF-6 cells (T23-3 and T73-3 cells) had increased ACLY, pACLY, ACC1, and FASN protein expressions compared to passage-matched control WTHBF-6 cells (C52-2 cells). pACC expression was decreased in the T23-3 and T73-3 cells compared to their passage-matched control WTHBF-6 cells. β-actin was used as a loading control.
Figure 3.11. Chromium(VI)-Transformed WTHBF-6 Cells Have Increased Palmitic Acid Levels. Cr(VI)-transformed WTHBF-6 cells (BPD-CrT cells) had increased palmitic acid levels compared to passage-matched control WTHBF-6 cells (C52-2 cells) (Data presented as percent increase of passage-matched control). Data represent 3 experiments, errors bars represent the SEM, *p<0.05 compared to passage-matched control cells.
Figure 3.12. Chromium(VI)-Transformed WTHBF-6 Cells Do Not Have Increased Fatty Acid Oxidation. Endogenous and exogenous fatty acid oxidation were measured on the Seahorse Analyzer. Cr(VI)-transformed WTHBF-6 cells (T23-3 and T73-3 cells) did not have increased fatty acid oxidation as compared to their passage-matched control WTHBF-6 cells. Data represent 3 experiments, errors bars represent the SEM, *p<0.05 compared to passage-matched control cells.
A

B

Lipid Droplet Formation

Abosrbance (490nM)

0.0  0.5  1.0  1.5  2.0

C52-2  T23-3  T73-3

Cell Line
Figure 3.13. Chromium(VI)-Transformed WTHBF-6 Cells Do Not Have Increased Lipid Droplets. Cr(VI)-transformed WTHBF-6 cells (T23-3 and T73-3 cells) had no increases in lipid droplet formation (Oil Red O staining) when compared to passage-matched control WTHBF-6 cells (C52-2 cells). Data represent 3 experiments, errors bars represent the SEM, *p<0.05 compared to passage-matched control cells.
3.6. Increased \textit{de novo} Lipogenesis Is Important for Chromium(VI)-Transformed Cells

To determine if the increases in lipogenesis is important in Cr(VI)-transformed cell survival and cancer properties, we examined soft agar colony formation, proliferation, and tumor growth after drug inhibition (C75) of FASN. We focused on just the BEAS-2B cells, since all three cell types showed the same metabolism changes following neoplastic transformation by chromium. Multiple reports have shown that inhibition of FASN leads to decreased cancer cell and tumor growth. Following, a 24 h treatment with 0, 1, 5, and 10 µM C75, BEAS-2B and B2B-CrT, C75 treatment decreased FASN expressions (3.14). Using a growth curve assay, we found that C75 negatively impacted the growth of B2B-CrT more than the BEAS-2B cells (Figure 3.15). Treatment with C75 also decreased the soft agar colony formation properties of B2B-CrT cells (Figure 3.16).
Figure 3.14. Drug Inhibition Decreased FASN Protein Expression in Cr(VI)-Transformed BEAS-2B Cells. 24 h treatment with 0, 1, 5, and 10 µM C75 (FASN inhibitor) decreased FASN protein expression in Cr(VI)-transformed BEAS-2B cells (B2B-CrT cells) and passage-matched BEAS-2B cells. DMSO was used as a vehicle control (VC). β-actin was used as a loading control.
Figure 3.15. Increased De Novo Lipogenesis Is Important for Cr(VI)-transformed Cells Growth. 24 h treatment with 0, 1, 5, and 10 µM C75 (FASN inhibitor) decreased Cr(VI)-transformed BEAS-2B cells (B2B-CrT cells) growth as measured with a growth curve [Data presented as number of cells (percent of untreated control)]. C75 reduced the growth of B2B-CrT cells more than passage-matched BEAS-2B cells. DMSO was used a vehicle control. Data represent 3 experiments, errors bars represent the SEM, *p<0.05 compared to untreated cells.
Figure 3.16 Increased de novo Lipogenesis Is Important for Cr(VI)-transformed Cells Growth in Soft Agar. 24 h treatment with 0, 1, 5, and 10 µM C75 (FASN inhibitor) decreased BEAS-2B cells (B2B-CrT cells) soft agar colony formation. (A) Representative soft agar image. (B) Average number of colonies per well for treatments. DMSO was used as a vehicle control. Data represent 3 experiments, errors bars represent the SEM, *p<0.05 untreated cells.
3.7. Chromate-Induced Lung Tumors Show Increased Lipogenesis Protein Expressions

Formalin-fixed lung tissue slides from tumor and normal adjacency obtained from a worked exposed to Cr(VI) were subjected to fluorescence immunostaining with antibodies against pACLY (red), ACLY (red), ACC1 (green), and FASN (red) and DAPI (nuclear control, blue). We observed increased staining of total ACLY and pACLY in Cr(VI)-tumor lung tissues (Figure 3.17E,K and 3.18G,Q, respectively) as compared to the adjacent normal lung tissues (Figure 3.17A,G and 3.18B,L, respectively). ACC1 and FASN protein expressions were also increased in the Cr(VI)-induced lung tumor tissues (Figure 3.18H,R and 3.19E,K, respectively) as compared to normal adjacent lung tissue (Figure 3.18C,M and 3.19B,H, respectively). These results confirm our cell culture findings and show that lipogenesis protein expressions are increased in Cr(VI)-induced lung tumor tissues.
Figure 3.17. Chromate Lung Tumors Show Increased ACLY Protein Expression. Formalin-fixed normal lung tissue from a healthy patient and lung tumor tissue and its adjacent normal tissue from a worker diagnosed with stage I and II lung adenocarcinomas due to occupational exposure to Cr(VI) were subjected to immunofluorescence staining for examination of expressions of ACLY (red) and DAPI (blue) was used for nuclear control. Scale bar is 20 µM.
Figure 3.18. Chromate Lung Tumors Show Increased pACLY And ACC1 Protein Expressions. Formalin-fixed normal lung tissue from a healthy patient and lung tumor tissue and its adjacent normal tissue from a workers diagnosed with stage I and II lung adenocarcinomas due to occupational exposure to Cr(VI) were subjected to immunofluorescence staining for examination of expressions of p-ACLY (red), ACC1 (green), and DAPI (blue) was used for nuclear control. Scale bar is 20 µM.
Figure 3.19. Chromate Lung Tumors Show Increased FASN Protein Expression. Formalin-fixed normal lung tissue from a healthy patient and lung tumor tissue and its adjacent normal tissue from a workers diagnosed with stage I and II lung adenocarcinomas due to occupational exposure to Cr(VI) were subjected to immunofluorescence staining for examination of expressions of FASN (red) and DAPI (blue) was used for nuclear control. Scale bar is 20 µM.
3.8. Importance

The results from this aim demonstrate that during prolong Cr(VI) exposure can induce lipogenesis-related proteins. Further, these resulted obtained for this chapter show that Cr(VI)-induced malignant transformation of human lung cells (normal cells to malignantly transformed cells) results in increased lipogenesis. Further, inhibition of a key protein in this pathway demonstrated that this pathway is important for Cr(VI)-transformed cells. Additionally, these results were not cell type specific (fibroblast versus epithelial cells). Lastly, results obtained using chromate-induced lung tumor tissues from human samples confirmed that these proteins were increased in this tumors compared to normal adjacent tissue, indicating these results are translational to chromate exposures in humans.
Chapter 4 Elucidating a Role of Mitochondrial Respiratory Dysfunction During Hexavalent Chromium-Induced Lung Carcinogenesis

This chapter is reproduced with permission from Begell House Inc. The original text and data can be found: Wise JTF, Wang L, Alstott MC, Ngalame NNO, Wang Y, Zhang Z, and Shi X. Investigating the Role of Mitochondrial Respiratory Dysfunction During Hexavalent Chromium-Induced Lung Carcinogenesis. *Journal of Environmental Pathology, Toxicology and Oncology.* 37(4);317-329. doi: 10.1615/JEnvironPatholToxicolOncol.2018028689.

4.1. Overview

The first part of this study investigates whether mitochondrial respiratory dysfunction occurs during Cr(VI)-induced lung carcinogenesis. These data show that three different sets of Cr(VI)-transformed lung cells do not have mitochondrial respiratory dysfunction as compared to their passage-matched control cells. Furthermore, we observed Xenograft tumor-derived cells did have mitochondrial respiratory dysfunction.

4.2. Chromium(VI)-Transformed BEAS-2B Cells Do Not Exhibit Mitochondrial Respiratory Dysfunction

BEAS-2B cells chronically treated with 0.5 μM Cr(VI) [6 months (180 days)] generated colonies in soft agar (data not shown). We developed Cr(VI)-transformed cells (B2B-CrT) from an isolated colony collected from soft agar. Passage-matched control BEAS-2B cells and B2B-CrT were analyzed for mitochondrial respiration using the Seahorse Analyzer and the mitochondrial stress test assay. The basal respiration, maximal respiration, and spare respiratory capacity of the Cr(VI)-transformed cells (B2B-CrT) were not different from passage-matched control BEAS-2B cells (Figure 4.1A-C). The proton leak, non-mitochondrial oxygen consumption and coupling efficiency were unchanged in the transformed cells compared to the passage-matched control cells (Figure 4.1D-E).
Interestingly, the transformed cells had a higher coupled respiration (Fig. 4.1E). These data indicated that Cr(VI)-transformed BEAS-2B cells did not display mitochondrial respiratory dysfunction and may be more metabolically active.
Figure 4.1. Chromium(VI)-Transformed Bronchial Airway Epithelial Cells (BEAS-2B) Do Not Have Mitochondrial Respiratory Dysfunction. (A) Mitochondrial Respiration Profile for BEAS-2B cells with the relevant injection strategy for the Seahorse Analyzer Mitochondrial Stress Test. (B) Oxygen consumption data for the BEAS-2B and B2B-CrT cells, presented as a baseline percentage to the third oxygen consumption read. (C) Basal respiration, maximal respiration, and spare respiratory capacity for the BEAS-2B and B2B-CrT cells. (D) Proton Leak, Non-mitochondrial oxygen consumption, and Coupled respiration for the BEAS-2B and B2B-CrT cells. (E) Mitochondrial coupling efficiency for the BEAS-2B and B2B-CrT cells. Data are the average of at least three experiments ± the SEM. *P < 0.05.
4.3. Chromium(VI)-Transformed BEP2D Cells Do Not Exhibit Mitochondrial Respiratory Dysfunction

Since cancer cells usually display some mitochondrial respiratory dysfunction, we tested whether our findings were cell-specific by comparing to other Cr(VI)-transformed cell types. We received HPV (E6 and E8) immortalized bronchial epithelial airway cells (BEP2D cells) and Cr(VI)-transformed BEP2D cells (BPD-CrT) from the laboratory of Dr. John P. Wise, Sr. at the University of Louisville, KY (Xie et al., 2007). Passage-matched control BEP2D cells and BPD-CrT were analyzed for mitochondrial respiration using the Seahorse Analyzer and the mitochondrial stress test assay.

The basal respiration, maximal respiration, and spare respiratory capacity of the transformed cells were not different from passage-matched control BEP2D cells (Figure 4.2A-C). The proton leak and non-mitochondrial oxygen consumption were unchanged in the transformed cells compared to the passage-matched control cells (Figure 4.2D). The coupled respiration and coupling efficiency were not statically different from passage-matched control cells (Figure 4.2D and E). These data indicated that BPD-CrT cells did not display mitochondrial respiratory dysfunction. These results are consistent with results from BEAS-2B cells.
A

Mitochondrial Respiration (BEP2D Profile)

- Oligomycin
- FCCP
- AA/Rotenone

- Basal Respiration
- Coupled Respiration
- Proton Leak
- Maximal Respiration
- Non-Mitochondrial Oxygen Consumption

- Spare Capacity

Time (min)
Oxygen Consumption Rate (%)

B

Mitochondrial Respiration

- BEP2D
- BPD-CrT

Time (min)
Oxygen Consumption Rate (%)
Figure 4.2. Chromium(VI)-Transformed Bronchial Airway Epithelial Cells (BEP2D) Do Not Have Mitochondrial Respiratory Dysfunction. (A) Mitochondrial Respiration Profile for BEP2D cells with the relevant injection strategy for the Seahorse Analyzer Mitochondrial Stress Test. (B) Oxygen consumption data for the BEP2D and BPD-CrT cells, presented as a baseline percentage to the third oxygen consumption read. (C) Basal respiration, maximal respiration, and spare respiratory capacity for the BEP2D and BPD-CrT cells. (D) Proton Leak, Non-mitochondrial oxygen consumption, and Coupled respiration for the BEP2D and BPD-CrT cells. (E) Mitochondrial coupling efficiency for the BEP2D and BPD-CrT cells. Data for B-E are the average of at least three experiments ± the SEM. *P < 0.04.
4.4. Chromium(VI)-Transformed Lung Fibroblasts Do Not Exhibit Mitochondrial Respiratory Dysfunction

As mentioned before, we were surprised to find no major respiration changes in our Cr(VI)-transformed cells. To further show that our findings were not unique to immortalization factor or cell type, we compared our results to h-TERT immortalized fibroblasts. We obtained immortalized human lung fibroblast cells (WTHBF-6 cells) and Cr(VI)-transformed WTHBF-6 cells (T23-3 and T73-3) from the laboratory of Dr. John P. Wise Sr. at the University of Louisville, KY (Wise, S.S. et al., 2018). These three different cell lines allowed us to determine if immortalization or cell type plays a role in our observed results. Passage-matched control WTHBF-6 cells (C52-2 cells), T23-3, and T73-3 cells were analyzed for mitochondrial respiration using the Seahorse Analyzer and the mitochondrial stress test assay. The basal respiration, maximal respiration, and spare respiratory capacity of the Cr(VI)-transformed fibroblasts were not different from passage-matched control fibroblast cells (Figure 4.3.A-C). The proton leak, non-mitochondrial oxygen consumption, coupling efficiency, and coupled respiration were unchanged in the transformed cells compared to the passage-matched control cells (Figure 4.3.D and E). These data indicated that Cr(VI)-transformed fibroblasts did not display mitochondrial respiratory dysfunction and confirmed the results of the epithelial cell lines, suggesting that immortalization factors did not play any role.
Figure 4.3. Chromium(VI)-Transformed Human Lung Fibroblasts (WTHBF-6) Do Not Have Mitochondrial Respiratory Dysfunction. (A) Mitochondrial Respiration Profile for WTHBF-6 cells with the relevant injection strategy for the Seahorse Analyzer Mitochondrial Stress Test. (B) Oxygen consumption data for the control WTHBF-6 cells (C52-2 cells) and Cr(VI)-transformed WTHBF-6 cells (T23-3 and T73-3 cells), presented as a baseline percentage to the third oxygen consumption read. (C) Basal respiration, maximal respiration, and spare respiratory capacity for the C52-2, T23-3, and T73-3 cells. (D) Proton Leak, Non-mitochondrial oxygen consumption, and Coupled respiration for the C52-2, T23-3, and T73-3 cells. (E) Mitochondrial coupling efficiency for the C52-2, T23-3, and T73-3 cells. Data the average of at least three experiments ± the SEM. *P < 0.04.
4.5. Cr(VI)-Transformed Cells Grow Tumors in Nude Mice

It was important to further demonstrate our Cr(VI)-transformed cells were malignantly transformed because we did not observe any changes in mitochondrial respiratory dysfunction and most cancer cell lines and tumors reported in the literature exhibit mitochondrial dysfunction (Hsu et al., 2016). We used the xenograft tumor growth assay to demonstrate that all sets of Cr(VI)-transformed cells were malignantly transformed and were able to induce tumors in nude mice. We injected all sets of the transformed cells and their passage-matched control cells into nude mice. Our results showed that B2B-CrT, BPD-CrT, T23-3, and T73-3 cells were able to grow tumors in nude mice (Figures 4.4-4.6). None of the passage-matched control cells grew tumors in nude mice. These data from the tumorigenesis experiments confirm that our Cr(VI)-transformed cells were malignantly transformed.
### A

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Incidence</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEAS-2B</td>
<td>(0/8) 0%</td>
<td></td>
</tr>
<tr>
<td>B2B-CrT</td>
<td>(6/8) 75%</td>
<td>0.007</td>
</tr>
</tbody>
</table>

### B

![Graph showing tumor volume comparison between BEAS-2B and B2B-CrT](graph.png)
Figure 4.4. Chromium(VI)-Transformed Human Lung Bronchial Cells (BEAS-2B) Grow Tumors in Nude Mice. Passage-matched control BEAS-2B cells and Cr(VI)-transformed BEAS-2B cells (B2B-CrT) were injected into the flanks of 6-week old athymic nude mice (1 x 10^6 cells per mouse) and checked weekly for tumor appearance. There were 8 s.c. injection sites per cell line. (A) Tumor incidences. (B) Tumor volume was measured after animal euthanasia. Tumor volume was determined by calipers, following the formula A x B^2 x 0.52, where A is the longest diameter of tumor and B is the shortest diameter. (C) Tumor weights. These data are expressed as the mean ± SEM.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Incidence</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEP2D</td>
<td>(0/8) 0%</td>
<td></td>
</tr>
<tr>
<td>BPD-CrT</td>
<td>(3/8) 37.5%</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**B**

Tumor Volume (mm³)

- BEP2D: 0/8
- BPD-CrT: 3/8

p = 0.057
Figure 4.5. Chromium(VI)-Transformed Human Lung Bronchial Cells (BEP2D) Grow Tumors in Nude Mice. Passage-matched control BEP2D cells and Cr(VI)-transformed BEP2D cells (BPD-CrT) were s.c. injected into the flanks of 6-week old athymic nude mice (1 x 10⁶ cells per mouse) and checked weekly for tumor appearance. There were 8 s.c. injection sites per cell line. (A) Tumor incidences. (B) Tumor volume was measured after animal euthanasia. Tumor volume was determined by calipers, following the formula A x B² x 0.52, where A is the longest diameter of tumor and B is the shortest diameter. (C) Tumor weights. These data are expressed as the mean ± SEM.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Incidence</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C52-2</td>
<td>(0/8) 0%</td>
<td></td>
</tr>
<tr>
<td>T23-3</td>
<td>(5/8) 62.5%</td>
<td>0.03</td>
</tr>
<tr>
<td>T73-3</td>
<td>(3/8) 37.5%</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**B**

![Graph showing tumor volume comparison between cell lines C52-2, T23-3, and T73-3. The graph indicates that T23-3 has the highest tumor volume, with a value of 5/8, compared to C52-2 and T73-3.](image-url)
Figure 4.6. Chromium(VI)-Transformed Human Lung Fibroblasts (WTHBF-6) Grow Tumors in Nude Mice. Passage-matched control WTHBF-6 cells (C52-2 and Cr(VI)-transformed WTHBF-6 cells (T23-3 and T73-3) were s.c. injected into the flanks of 6-week old athymic nude mice (1 x 10^6 cells per mouse) and checked weekly for tumor appearance. There were 8 s.c. injection sites per cell line. **(A)** Tumor incidences. **(B)** Tumor volume was measured after animal euthanasia. Tumor volume was determined by calipers, following the formula A x B^2 x 0.52, where A is the longest diameter of tumor and B is the shortest diameter. **(C)** Tumor weights. These data are expressed as the mean ± SEM.
4.6. Cr(VI)-Lung Fibroblast Xenograft Tumor-Derived Cells Have Mitochondrial Respiratory Dysfunction

Carcinogenesis is considered a multi-step process. The mechanism of metal-induced carcinogenesis can be conceptualized into two stages. During the first stage, normal cells undergo neoplastic transformation into malignantly transformed cells. In this stage, Cr(VI)-induced cellular transformation did not induce mitochondrial respiratory dysfunction. The second stage is the progression from malignantly transformed cells into tumors (tumorigenesis) (Xu et al., 2017). We assessed if xenograft tumor-derived cells have mitochondrial respiration changes. This would further provide evidence that the tumor microenvironments may drive some of the mitochondrial dysfunctions found in cancer cells reported in the literature. From the WTHBF-6 Cr(VI)-transformed cell lines’ xenograft tumor tissues we isolated cells. Specifically, we isolated six cell lines from the T23-3 xenograft tumor tissues and two cell lines from the T73-3 xenograft tumor tissues. Passage-matched control WTHBF-6 cells (C52-2) and the two Cr(VI)-transformed colonies (T23-3 and T73-3), and the xenograft tumor-derived cell lines (T23-3-X2, T23-3-X3, T23-3-X4, T23-3-X5, T23-3-X6, T23-3-X7, T73-3-X2, and T73-3-X3) were analyzed for mitochondrial respiration. Again, mitochondrial respiration was measured using the Seahorse Analyzer.

The basal respiration of xenograft tumor-derived cells was not different between passage-matched control cells (C52-2 cells) and Cr(VI)-transformed cells (T23-3 and T73-3 cells) (Figure 4.7A, B, E, and F). Xenograft tumor-derived cells showed decreases in maximal respiration and spare respiratory capacity compared to the passage-matched control cells and the transformed cell lines (except T23-3-X3 which had increased...
respiration). The proton leak was higher in T23-3-X5, T23-3-X6, T23-3-X7, T73-3-X3, and T73-3-X3 compared to passage-matched control cells (C52-2) (Fig. 7C and G). In most of the xenograft tumor-derived cell lines (T23-3-X2, T23-3-X4, T23-3-X5, T23-3-X6, T23-3-X7, T73-3-X2, and T73-3-X3) coupled respiration was higher than the passage-matched control cells (Figure 4.7C and G). Non-mitochondrial oxygen consumption was lower in all the xenograft tumor-derived cells except T73-3-X2. All of the xenograft tumor-derived cells showed decreased mitochondrial coupling efficiency when compared to passage-matched control fibroblasts and Cr(VI)-transformed cells. These data demonstrate that xenograft tumor-derived cells showed mitochondrial respiratory dysfunction and provided evidence that mitochondrial dysfunction observed in cancer may be, in part, due to tumor microenvironments.
Figure 4.7. Xenograft Tumor-derived Cells Have Dysfunctional Mitochondrial Respiration. (A) Oxygen consumption data for the control WTHBF-6 cells (C52-2) and Cr(VI)-transformed WTHBF-6 cells (T23-3) and xenograft tumor-derived cells (T23-3-X2, T23-3-X3, T23-3-X4, T23-3-X5, T23-3-X6, and T23-3-X7) presented as a baseline percentage to the third oxygen consumption read. (B) Basal respiration, maximal respiration, and spare respiratory capacity for the C52-2, T23-3, T23-3-X2, T23-3-X3, T23-3-X4, T23-3-X5, T23-3-X6, and T23-3-X7 cells. (C) Proton Leak, Non-mitochondrial oxygen consumption, and Coupled respiration for C52-2, T23-3, T23-3-X2, T23-3-X3, T23-3-X4, T23-3-X5, T23-3-X6, and T23-3-X7 cells. (D) Mitochondrial coupling efficiency for the C52-2, T23-3, T23-3-X2, T23-3-X3, T23-3-X4, T23-3-X5, T23-3-X6, and T23-3-X7 cells. (E) Oxygen consumption data for the control WTHBF-6 cells (C52-2) and Cr(VI)-transformed WTHBF-6 cells (T73-3) and xenograft tumor-derived cells (T73-3-X2, and T73-3-X3) presented as a baseline percentage to the third oxygen consumption read. (F) Basal respiration, maximal respiration, and spare respiratory capacity for the C52-2, T73-3, T73-3-X2, and T73-3-X3 cells. (G) Proton Leak, Non-mitochondrial oxygen consumption, and Coupled respiration for C52-2, T73-3, T73-3-X2, and T73-3-X3 cells. (H) Mitochondrial coupling efficiency for the C52-2, T73-3, T73-3-X2, and T73-3-X3 cells. Data are the average of at least three experiments ± the SEM. *P < 0.05 from C52-2. #P < 0.05 from either T23-3 or T73-3.
4.7. Importance

The results from this aim demonstrate that during the early stages of metal-induced carcinogenesis (normal cells to malignantly transformed cells) malignantly transformed human lung cells [due to exposure to Cr(VI)] do not have mitochondrial respiratory dysfunction. Additionally, these results were not cell type specific (fibroblast versus epithelial cells). In the later stages of metal-induced carcinogenesis (transformed cells to tumor), mitochondrial respiratory dysfunction was observed. These results suggest that mitochondrial respiratory dysfunction occurs during the later stages of Cr(VI)-induced carcinogenesis.
Chapter 5 Anaerobic Glycolysis or the ‘Warburg effect’ is not Important for Hexavalent Chromium-induced Lung Carcinogenesis

5.1. Overview

The first part of this study investigates whether the “Warburg effect” or increased anaerobic glycolysis is important to Cr(VI)-induced lung carcinogenesis. These resulted obtained for this chapter show that three different sets of Cr(VI)-transformed lung cells do not increase anaerobic glycolysis as compared to their passage-matched control cells. Furthermore, the same results were observed in xenograft tumor-derived cells.

5.2. Chromium(VI)-Transformed BEAS-2B Cells Do Not Exhibit the ‘Warburg Effect’

Previously we developed a malignantly transformed cell lines from a colony collected from agar for 6 month Cr(VI)-treated BEAS-2B cells (Wise, J et al., 2018). The endpoints to measure properly investigate the “Warburg effect” or increased anaerobic glycolysis include glycolysis measurements and extracellular lactate production. Therefore to investigate this pathway we used passage match control BEAS-2B cells and the Cr(VI)-transformed colony (B2B-CrT) and analyzed functional glycolysis (using the Seahorse Analyzer) and L-lactate levels in the media. We also measured the rate-limiting glycolytic enzymes by western blot analysis [hexokinase I, hexokinase II, lactate dehydrogenase a (LDHA), phosphofructokinase (PFKP), pyruvate dehydrogenase (PDH), pyruvate kinase isomers M1/M2 (PKM 1/2), and pyruvate kinase isomer M2 (PKM2)].

Using the glycolytic stress test, we observed that glycolysis, maximal glycolysis, and glycolytic reserve were not increased in the B2B-CrT compared to BEAS-2B cells (Figure 5.1A-C). To confirm this functional assay, we performed a second glycolytic test,
the glycolytic rate assay. We found that basal glycolysis and compensatory glycolysis (this is the glycolysis following mitochondrial inhibition) was not increased in the Cr(VI)-transformed BEAS-2B cells compared to their passage-matched cells (Figure 5.1D-E). Next, we measured the extracellular L-lactate levels in the media and found that these levels were not statistically different in the transformed cells as compared to the passage-matched control cells (Figure 5.1F). Lastly, we measured the rate-limiting enzyme of glycolysis by western blot and saw no major increases in most the glycolytic enzymes in the B2B-CrT cells, similarly, we did not observe in PDH, LDHA, and LDHB (Figure 5.1G). These data demonstrate that Cr(VI)-transformed BEAS-2B cells do not exhibit the ‘Warburg effect’.
A

Glycolytic Function (BEAS-2B)

Extracellular Acidification Rate (mP/sec/min)

Time (min)

Glucose  Oligomycin  2-DG

Glycolytic Reserve

Glycolytic Capacity

Glycolysis

Non-glycolytic Acidification

B

Glycolytic Function

Extracellular Acidification Rate (mP/sec/min) vs. Time (min)

BEAS-2B
B2B-CrT
D

Glycolytic Rate Assay Profile for BEAS-2B

- Total Proton Efflux
- Glycolytic Proton Efflux

Proton Efflux Rate (pmol/min)

Time (min)

Basal Glycolysis
Compensatory Glycolysis
Rot/AA
2-DG
Mito Acidification
Post 2-DG Acidification
L-Lactate Levels in the Media

L-Lactate (mM)

BEAS-2B

B2B-CrT

141
Figure 5.1. Chromium(VI)-Transformed BEAS-2B Cells Do Not Exhibit the ‘Warburg Effect’. (A) Glycolytic Function Profile for BEAS-2B cells with the relevant injection strategy for the Seahorse Analyzer Glycolytic Stress Test. (B) Glycolytic stress test data for the BEAS-2B and B2B-CrT cells. (C) Glycolysis, Glycolytic capacity, and glycolytic reserve for the BEAS-2B and B2B-CrT cells. (D) Glycolytic Rate Profile for the BEAS-2B cells with the relevant injection strategy for the Seahorse Analyzer Glycolytic Rate Assay. (E) Basal glycolysis and compensatory glycolysis for the BEAS-2B and B2B-CrT cells. (F) Extracellular L-lactate levels for BEAS-2B and B2B-CrT cells. (G) Hexokinase I, Hexokinase II, phosphofructokinase (PFKP), pyruvate kinase isozymes M1/M2 (PKM 1/2), PKM 2, pyruvate dehydrogenase (PDH), lactate dehydrogenase A and B (LDHA, LDHB) protein expressions in BEAS-2B and B2B-CrT cells. Histone 3 (H3) was used as a loading control. Data are the average of at least three experiments ± the SEM. *P < 0.05. Data normalized to theoretical cell numbers based on doubling time in 96 well plates.
5.3. Chromium(VI)-Transformed BEP2D Cells Do Not Exhibit the ‘Warburg Effect’

Given, that most cancer cells exhibit increases to their anaerobic glycolysis. We wanted to confirm our findings with the BEAS-2B Cr(VI)-transformed cells. Therefore using immortalized bronchial epithelial airway cells (BEP2D cells) and a Cr(VI)-transformed BEP2D cells (BPD-CrT) (from the Laboratory of Dr. John P. Wise at the University of Louisville), these cells were previously described (Xie et al., 2007). Passage match control BEP2D cells and the BPD-CrT cells were analyzed for functional glycolysis, L-lactate levels, and glycolytic enzymes. Again, using the glycolytic stress test, we saw no increase in glycolysis, maximal glycolysis, and the glycolytic reserve of Cr(VI)-transformed BEP2D cells compared to the passage-matched control BEP2D cells (Figure 5.2A-C). Using the glycolytic rate assay, we observed no increase in basal glycolysis or compensatory glycolysis of Cr(VI)-transformed BEP2D cells compared to the passage-matched control BEP2D cells (Figure 5.2D-E). We also found no change in the L-lactate levels of Cr(VI)-transformed BEP2D cells compared to the passage-matched control BEP2D cells (Figure 5.2F). Lastly, we saw no increase in the rate-limiting glycolytic enzymes, PDH, LDHA, and LDHB in the Cr(VI)-transformed BEP2D cells compared to the passage-matched control BEP2D cells (Figure 5.2G). Combined with the Figure 5.1 data, these data indicate that Cr(VI)-transformed lung epithelial cells do not exhibit the ‘Warburg effect’.
Glycolytic Rate Assay Profile for BEP2D

- Glycolytic Proton Efflux
- Total Proton Efflux

Proton Efflux Rate (pmol/min)

Time (min)

Rot/AA

2-DG

Compensatory Glycolysis

Mito Acidification

Basal Glycolysis

0 10 20 30 40 50 60 70 80

250

200

150

100

50

0
Figure 5.2 Chromium(VI)-Transformed BEP2D Cells Do Not Exhibit the ‘Warburg Effect’. (A) Glycolytic Function Profile for BEP2D cells with the relevant injection strategy for the Seahorse Analyzer Glycolytic Stress Test. (B) Glycolytic stress test data for the BEP2D and BPD-CrT cells. (C) Glycolysis, Glycolytic capacity, and glycolytic reserve for the BEP2D and BPD-CrT cells. (D) Glycolytic Rate Profile for the BEP2D cells with the relevant injection strategy for the Seahorse Analyzer Glycolytic Rate Assay. (E) Basal glycolysis and compensatory glycolysis for the BEP2D and BPD-CrT cells. (F) Extracellular L-lactate levels for BEP2D and BPD-CrT cells. (G) Hexokinase I, Hexokinase II, phosphofructokinase (PFKP), pyruvate kinase isozymes M1/M2 (PKM1/2), PKM2, pyruvate dehydrogenase (PDH), lactate dehydrogenase A and B (LDHA, LDHB) protein expressions in BEP2D and BPD-CrT cells. Histone 3 (H3) was used as a loading control. Data are the average of at least three experiments ± the SEM. *P < 0.05. Data normalized to theoretical cell numbers based on doubling time in 96 well plates.
5.4. Chromium(VI)-Transformed Lung Fibroblasts Cells Do Not Exhibit the ‘Warburg Effect’

We wanted to further demonstrate that our results were not cell line specific or that immortalization factors affected our results. Immortalized lung fibroblasts (WTHBF-6) and a Cr(VI)-transformed WHTBF-6 cells (T23-3 and T73-3 cells) (from the Laboratory of Dr. John P. Wise at the University of Louisville) were used. These cells were previously described (S.S. Wise et al., 2018). He Passage match control cells (C52-2 cells) and the Cr(VI)-transformed cells (T23-3 and T73-3 cells) were analyzed for functional glycolysis, L-lactate levels, and glycolytic enzymes. Using the glycolytic stress test, we observed no increases in glycolysis, maximal glycolysis, and the glycolytic reserve of Cr(VI)-transformed WTHBF-6 cells compared to the passage-matched control WTHBF-6 cells (C52-2) (Figure 5.3A-C). With the glycolytic rate assay, we observed no increase in basal glycolysis or compensatory glycolysis of T23-3 and T73-3 cells compared to the passage-matched control C52-2 cells (Figure 5.3D-E). Similarly, we also found no change in the L-lactate levels of Cr(VI)-transformed WTHBF-6 cells compared to the passage-matched control WTHBF-6 cells (Figure 5.3F). Lastly, We did not observe any change in the rate-limiting glycolytic enzymes, PDH, LDHA, and LDHB in the Cr(VI)-transformed WHTBF-6 cells compared to the passage-matched control WHTBF-6 cells (Figure 5.3G). Combined with the Figure 1 and 2 data, these data demonstrate that Cr(VI)-transformed lung cells do not exhibit the ‘Warburg effect’ and further shows that cell type and cell immortalization did not affect our results.
L-Lactate Levels in the Media

L-Lactate (mM)

C52-2  T23-3  T73-3

0  10  20  30  40
Figure 5.3. Chromium(VI)-Transformed WTHBF-6 Cells Do Not Exhibit the ‘Warburg Effect’. (A) Glycolytic Function Profile for WTHBF-6 cells with the relevant injection strategy for the Seahorse Analyzer Glycolytic Stress Test. (B) Glycolytic stress test data for the WTHBF-6 cells (C52-2 cells) and Cr(VI)-transformed WTHBF-6 cells (T23-3 and T73-3 cells). (C) Glycolysis, Glycolytic capacity, and glycolytic reserve for the C52-2, T23-3, and T73-3 cells. (D) Glycolytic Rate Profile for the WTHBF-6 cells with the relevant injection strategy for the Seahorse Analyzer Glycolytic Rate Assay. (E) Basal glycolysis and compensatory glycolysis for the C52-2, T23-3, and T73-3 cells. (F) Extracellular L-lactate levels for C52-2, T23-3, and T73-3 cells. (G) Hexokinase I, Hexokinase II, phosphofructokinase (PFKP), pyruvate kinase isozymes M1/M2 (PKM1/2), PKM2, pyruvate dehydrogenase (PDH), lactate dehydrogenase A and B (LDHA, LDHB) protein expressions in C52-2, T23-3, and T73-3 cells. Histone 3 (H3) was used as a loading control. Data are the average of at least three experiments ± the SEM. *P < 0.05. Data normalized to theoretical cell numbers based on doubling time in 96 well plates.
5.5. Cr(VI)-WTHBF-6 Xenograft-derived Cells Do Not Exhibit the “Warburg Effect”

Since we saw did observe increases in anaerobic glycolysis in Cr(VI)-transformed cells compared to passage-matched control cells, we wanted to establish that the ‘Warburg effect’ is a factor from the tumor microenvironment. Therefore using a mouse xenograft tumor model, we isolated 6 cell lines from the T23-3 xenograft tumors and 2 cell lines from the T73-3 xenograft tumors (Wise J et al., 2018). Passage match control WTHBF-6 cells (C52-2) and the two Cr(VI)-transformed colonies (T23-3 and T73-3), and the xenograft-derived cell lines (T23-3-X2, T23-3-X3, T23-3-X4, T23-3-X5, T23-3-X6, T23-3-X7, T73-3-X2, and T73-3-X3) were analyzed for functional glycolysis, glycolytic rate, L-lactate levels, and rate-limiting glycolytic enzymes. Results from the glycolytic stress test revealed that some xenograft tumor-derived cells (T23-3-X5 and T23-3-X6) had an increase in their glycolysis, maximal glycolysis, and glycolytic reserve compared to the C52-2, T23-3, and T73-3 cells (Figure 5.4A-D). Interestingly, we observed no major increase in basal glycolysis or compensatory glycolysis when using the glycolytic rate assay (Figure 5.4E-F). The L-lactate levels in the media were unchanged in the xenograft tumor-derived cells as compared to transformed cell counterparts (Figure 5.4G-H). Lastly, we found no overall increase in the expressions of glycolytic rating limiting enzymes, PDH, LDHA, and LDHB (Figure 5.4I). These data indicate that Cr(VI)-xenograft tumor-derived cells do not exhibit the ‘Warburg effect’ but have some xenograft tumor-derived cells had increased glycolysis.
B

Extracellular Acidification Rate
(units: mM/min/10^6 Cells)

Glycolysis | Glycolytic Capacity | Glycolytic Reserve

- C52-2
- T23-3
- T23-3-X3
- T23-3-X4
- T23-3-X5
- T23-3-X6
- T23-3-X7

* #

159
L-Lactate Levels in the Media

G

H

163
(A) Glycolytic stress data for the control WTHBF-6 cells (C52-2) and Cr(VI)-transformed WTHBF-6 cells (T23-3) and xenograft tumor-derived cells (T23-3-X2, T23-3-X3, T23-3-X4, T23-3-X5, T23-3-X6, and T23-3-X7). (B) Glycolysis, glycolytic capacity, and glycolytic reserve for the C52-2, T23-3, T23-3-X2, T23-3-X3, T23-3-X4, T23-3-X5, T23-3-X6, and T23-3-X7 cells. (C) Glycolytic stress test data for C52-2 cells and Cr(VI)-transformed WTHBF-6 cells (T73-3) and xenograft tumor-derived cells (T73-3-X2, and T73-3-X3). (D) Glycolysis, glycolytic capacity, and glycolytic reserve for the C52-2, T73-3, T73-3-X2, and T73-3-X3 cells. (E) Basal glycolysis sand compensatory glycolysis for C52-2, T23-3, T23-3-X2, T23-3-X3, T23-3-X4, T23-3-X5, T23-3-X6, and T23-3-X7 cells. (F) Basal glycolysis sand compensatory glycolysis for C52-2, T73-3, T73-3-X2, and T73-3-X3, cells. (G) Extracellular L-lactate levels for C52-2, T23-3, T23-3-X2, T23-3-X3, T23-3-X4, T23-3-X5, T23-3-X6, and T23-3-X7 cells. (H) Extracellular L-lactate levels for C52-2, T73-3, T73-3-X2, and T73-3-X3 cells. (I) Hexokinase I, Hexokinase II, phosphofructokinase (PFKP), pyruvate kinase isozymes M1/M2 (PKM 1/2), PKM 2, pyruvate dehydrogenase (PDH), lactate dehydrogenase A and B (LDHA, LDHB) protein expressions in the different cells listed above. Histone 3 (H3) was used as a loading control. Data are the average of at least three experiments ± the SEM. *P < 0.05 from C52-2. #P < 0.05 from either T23-3 or T73-3.
5.6. Chromate-induced Lung Tumors Do Not Show Increased Lactate Dehydrogenase A Protein Expression

Formalin-fixed lung tissue slides from tumor and normal adjacency obtained from a worked exposed to Cr(VI) were subjected to fluorescence immunostaining with antibodies against lactate dehydrogenase A (LDHA) in green and DAPI (nuclear control, blue). The LDHA expression was not increased in Cr(VI)-tumor lung tissues (Figure 5.29E and K) as compared to adjacent normal lung tissues (Figure 5.29B and H). These results confirm our cell culture findings and show that LDHA protein expression is not increased in Cr(VI)-induced lung tumor tissues.
Figure 5.5. Chromate Lung Tumors Compared Do Not Show Increased LDHA Protein Expression. (A-F) LDHA expression in normal tissue as compared to stage I lung tumor tissue (chromate-induced). (G-L) LDHA expression in normal tissue as compared to stage II lung tumor tissue (chromate-induced). LDHA expression is in red, DAPI is blue and was used for nuclear control. Scale bar is 20 µM.
5.7. ATP Production

Recently, it has been proposed that cancer cells normally do not produce more ATP, but rather are upregulating energy metabolism pathways for producing building block for cellular components. To tell if Cr(VI)-transformed cells have increased ATP production as compared to their passage-matched control cells, we used the ATP production assay on the Seahorse analyzer to measure the ATP production. Surprisingly the total ATP production results were mixed. We observed that the Cr(VI)-transformed BEAS-2B cells had lower total ATP production when compared to their passage-matched control BEAS-2B cells (Figure 5.6A). Conversely, we found that the Cr(VI)-transformed BEP2D and WTHBF-6 cells had no differences in their total ATP production when compared to their passage-matched control cells (Figures 5.6B-C). We continued in the investigation of the ATP production assays and measured the ATP production of the xenograft tumor-derived cells and compared them to the Cr(VI)-transformed cells (T23-3 and T73-3 cells) and the passage-matched control cells (C52-2). When examining the total ATP production, we saw a variation between the Xenograft tumor-derived cells (Figure 5.7A-B). Not all of the cell lines had increased total ATP production when compared to the passage-matched control cells. We found that T23-3-X2, T23-3-X3, T23-3-X4, and T23-3-X6 had increased total ATP, while T23-3-X7, T73-3-X3, T73-3-X2, and T73-3-X3 cells had decreased total ATP production. Interestingly, the Cr(VI)-transformed cells when used in these experiments showed lower total ATP when compared to the passage-matched control cells. This is different then what we noted in the section above. We propose this difference is likely due to subtle metabolic variations in the cells. It is important to note is the breakdown of their
ATP production does not change and matches the glycolysis and mitochondrial respiratory results (Wise J et al., 2018).
Total ATP Production

ATP Production Rate (pmol/min)

BEAS-2B

B2B-CrT

*
B

Total ATP Production

ATP Production Rate (pmol/min)

BEP2D

BPD-CrT
Figure 5.6. ATP Production of Chromium(VI)-Transformed Cells. (A) Total ATP production for the BEAS-2B and B2B-CrT cells as measured by the Seahorse Analyzer. (B) Total ATP production for the BEP2D and BPD-CrT cells. (C) Total ATP production for the WTHBF-6 Cells (C52-2) and Cr(VI)-transformed WTHBF-6 cells (T23- and T73-3 cells). Data are the average of at least three experiments ± the SEM. *P < 0.05.
Figure 5.7. ATP Production of Xenograft Tumor-Derived Cells. (A) Total ATP production for control WTHBF-6 cells (C52-2) and Cr(VI)-transformed WTHBF-6 cells (T23-3) and xenograft tumor-derived cells (T23-3-X2, T23-3-X3, T23-3-X4, T23-3-X5, T23-3-X6, and T23-3-X7). (B) Total ATP production Total ATP production for control WTHBF-6 cells (C52-2) and Cr(VI)-transformed WTHBF-6 cells (T73-3) and xenograft tumor-derived cells (T73-3-X2 and T73-3-X3). Data are the average of at least three experiments ± the SEM. *P < 0.05 from C52-2. #P < 0.05 from either T23-3 or T73-3.
5.8. Importance

The results from this aim demonstrate that during Cr(VI)-induced carcinogenesis (normal cells to malignantly transformed cells), malignantly transformed human lung cells due to exposure to Cr(VI) do not exhibit increased anaerobic glycolysis (“Warburg effect”). Additionally, these results were not cell type specific (fibroblast versus epithelial cells). Also, xenograft tumor-derived cells did not have increased anaerobic glycolysis (“Warburg effect”). These data are consistent with results from the chromate-tumor data. The lack of observed “Warburg effect” is important, because these data suggest the possibility that not all energy metabolism dysfunction occur for all forms of cancer. Further these cells could be used to investigate changes independent of the “Warburg effect”.

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Chapter 6 Discussion

6.1. Overview

Hexavalent chromium is a well-established human carcinogen and has been shown to cause respiratory cancers including lung cancer. After decades of research, the underlying mechanisms for its carcinogenicity remain elusive, though many hypotheses have been proposed and multiple pieces of the mechanism are known (Figure 1.4). It is clear from the literature that ROS, redox imbalances, Cr(III)-DNA adducts, Cr(III) and cellular components interactions, epigenetics, genomic instability, cell death pathway evasion, and altered DNA are all key to the mechanism underlying Cr(VI) carcinogenesis. However, other hallmarks of cancer such as dysregulated cellular energetics, remain under investigated. This project investigated the hypothesis that \textit{Cr(VI)}-induced transformation of human lung cells will cause altered cellular energetics, specifically resulting in increased de novo lipogenesis, the “Warburg effect”, and mitochondrial respiratory dysfunction. The rational for these aims were the limit of literature on energy metabolism in Cr(VI) carcinogenesis and the ability of Cr(VI) to activate upstream pathways of energy metabolism. Similarly, some report in the literature suggested acute exposures to Cr(VI) in cell culture studies could alter cellular energetics. Therefore we chose to investigate some of these prominent energy pathways in carcinogenesis in an applied setting, in Cr(VI)-induced carcinogenesis. Using the model Cr(VI)-transformed human lung cells, we chose to examine three specific aims:

\textit{Specific Aim #1: Cr(VI)}-transformed cells exhibit increased de novo lipogenesis, which is important to their carcinogenic properties.

\textit{Specific Aim #2: Mitochondrial respiratory dysfunction does not occur during Cr(VI)}-transformation but rather during tumorigenesis.
Specific Aim #3: Cr(VI)-transformed cells do not exhibit increased anaerobic glycolysis or the ‘Warburg effect’.

In brief, we studied malignant transformed [due to Cr(VI) exposures] human lung cells. Human bronchial epithelial airway cells (BEAS-2B cells) were treated to sodium chromate for 180 days to induced transformation and we isolated an individual colony from soft agar [BEAS-2B Cr(VI)-transformed cells]. Parallel untreated control BEAS-2B cells were passaged in parallel. We then compared the results from BEAS-2B Cr(VI)-transformed cells to two other sets of Cr(VI)-transformed lung cell line sets, human bronchial epithelial airway cells (BEP2D cells) and human lung fibroblasts (WTHBF-6 cells). Further, we also employed a tumor growth assay using the xenograft nude mouse model and isolated xenograft tumor-derived cells from the Cr(VI)-transformed fibroblasts. We observed increased lipogenesis enzymes and activity in all Cr(VI)-transformed cells as compared to their passage-matched control cells. This pathway was demonstrated to be important for the Cr(VI)-transformed BEAS-2B cells. We reported no mitochondrial respiratory dysfunction in the Cr(VI)-transformed cells as compared to their passage-matched control cells, but observed mitochondrial respiratory dysfunction in xenograft tumor-derived cells. We found no increased anaerobic glycolysis or “Warburg effect” in the Cr(VI)-transformed cells or the xenograft-tumor derived cells. Lastly, we found that human chromate-induced lung cancer tissues had increased pACL, ACLY, ACC1, and FASN expressions and lung tumor tissues had no change in LDHA expression. These results suggest that some energy metabolism changes (reported in cancers) occur from Cr(VI)-induced malignant transformation but others require the tumor microenvironment and some may not occur during the transformation process.
6.2. Chromium and Lipid

In chapter 3, we reported on lipid metabolism changes in Cr(VI)-induced transformation, these results are also summarized in table 6.1. We reported that key lipogenesis proteins (ACLY, ACC1, and FASN) were increased in Cr(VI) treated human lung cells for 120 h. We observed that key lipogenesis proteins (ACLY, ACC1, and FASN) were increased in three sets of Cr(VI)-transformed lung cells when compared to their passaged matched control cells. Functionally, we saw these cells were increasing free fatty acid levels (palmitate). Additionally, we observed no changes in lipid droplet formation or fatty acid oxidation of the Cr(VI)-transformed cells. Lastly, we confirmed this pathway was important to Cr(VI)-transformed BEAS-2B cells and these proteins were increased in chromate lung tumors.

Our data in Cr(VI)-transformed cells is consistent with the literature, showing cancer cells have increased lipogenesis proteins (Santos and Schulze et al. 2012). Some studies have implicated that overexpression of these lipogenesis proteins (ACC1 and FASN) are important to the carcinogenesis mechanism and can induce neoplastic cellular transformation. In murine skin cells overexpression of ACC1 drove the neoplastic transformation of these cells (Li et al., 2016). Further, using withaferin A, the authors were able to block the cancer properties from the overexpression of ACC1 (Li et al., 2016). Similarly overexpression of RAS or EGFR led to increased FASN in mammary epithelial cells which drove neoplastic transformation furthermore, this FASN overexpression has been shown to be important to the carcinogenesis of mammary epithelial cells (Yang et al., 2002). A study from Vazquez-Martin et al. (2008) reported that overexpression of FASN activated the HER1/HER2 tyrosine kinase receptors in human mammary epithelial cells and these cells underwent neoplastic transformation. Additionally, Zaytseva et al. (2014)
demonstrated that in colorectal cancer cells with a low expression of FASN, overexpression of FASN led to increased angiogenesis properties. In prostate epithelial cells overexpression of FASN caused neoplastic transformation and transgenic expression of FASN in mice resulted in prostate intraepithelial neoplasia (Miigita et al., 2009).

Our prolonged (120 h) Cr(VI) treatment of human lung cells increased lipogenesis protein expressions, pACLY, ACLY, ACC1, and FASN (Figure 3.1.). It appears that there is a dose dependent increase, additional work is needed to see if this is also time dependent. Guo et al. (2013) reported that acute Cr(VI) treatment of human skin fibroblasts could induce SREBP-1 proteins and change the cholesterol profile of these cells. Additionally, it has been reported that Cr(VI) induced ROS can cause lipid oxidation, specifically causing lipid peroxidation (Leonard et al., 2004). In humans, chromate workers exposed had increased lipid peroxidation in their blood plasma (Elis et al., 2001).

During acute and prolonged exposures to Cr(VI), the cell may respond to upregulate lipogenesis related proteins due to endoplasmic reticulum stress, as endoplasmic reticulum stress can lead to lipid accumulation (Fang et al., 2013). Along with this, it may be upregulating the lipogenesis pathway to increase the amount of free fatty acids to increase the amount of saturated and monounsaturated fatty acids to protect against lipid peroxidation from the acute toxicity of ROS. Polyunsaturated acyl-chains easily undergo peroxidation as compared to saturated and monounsaturated fatty acids. Increased rates of de novo lipogenesis in cancer cells has been linked to a high rate of saturated and monounsaturated fatty acids (Santos and Schuzle, 2012). However more studies are needed to clarify if the cells are upregulating lipid proteins in response to Cr(VI) exposure in order to make more saturated and monounsaturated fatty acids.
Following neoplastic transformation with Cr(VI), we found that Cr(VI)-transformed BEAS-2B, BEP2D, and WTHBF-6 cells compared to their passage-matched control cells had increased pACLY ACLY, ACC1, FASN protein expression and increased free fatty acid levels (palmitic acid). Given, that cancer cells may upregulate de novo lipogenesis for energy usage or energy storage, we measured β-oxidation for energy usage and lipid droplet formation for energy storage. We saw no increases in either endpoint in the Cr(VI)-transformed cells as compared to their passage-matched control cells. Additional investigations are needed to determine if these Cr(VI)-transformed cells are upregulating de novo lipogenesis for changing the lipid profile or for stabilizing EGFR.

The literature supports a positive feedback loop between EGFR and FASN overexpression in cancer. Specifically, a palmitate attached to the EGFR receptor to stabilize it on the membrane and leading to palmitoylation, the EGFR then upregulates the FASN and a positive feedback loop is achieved (Ali et al., 2018; Bollu et al., 2015). Cr(VI)-transformed cells have increased FASN and EGFR expressions and therefore this feedback loop may be highly probable. Cancer cells will change the lipid profile of the cellular membrane for multiple reasons, one being protection against lipid peroxidation (discussed above). Previous reports have implicated lower ROS in Cr(VI)-transformed cells compared to passage-matched control cells (Xu et al., 2018), it is possible this is due to a change in the lipid profile. Future investigations are aimed at examining these two endpoints in Cr(VI)-transformed cells.

Next, we demonstrated that drug inhibition (C75) of FASN caused FASN protein expression, cell proliferation, and soft agar colony formation of Cr(VI)-transformed BEAS-2B cells. These results established that increased de novo lipogenesis is important
for Cr(VI)-transformed cells and overexpression of FASN is a key protein for some cancer properties of these cells. Relat et al. (2012) reported that in A549 cells C75 inhibition inhibited FASN and cancer properties of these cells, our data match these results. C75 has the potential to have an off target effect on the carnitine transporter and negatively affect fatty acid oxidation and therefore is not appropriate for animal studies. These data match reports in lung cancer where overexpression of these lipogenesis proteins is seen in lung cancer cell lines and in patient tumors (Conde et al; 2007; Hess and Igal 2011; Jin et al., 2014; Migita et al., 2008; Orita et al., 2007; Osugi et al., 2015; Piyathilake et al., 2000; Relat et al., 2012; Visca et al., 2004). From the C75 inhibition data it is not clear if the lack of growth in the growth curve experiments is due to cell death or cell cycle arrest. However, it has been reported that inhibition of overexpressed FASN in cancer leads to a cytotoxic buildup of malonyl-CoA (Swinnen et al., 2006). Additionally, C75 treatment was shown to induce apoptosis of human melanoma cells, human breast cancer cells, and human lung cancer cells, (Ho et al., 2007; Puig et al., 2008; Relat et al., 2012). Therefore, it is likely that the inhibition of FASN in Cr(VI)-transformed cells is resulting in cell death. Further, it has been demonstrated that FASN is important for lung cancer cells’ tumorigenesis (Relat et al., 2012). Therefore in our future experiments, we will investigate shRNA knockdown of FASN to demonstrate that FASN is important for Cr(VI)-transformed cell tumorigenesis.

In lung cancer patients overexpression of ACLY and FASN in tumor tissues has been observed and associated with a poor post treatment survival rate (Migita et al., 2008; Orita et al., 2007; Osugi et al., 2015; Piyathilake et al., 2000; Visca et al., 2004). Similarly, increased human lung tumor expression of p-ACC (phosphorylation of ACC1/ACC2
leads to inhibition) is linked to increased patient survival (Conde et al.; 2007). These studies demonstrate that the change in lipogenesis as measured in cell culture studies translates to lung cancer patients. We stained tumor and adjacent normal tissue from 2 chromate-induced lung cancers from chromate workers. One subject was stage I and one subject was stage II, both tumors with squamous cells. We observed that total ACLY, pACLY, ACC1, and FASN expressions were increased in the Cr-tumor tissue as compared to the normal adjacent tissues. We did see the possibility of a link between lung cancer staging and ACC1 and FASN expressions, unfortunately our sample size for these two stages is 1, and therefore a clear link remains unknown. Furthermore these visual protein expression data are important because they demonstrate our cell culture results are consistent with chromate-induced lung cancers in humans.

Additional future studies are needed to investigate the interactions of upstream factors and the change in lipogenesis in Cr(VI)-transformed cells. Specifically, the role of Nrf2 in the lipogenesis changes needs to be investigated, given that Nrf2 is upstream of certain metabolism pathways and that this transcription factor becomes constitutively activated in Cr(VI)-transformed cells compared to passage-matched control cells (Kim et al., 2015; Kim et al., 2016; Kitteringham et al., 2008; Pratheeshkumar et al., 2016; Wang Y et al., 2018). When Nrf2 becomes constitutively activated, it plays an oncogenic role in cancer development (Wang Y et al., 2018). It is likely that Nrf2 may not properly regulate lipid metabolism endpoints as previously described in the literature and may be a major cause for lipogenesis enzyme shifts (Kitteringham et al., 2008; Wang Y et al., 2018). However, given lipid signaling is different in murine livers as compared to humans, it adds further complexity to the role Nrf2 is playing here (Bergen and Mersmann 2005). Lastly,
as discussed above, EGFR and FASN form a positive feedback loop in cancer cells and this positive feedback loop needs to be investigated in Cr(VI)-transformed lung cells (Ali et al., 2018; Bollu et al., 2015).

In conclusion for lipogenesis endpoints, we found that Cr(VI)-transformed cells have increased lipogenesis proteins and increased lipogenesis as compared to passage-matched control cells. These cells are not producing more lipids for energy usage or energy storage. This pathway change is not unique to lung cell type and immortalization factor for the lung cell line does not affect the results. Drug inhibition demonstrated that FASN overexpression is important for Cr(VI)-transformed cells’ survival and cancer properties. Lastly, we saw that pACLY, ACLY, ACC1, and FASN expressions were increased in chromate-induced human lung tumors as compared to adjacent normal lung tissue. Increased lipogenesis and associated enzymes may be a potential therapeutic target in Cr(VI)-induced lung carcinogenesis.
Table 6.1 Results of Lipogenesis Experiments

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>120 h Cr(VI) Exposure</th>
<th>BEAS-2B Cr(VI) Transformed Cells</th>
<th>BEP2D Cr(VI) Transformed Cells</th>
<th>WTHBF-6 Cr(VI) Transformed Cells</th>
<th>Chromate Lung Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth in Agar</td>
<td>---</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>---</td>
</tr>
<tr>
<td>pACLY/ACLY Expressions</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>FASN Expression</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>ACC1 Expressions</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Fatty Acid Oxidation</td>
<td>---</td>
<td>No Change</td>
<td>No Change</td>
<td>No Change</td>
<td>---</td>
</tr>
<tr>
<td>Lipid Droplets</td>
<td>---</td>
<td>No Change</td>
<td>No Change</td>
<td>No Change</td>
<td>---</td>
</tr>
<tr>
<td>FASN inhibition – Soft Agar Colonies</td>
<td>---</td>
<td>Decreased</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>FASN Inhibition Cell Growth</td>
<td>---</td>
<td>Decreased</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
6.3. Chromium and Mitochondria

In chapter 4, we reported on mitochondrial respiratory changes in Cr(VI)-induced transformation, these results are also summarized in table 6.2. Interestingly, we found that xenograft tumor-derived cells have mitochondrial respiratory dysfunction but Cr(VI)-transformed cells did not exhibit mitochondrial respiratory dysfunction.

Currently, there is limited literature on the role of mitochondrial dysfunction during the process of malignant cell transformation. In one report, it was shown that human skin fibroblasts treated with a high concentration (5 µM) of Cr(VI) generated a small number of cell populations that were resistant to Cr(VI) toxicity and survived this acute high Cr(VI) treatment. These survivor cells exhibited no mitochondrial DNA damage compared to control passage-matched cells. Interestingly, these cells had a decreased spare respiratory capacity compared to control (Nickens et al., 2012). It is important to note that it was a very small number of cells that had resistance to high dose acute Cr(VI) exposure. Additionally, in Cr(VI)-transformed cells, we did not observe major changes in their spare respiratory capacity as was found with skin fibroblasts in the aforementioned study. There are a couple of possible explanations for these differences: (1) the previous study focused on acute, high-Cr(VI) exposures and the respiration change could be related to survival during acute Cr(VI) exposure; and (2) another report found that human lung fibroblasts and skin fibroblasts have different sensitivities to acute Cr(VI) exposures (Xie et al., 2015).

In chapter 4, we reported on mitochondrial respiration endpoints in multiple sets of Cr(VI)-transformed lung cells as compared to their passage-matched control cells. We also described the same mitochondrial respiration endpoints of xenograft tumor-derived cells. It was found previously, that Cr(VI) cancer stem-like cells had decreased oxygen consumption when compared to passage-matched control BEAS-2B cells and BEAS-2B
Cr-(VI)-transformed cells (Dai et al., 2017). It is recognized from multiple studies that cancer stem cells have different metabolism compared to cancer cells, which would explain the difference in results obtained from Cr(VI) cancer stem-like cells and our current results (Deshmukh et al., 2016; Dong et al., 2017; Peiris-Pagès et al., 2016). Another report found that acute exposures to Cr(VI) could cause mitochondrial dysfunction (Abreu et al., 2013), this difference from our results could be due to toxicity of Cr(VI) at 48 h. Further, these mitochondrial dysfunction response may represent a survival response to acute Cr(VI) toxicity or the changes reported are early response part of Cr(VI)-induced carcinogenic mechanism.

Our results indicate that mitochondrial respiration is not affected negatively after malignant transformation from Cr(VI) exposures, as evident from comparing Cr(VI)-transformed lung cells to their passage-matched control cells. We found a general trend that some of Cr(VI)-transformed cells have increased coupled respiration, yet some Cr(VI)-transformed cells do not. The differences in some of the respiration functions between the transformed cells could be due to some mitochondrial changes in the cells that are not uniform during cellular transformation and other metabolic endpoints (e.g., increased lipogenesis, mitochondrial membrane potential, alterations to mitochondrial complexes, and mitochondrial DNA changes) may also be involved. We reported that xenograft tumor-derived cells had significant inhibition of their max mitochondrial respiration (following FCCP treatment) and their spare respiratory capacities. These xenograft tumor-derived cells also had decreased coupling efficiency, another important endpoint showing mitochondrial respiratory dysfunction, as it shows these cells are making less ATP to total oxygen used. Interestingly, xenograft tumor-derived cells have increased coupled
respiration compared to their Cr(VI)-transformed cells. Contrary to these results, one xenograft tumor-derived cell line (T23-3-X2) showed increased mitochondrial respiration, providing evidence that not every cell from a tumor undergoes mitochondrial dysfunction. These data indicate that cellular transformation does not cause mitochondrial dysfunction, rather it may be due to the tumor microenvironment.

Ethidium bromide treatment of immortalized prostate epithelial cells caused a depletion of mitochondrial DNA, which is critical factor in various cancer endpoints (e.g. glycolytic metabolism, migration, and survival (Moro et al., 2009). Mutations in mitochondrial DNA were found in patient-derived pancreatic cancer cell lines and further examination of the metabolic profiling led to the finding that complex I, III, and IV were inhibited in some of these patient-derived pancreatic cell lines (Hardie et al., 2017). In the same study, the Hardie et al. (2017) showed that, that in the patient-derived pancreatic cell lines (with complex I inhibition) oxygen consumption was lower than immortalized pancreatic cells. These patient-derived xenografts cell line results are in agreement with our results using the xenograft tumor-derived cells. In human lung cells (BEAS-2B) chronically (6 month) exposed to arsenic were reported to have mitochondrial dysfunction. Additionally, the authors measured the endpoints of gene expressions and found multiple genes associated with mitochondrial dysfunction were lower in the chronically arsenic-treated cells compared to the passage-matched control BEAS-2B cells. Specifically, the expressions of genes associated with the electron transport chain were lower (Stueckle et al., 2012). Although the authors did not isolate a single phenotype in their study of chronic arsenic treatment, these data do indicate a possible difference in mitochondrial response between arsenic and chromium. However, it is possible that there may be mitochondrial
gene changes in Cr(VI)-transformed cells, but more investigations are warranted to confirm this.

Even though there was no mitochondrial respiratory dysfunction in Cr(VI)-transformed cells, other endpoints of mitochondrial dysfunction may be present, including mitochondrial DNA damage, mitophagy dysfunction, and mitochondrial membrane potential changes. However, respiration is unaffected in these cells so it is plausible that the mitochondrial membrane potential is also unaffected. Additional studies are warranted to examine the cause of mitochondrial respiration changes in the xenograft tumor-derived cells. Specifically, Nrf2 role in mitochondrial changes needs to be studied, given that Nrf2 is upstream of many metabolism pathways and Nrf2 becomes constitutively activated in Cr(VI)-transformed cells compared to passage-matched control cells (Kim et al., 2015, 2016; Pratheeshkumar et al., 2016, 2017; Wang Y et al., 2018). Nrf2 when constitutively activated, will play an oncogenic role in cancer development. It is likely that Nrf2 may not properly regulate its metabolism endpoints and may be a major contributing factor for metabolism shifts (Wang Y et al., 2018). Further investigation on the changes in the mitochondrial membrane potential, mitochondrial DNA and mitophagy (mitochondria autophagy) during Cr(VI)-transformation and in Cr(VI)-transformed cell tumorigenesis remains to be investigated.

The results from the chapter 4 demonstrate that during the early stages of metal-induced carcinogenesis (normal cells to malignantly transformed cells) malignantly transformed human lung cells [due to exposure to Cr(VI)] do have mitochondrial respiratory dysfunction. As with the lipogenesis results, these results were not cell type specific (fibroblast versus epithelial cells). In the later stages of metal-induced
cancerogenesis (transformed cells to tumor) we observed mitochondrial respiratory dysfunction. Thus, these results suggest that mitochondrial respiratory dysfunction occurs late in Cr(VI)-induced carcinogenesis.
Table 6.2 Results of Mitochondria Respiration Experiments

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>BEAS-2B Cr(VI) Transformed Cells</th>
<th>BEP2D Cr(VI) Transformed Cells</th>
<th>WTHBF-6 Cr(VI) Transformed Cells</th>
<th>Xenograft Tumor-derived Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth in Nude Mice</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>---</td>
</tr>
<tr>
<td>Basal Respiration</td>
<td>No Difference</td>
<td>No Difference</td>
<td>No Difference</td>
<td>Decreased</td>
</tr>
<tr>
<td>Maximal Respiration</td>
<td>No Difference</td>
<td>No Difference</td>
<td>No Difference</td>
<td>Decreased</td>
</tr>
<tr>
<td>Spare Respiratory Capacity</td>
<td>No Difference</td>
<td>No Difference</td>
<td>No Difference</td>
<td>Increased</td>
</tr>
<tr>
<td>Proton Leak</td>
<td>No Difference</td>
<td>No Difference</td>
<td>No Difference</td>
<td>Decreased</td>
</tr>
<tr>
<td>Non-Mitochondrial Respiration</td>
<td>No Difference</td>
<td>No Difference</td>
<td>No Difference</td>
<td>Decreased</td>
</tr>
<tr>
<td>Coupled Respiration</td>
<td>No Difference</td>
<td>No Difference</td>
<td>No Difference</td>
<td>Decreased</td>
</tr>
<tr>
<td>Coupling Efficiency</td>
<td>No Difference</td>
<td>No Difference</td>
<td>No Difference</td>
<td>Decreased</td>
</tr>
</tbody>
</table>
6.4. Chromium and ‘Warburg Effect’

In chapter 5, we reported on the glycolysis and L-lactate levels in chromium-transformed lung cells as compared to their passaged match control cells. These results are summarized in table 6.3. Our results clearly indicate through functional glycolysis and glycolytic rates that glycolysis is not increased in Cr(VI)-transformed lung cells as compared to passage matched control cells. We also observed no changes in extracellular lactate levels. The same results occurred in Cr(VI)-transformed fibroblast xenograft tumor-derived cells. We also observed a general trend of no increases in expressions in the glycolytic rate-limiting enzymes (Hexokinase, PFKP, PKM 1, and PKM 2), PDH, LDHA, LDHB. These data indicate that there are no increased in anaerobic glycolysis or ‘Warburg effect’ during Cr(VI)-induced lung carcinogenesis in vivo.

In previous report, it was shown that Cr(VI)-cancer stem like cells had increased anaerobic glycolysis as compared to the normal-stem cells and Cr(VI)-transformed cells (Dai et al., 2017). Differences have been found between the energy metabolism of cancer stem like cells and cancer cells (Deshmukh et al., 2016; Peiris-Pagès et al., 2016). Moreover, cancer stem like cells have been shown to have higher levels of anaerobic glycolysis, specifically have higher glycolysis levels, glycolytic enzymes, and L-lactate levels compared to cancer cells (Deshmukh et al., 2016; Hammoundi et al., 2011; Liu et al., 2013; Palorini et al., 2014). We suspect if we isolated Cr(VI)-cancer stem like cells for these Cr(VI)-transformed cell lines that we would see a similar trend as reported by Dai et al. (2017).

It has become well established that most cancer cells and tumors have permanent shift towards anaerobic glycolysis versus aerobic glycolysis, even in the presence of
oxygen, which has been termed the ‘Warburg effect’. Previous studies have looked at L-lactate levels and glycolysis intermediate levels during transformation of mammary epithelial cells and reported increases in lactate levels and glycolysis sugars (Chang et al., 2016; Janzer et al., 2014; Zou et al., 2017). In human lung epithelial cells transformation from radon was shown to increase lactate in late passages (Liu et al. 2016). In human fibroblasts transformation from RAS mutations resulted in increased glycolysis measured by the Seahorse Analyzer but no increases in lactate. In neural stem cells, causing transformation by interfering with metabolism proteins results in increases in glycolysis and lactate (Peruzzo et al., 2016). While, these studies state the “Warburg effect” is result of carcinogenesis (excluding the fibroblast study). They all have their shortcomings when looking at experimental design and their results are over-stated. Each study used a mixed phenotype population of cells when measuring endpoints of anaerobic glycolysis. It is important to use a single phenotype when investigating transformation, by either isolating colonies from soft agar or sub-cloning and selecting colonies that are transformed and grow in agar. Otherwise within the cells tested some not transformed and it is not clear if the results seen in these studies are due to changes in the stably transformed cells in their studies or cells that have a non-stable phenotype. In our study, transformed cells tested were either selected from soft agar or sub-clones that grew in soft agar, we also confirmed transformation of all our transformed cell lines by xenograft tumor growth. Therefore, we consider from our results that increased anaerobic glycolysis in the presence of oxygen or the “Warburg effect” (as measured by functional glycolysis assays, glycolytic enzymes, and lactate levels) does not occur in Cr(VI)-induced carcinogenesis and this may be true for carcinogenesis as a whole in vivo. Interestingly, using chromate lung tumor tissues and
immunofluorescence, we noticed LDHA was not increased in the tumor tissues as compared to the normal lung tissue. We can speculate that it is possible chromate lung tumors do not have the ‘Warburg effect’, however more studies using fresh patient samples are need to measure L-lactate levels and functional glycolysis to confirm this finding. We also found that LDHB (responsible for conversion of pyruvate to lactate) was not increased in the Cr(VI)-transformed cells as compared to the passage-matched control cells, these data provide evidence that the lactate is not being used differently between the Cr(VI)-transformed cells and passage-matched control cells, however using stable isotope labeled lactate is needed to confirm these results.

ATP production was not increased in the Cr(VI)-transformed cells as compared to their passaged matched control cells. There was variability in ATP production and the source of ATP for the xenograft tumor-derived cells. We suspect this variability is likely due to the fact that not all cells in a tumor have the same access to ATP. At this time without further investigation a pattern cannot be discerned. However, overall these ATP data fit with the hypothesis that cancer cells’ dysregulated cellular energetics is for ‘building blocks’ versus increasing total ATP.

A future study is aimed at using the Cr(VI)-transformed cells from this aim to solve a fundamental cancer biology question on if the ‘Warburg effect’ is a consequence or a contributing factor for tumor development. We will aim to knockout LDHA and block the ability of these transformed cells to upregulate anaerobic glycolysis and investigate this chicken or egg question. Furthermore, these cells that don’t overexpress LDHA allow us to investigate dysregulated cellular energetics that are independent of the ‘Warburg effect’. Additionally, isolating cancer stem like cells from these malignantly transformed cells may
show that the cancer stem like cells have undergone ‘Warburg effect’. We may also investigate if the ‘Warburg effect’ occur after putting these malignantly transformed cells in physiological oxygen. Liu et al. (2016) reported their radon-transformed cells handled lower oxygen environments more efficiently than passage-matched control cells and had a higher upregulation of L-lactate.

Lastly, our data demonstrated that in a laboratory setting, the ‘Warburg effect’ does not occur during Cr(VI)-induced transformation of lung cells and does not occur during Cr(VI)-transformed cells’ tumorigenesis. Interestingly, these results may carry over to chromate lung tumors. More studies are needed to establish if the ‘Warburg effect’ occurs in chromate lung tumors in humans or if using a chromate exposure in mice, does the ‘Warburg effect’ occur.
<table>
<thead>
<tr>
<th>Endpoint</th>
<th>BEAS-2B Cr(VI) Transformed Cells</th>
<th>BEP2D Cr(VI) Transformed Cells</th>
<th>WTHBF-6 Cr(VI) Transformed Cells</th>
<th>Xenograft Tumor-derived Cells</th>
<th>Chromate Lung Tumors</th>
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</thead>
<tbody>
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<td>No Change</td>
<td>No Change</td>
<td>No Change</td>
<td>---</td>
</tr>
<tr>
<td>Maximal Glycolysis</td>
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<td>No Change</td>
<td>No Change</td>
<td>No Change</td>
<td>---</td>
</tr>
<tr>
<td>Glycolytic Capacity</td>
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<td>No Change</td>
<td>No Change</td>
<td>No Change</td>
<td>---</td>
</tr>
<tr>
<td>L-Lactate Levels</td>
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<td>No Change</td>
<td>No Change</td>
<td>No Change</td>
<td>---</td>
</tr>
<tr>
<td>Glycolytic Rate</td>
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<td>No Change</td>
<td>No Change</td>
<td>---</td>
</tr>
<tr>
<td>Rate Limiting Glycolytic Enzymes</td>
<td>Variable</td>
<td>Variable</td>
<td>Variable</td>
<td>Variable</td>
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<tr>
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<td>No Change</td>
<td>No Change</td>
</tr>
<tr>
<td>Total ATP Production</td>
<td>No Change</td>
<td>No Change</td>
<td>No Change</td>
<td>No Change</td>
<td>---</td>
</tr>
</tbody>
</table>
6.5. Overall Role of Dysregulated Cellular Energetics in Cr(VI) Carcinogenesis

Reports have shown that acute exposure to Cr(VI) can alter cellular energetics in human cells. Specifically, acute exposure to Cr(VI) can alter the cholesterol proteins and SREBP-1 (Guo et al., 2013), increase glycolysis (Abreu et al., 2014), and alter mitochondrial respiratory function (Abreu et al., 2014; Nickens et al., 2012). Additionally, ROS from Cr(VI) could cause lipid peroxidation (Leonard et al., 2004) and in chromate exposed humans, blood plasma had increased levels of lipid peroxidation (Elis et al., 2001).

The data presented in chapters 3, 4, and 5 demonstrated that dysregulated cellular energetics occur for Cr(VI) carcinogenesis. First in prolonged (120 h) exposures we saw that Cr(VI) could induce lipogenesis protein expression in a concentration dependent manner. Next, we found increased lipogenesis related protein expressions (ACLY, ACC1, and FASN) in Cr(VI)-transformed cells and chromate lung tumors. Additionally, we demonstrated the overexpression of FASN was important for Cr(VI)-transformed cell cancer properties. Interestingly, we also found that Cr(VI)-transformed human lung cells (BEAS-2B, BEP2D, and WTHBF-6 cells) had no major changes in their glycolytic function, glycolytic rate, L-lactate levels or mitochondrial respiration when compared to their passaged matched control cells. Similarly, xenograft tumor-derived cells did not exhibit the ‘Warburg effect’, but did have mitochondrial respiratory dysfunction.

Combined with the literature and our data presented in chapter 3, 4, and 5 it is clear that Cr(VI) exposure and Cr(VI)-induced carcinogenesis results in dysregulated cellular energetics. Acute or prolonged exposures to Cr(VI) elicits a cellular response in the form of an increase in glycolysis, mitochondrial respiration response, and increased lipogenesis, the reasons for which are unclear. These data show that certain energy pathways (lipogenesis and mitochondria respiration) have dysregulated function after malignant
transformation from Cr(VI) exposure. In the case of increased de novo lipogenesis, it is an early carcinogenesis step, while mitochondrial respiration occurs in later steps in Cr(VI) carcinogenesis.

In Warburg’s original findings, he speculated that an insult would cause a cellular energetic response, which later would become permanent after prolonged exposure to this insult and would result in a permanent phenotypic change in energy metabolism. It is possible that this occurs in Cr(VI) carcinogenesis, given the initial changes in energy metabolism due to acute or prolonged Cr(VI) exposures. Then some of these changes in energy metabolism become permanent after malignant transformation from Cr(VI) and further drive the dysregulated cellular energetics. Future studies are needed to investigate other metabolism pathways in Cr(VI)-induced carcinogenesis and to confirm these energy changes are an initial response to Cr(VI) exposures and later become permanent. To conclude some metabolism pathways are important to Cr(VI)-induced carcinogenesis, while others appear not to be.

6.6. Proposed Cr(VI) Carcinogenesis Mechanism

When using models in the laboratory, different changes in human cells occur at different times during Cr(VI) carcinogenesis (figure 6.1). It has been established that after Cr(VI) exposures human lung cells upregulate p62, Nrf2, HIF-1α, and EGFR in response to the Cr(VI) (Kim et al., 2015, 2016; Pratheeshkumar et al., 2016, 2017). Cells also upregulate Nrf2 in response to the ROS from Cr(VI) (Wang et al., 2011; Wang et al., 2016). Additionally, reports have shown that acute exposure to Cr(VI) leads to responses from energetic pathways (Abreu et al., 2014; Nickens et al., 2012, Guo et al., 2013), however the reasons for this is unclear. Acute Cr(VI) exposures also cause genomic instability,
epigenetic changes, and altered DNA responses (Wise and Speer, 2018; Wise and Wise, 2018). Following chronic exposure to Cr(VI), cells undergo neoplastic transformation to malignantly transformed cells. These cells have constitutively activated p62, Nrf2, HIF-1a, and EGFR protein expressions, cell death evasion, and epigenetic changes (Kim et al., 2015, 2016; Pratheeshkumar et al., 2016, 2017). Additionally, Cr(VI)-transformed cells have altered DNA repair and genomic instability (Wise et al., 2018). In chapters 3, 4, and 5 we found Cr(VI)-transformed cells have dysregulated cellular energetics. Next, following tumorigenesis using a xenograft tumor model in nude mice, Cr(VI)-transformed cells developed tumors. Cell lines insulated from these tumors (xenograft tumor-derived cells) had additional cancer properties, including dysregulated cellular energetics (chapter 4 and 5) and preliminary data suggests a near-tetraploid karyotype (data not shown).
Human Lung Cells
- Basal p62, Nrf2, HIF-1α, EGFR
- Respond to cell death pathways
- Regulated cellular energetics
- Normal karyotype
- Functional DNA repair

Cr(VI) Exposure

Cr(VI)-Transformed Cells
- Constitutive p62, Nrf2, HIF-1α, EGFR
- Epigenetic changes
- Cell death evasion
- Dysregulated cellular energetics
  - Increased lipogenesis
  - No increased anaerobic glycolysis
- Genomic instability
- Altered DNA repair

Tumor Development in Nude Mice

Xenograft Tumor-derived Cells
- Dysregulated cellular energetics
  - Mitochondrial respiratory dysfunction
  - Some increases in glycolysis
  - No increased lactate
- Near-tetraploid karyotype
Figure 6.1. Chromium(VI) Transformation Changes.
Human lung cells have basal or physiological levels of p62, Nrf2, HIF-1α, and EGFR. Additionally, these cells have regulated cellular energetics, functional DNA repair, and a normal karyotype. Following Cr(VI) exposure inducing transformation, transformed cells have constitutively activated p62, Nrf2, HIF-1α, and EGFR. These cells also have ability to exhibit epigenetic changes, genomic instability, altered DNA repair, and dysregulated cellular energetics, and evade cell death pathways. Following Cr(VI)-transformed cell tumor development in nude mice, these xenograft tumor-derived cells have a small increase in glycolysis, mitochondrial respiratory dysfunction, and a near tetraploid karyotype.
Next, updating proposed Cr(VI) carcinogenesis mechanism from the Wise Laboratory of Environmental Health and Toxicology, we propose two additional parallel mechanism to be included in this over mechanism: cell death pathway evasions and dysregulated cellular energetics (figure 6.2). The steps between and how the cells are evading cell death and responding with dysregulated cellular energetic remains to be determined. In the case of increased lipogenesis protein the Cr(VI) treated cells may be upregulating the level of monounsaturated and saturated fatty acids to protect against lipid peroxidation. It is also possible that dysregulated energetics may have interactions with cell death, epigenetics, and genomic instability by providing signaling materials or other important molecules (e.g., nucleotides, amino acids, carbohydrates, etc.). However, more studies are needed to investigate this.
Figure 6.2. Updated Cr(VI) Carcinogenesis Mechanism.
Using the mechanism slide from the introduction, here is an updated proposed Cr(V) carcinogenesis mechanism. Two other mechanisms may occur in parallel to the genomic instability and DNA repair changes. Cr(VI) treated cells with damage may evade cell death pathways. Additionally, Cr(VI) appears to induced dysregulated cellular energetics.
6.7. Potential Future Directions

Future directions could be used to address the chicken and egg question in cancer research on dysregulated cellular energetics, what comes first the ‘Warburg effect’ or the tumor? Using the Cr(VI)-transformed cells in this study, we could knockout lactate dehydrogenase A and determine if malignantly transformed cells not relying on this pathway are able to still grow tumors. We will also examine if the ‘Warburg effect’ occur after putting these malignantly transformed cells in physiological oxygen. Additionally, we can investigate metabolism changes independent of the ‘Warburg effect’. Investigating other energy metabolism pathways in Cr(VI)-induced carcinogenesis would be worthwhile, as well as determining FASN and EGFR have a positive feedback loop in Cr(VI)-transformed cells like other cancer cells and examining if Nrf2 has a role in the increased de novo lipogenesis of Cr(VI)-transformed cells. Other investigative avenues include determining other reasons for the increases in lipogenesis aside from signaling (i.e., lipid peroxidation) and what is causing the mitochondrial respiratory dysfunction. Lastly, more investigations with Cr(VI) exposures and energy pathways and key proteins are needed to further establish the role dysregulated cellular energetics are playing in the Cr(VI) carcinogenic mechanism.

6.8. Importance

Multiple studies have established that ACLY, ACC1, and FASN over expression are crucial to the increase in lipid of cancer cells and some malignant features of cancer cells. Additionally, changes in mitochondrial respiration and increased anaerobic glycolysis are characteristics of cancer cells and tumors. Our results to reveal metabolic reprogramming as a key piece in Cr(VI) carcinogenesis. Interestingly, some changes reported in cancer and cancer cells require the tumor microenvironment and may require
xenograft tumor cell lines when doing transformation studies for proper investigation. These results also further indicate dysregulated cellular energetics as a possible parallel mechanism in the proposed Cr(VI) carcinogenesis mechanism (Figures 1.4 and 6.2), however more detailed investigations are required as well as further studies to understand the overlap between the mechanisms. Lastly, these data open the door for the lipogenesis proteins as therapeutic targets in chromate induced cancers and in addition, chromium may be involved in other diseases involving dysregulated cellular energetics.

6.9. Conclusions

Our data demonstrated that increased lipogenesis occurs in malignant transformation of human lung cells [due to Cr(VI) exposure]. Our data also showed that mitochondrial respiratory dysfunction requires the tumor microenvironment for Cr(VI)-transformed cells and is a later event in Cr(VI) carcinogenesis. Lastly, increased anaerobic glycolysis does not occur in Cr(VI)-transformation. The lipogenesis results were able to translate to chromate lung tumors in humans. These data established that dysregulated energy metabolism occurs after Cr(VI)-induced cellular transformation.
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November 8, 2018

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References


Vita

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EDUCATION AND DEGREES:
2007-2013 Bachelor’s Degree in Biology with a Minor in Toxicology and Environmental Health from the University of Southern Maine.

2003-2007 Cape Elizabeth High School Cum Laude

APPOINTMENTS:
2014-2019 Graduate Research Assistantship, Department of Pharmacology and Nutritional Sciences, College of Medicine, University of Kentucky

2017-2019 Website and Social Media Manager, Wise Laboratory of Environmental and Genetic Toxicology, Wise Lab Field Research Program, a project of The Ocean Foundation, Washington, D.C.

2015-Present Field Project Volunteer of Wise Laboratory of Environmental and Genetic Toxicology, Wise Lab Field Research Program, a project of The Ocean Foundation, Washington, D.C.

2011-2015 Social Media Manager, Wise Laboratory of Environmental and Genetic Toxicology, Maine Center for Toxicology and Environmental Health, University of Southern Maine

2009-2013 President and Co-Founder of University of Southern Maine’s Environmental Health and Toxicology Club, University of Southern Maine, Portland, Maine

2008-2015 Website Manager of Wise Laboratory of Environmental and Genetic Toxicology, Maine Center for Toxicology and Environmental Health, University of Southern Maine, Portland, Maine

2007-2009 Team member (Involved in protocol development as well as lab work and zero gravity flying at NASA) of Dirigo (First team from Maine), a
team participating in NASA’s Reduced Gravity Student Flight Opportunities Program, Houston, TX

2007-2013 Undergraduate Student Researcher, Wise Laboratory of Environmental and Genetic Toxicology, University of Southern Maine, Portland, Maine

2006-2007 High School Student Researcher, Wise Laboratory of Environmental and Genetic Toxicology, University of Southern Maine, Portland, Maine

2005-Present Volunteer, Ocean Alliance, Gloucester, MA.

RESEARCH EXPERIENCE:

1. Five years of basic research (2014-Present), College of Medicine, University of Kentucky
2. Five years of basic research (2014-Present), Wise Laboratory of Environmental and Genetic Toxicology, Wise Lab Field Research Program, a project of The Ocean Foundation, Washington, D.C.
3. Six years of field research (2008-2013), Wise Laboratory of Environmental and Genetic Toxicology, University of Southern Maine, Portland, Maine
4. Seven years of basic research (2006-2013), Wise Laboratory of Environmental and Genetic Toxicology, University of Southern Maine, Portland, Maine

SCHOLASTIC AND PROFESSIONAL HONORS:

2018 Received Best Student Poster at 10th Conference on Metal Toxicity & Carcinogenesis

2016 Received Best Student Poster at 9th Conference on Metal Toxicity & Carcinogenesis

2016 American Association for the Advancement of Science 3 year Sponsored Membership through the AAAS/Science Program for Excellence in Science

2013 Dean’s List (Spring Semester)

2012 Dean’s List (Fall Semester)

2012 Maine Center for Environmental Health and Toxicology Outstanding Undergraduate Student Award

2012 USM’s Leadership Development Board’s Outstanding Student Leader Award

2011-2012 Maine Space Grant Academic Year Internship
2011 USM’s Leadership Development Board’s Outstanding Student Leader Award
2011 Dean’s List (Fall Semester)
2010 Dean’s List (Fall Semester)
2011 PRIMO16 Student Travel Award
2011 Maine Space Grant Academic Half-Year Internship
2009-2010 Maine Space Grant Academic Year Internship
2009 University of Southern Maine’s Thinking Matters Poster chosen to be presented at President Selma Botman’s Inauguration to University of Southern Maine
2008 Alternate for Undergraduate student travel award, Society of Toxicology
2007 Graduate Cape Elizabeth High School cum Laude
2004 Magna cum Laude – National Latin Exam

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