The Role of Extracellular Matrix Communication in Arsenic-Impaired Skeletal Muscle Stem Cell Determination

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Submitted to the Graduate Faculty of the

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2020

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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May 6, 2020

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Abstract

Hundreds of millions of individuals worldwide are exposed daily to pathogenic levels of arsenic in the environment. Anthropogenic exposures have caused morbidity, disease, and fatalities through millennia. Chronic arsenic exposures increase risks of cancer and non-cancer diseases, of which cardiovascular and metabolic diseases carry the most considerable disease burden. There is increasing realization that declines in skeletal muscle homeostasis and compositional quality underlie the etiology of cardiovascular and metabolic disease risk. However, the contribution and mechanisms of environmental factors, such as arsenic exposure, in promoting these declines are relatively unknown. Thus, this dissertation sought to fill this significant knowledge gap by investigating the hypothesis that arsenic promotes muscle dysfunction by disrupting intercellular communication within the muscle progenitor cell niche that is critical to muscle maintenance and determination of muscle composition. The studies used *in vivo* arsenic exposures with interventions combined with *ex vivo* cell culture experiments to demonstrate that arsenic imparts a dysfunctional memory of intercellular communication into the muscle extracellular matrix (ECM) that disrupts the differentiation fate of myogenic and fibro-adipogenic progenitor cells following injury. Human muscle progenitor cells exposed to low levels of arsenic

or to ECM elaborated by connective tissue fibroblasts exposed to arsenic *in vivo* were misdirected from their myogenic fate to either fibrogenic or adipogenic determination. The studies found that arsenic-directed Notch1 signaling, a master regulator of intercellular communication and cell fate, in the ECM promoted the fibro-adipogenic determination of the progenitor cells. Importantly, *in vivo* intervention with SS-31, a mitochondrial protective peptide, or *ex vivo* pharmacological inhibition of Notch activation reversed the impaired tissue regeneration and dysfunctional cell fate determination caused by arsenic exposures. These studies identify the connective tissue fibroblast mitochondria as a target of arsenic-promoted muscle pathogenesis. The findings may have a significant impact on public health as they would reveal a plausible therapeutic intervention that may reduce the severity and burden of disease caused by arsenic exposures.

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Preface

Acknowledgments

I would first like to thank Dr. Aaron Barchowsky for his time, patience, guidance, and support. This man graciously adopted the role of my Principal Investigator, performing his general scientific stewardship duties in addition to maintaining my sanity throughout the years. I may not have been the most ideal of doctoral candidates, but I hope to have been among the most interesting that he has had in his extensive record of graduate mentees. I wish him health and fortune in all of his future endeavors. He is a man that is very deserving of all that is good and more.

I would like to thank my committee members, who include Dr. Bruce Pitt of the Department of Environmental and Occupational Health, Dr. Fabrisia Ambrosio of the Department of Physical Medicine and Rehabilitation, and Dr. Claudette St. Croix of the Department of Cell Biology. I am thankful for Dr. Pitt's mild temperament and appreciation for science. Dr. Ambrosio, warmly known as Faby, is an admirable woman who exemplifies innovative thought and who motivates all those around her with this exuberant energy that she manifests. Finally, Dr. St. Croix is a woman of science with a killer sense of style and a genuinely kind demeanor capable of putting anyone at ease. I am grateful for having had her as my academic advisor and for taking her imaging course.

I would like to thank my parents, Jose and Socorro Anguiano - my infinite sources of emotional support. I am immensely proud to be the daughter of hardworking immigrants who've made countless sacrifices to build a sustainable life for their three children. I can only hope to have made them as proud of me as I am of them. I also thank my older sister, Yadira Guzman, and

brother, Ernesto Anguiano, for their humor and encouragement throughout the years. Les tengo mucho cariño y amor.

I thank past and current members of the Barchowsky laboratory. I especially thank Dr. Kevin Beezhold and Dr. Yesica Garciafigueroa for extending their friendship and support to me when I initially joined the lab. I would also like to thank all students, professors, and administrative staff in the Department of Environmental and Occupational Health.

Finally, I wish to thank my boyfriend, Stephen Thomas Packosky. Steve is a handsome man with piercing blue eyes and a beguiling crooked smile. He is one of the funniest and generous men I have ever encountered, and I was fortunate to have our paths cross when they did. I fell for his charm and gentle nature; I can only assume he appreciated my knowledge of dystopian literature and found my quirks endearing. His support and affection kept me going when times were stormy and speckled with uncertainty. I will forever consider him to be one of the greatest gifts the universe has granted me because together we are imperfectly perfect.

Chapter 1: Introduction

1.1 Overview of Arsenic

Arsenic is a naturally occurring toxic metalloid, ranking 20th as the most abundant element comprising the earth's crust (Mandal & Suzuki, 2002). Because arsenic is an element, it is ubiquitously distributed in nature (ATSDR, 2016; Oremland & Stolz, 2003). The amount of arsenic in the environment supplied by natural sources, however, is surpassed by the amount of arsenic released into the environment through various anthropogenic activities, of which include: extraction of drinking water from underground aquifers, the combustion of fossil fuels, smelting of multiple metals, hide tanning waste, runoff from mine tailings, pigment production for dyes and paints, preservation of wood, and glass and electronics industries (ATSDR, 2016). Presently, arsenic, in the form of arsine gas and gallium arsenide, is widely used in manufacturing electronics and semiconductor devices (ATSDR, 2016), and inorganic arsenic is commonly used in the manufacture of specialty metals and glasses.

Arsenic (atomic number 33; relative atomic mass 74.92) can be found in several chemical forms and varying oxidations states. There are three primary forms in which arsenic compounds can be found: organic, inorganic, and arsine gas (ATSDR, 2016). The environmentally relevant forms of arsenic, organic and inorganic, can exist in four oxidation states (-3, 0, +3, and +5) (ATSDR, 2016; Hughes *et al.*, 2011). Arsenic is rarely observed in its elemental state (with a valence state of 0) because it can readily form covalent bonds with carbon, hydrogen, oxygen, sulfur, and other elements (Hughes *et al.*, 2011; Oremland & Stolz, 2003). Common arsenic ores include orpiment (yellow arsenic, As2S3), realgar (red arsenic, AsS), and arsenopyrite (grey

arsenic, FeAsS) (Barchowsky, 2020). Arsenic also has the capability of forming alloys with certain metals such as gold, lead, or copper (Barchowsky, 2020; Oremland & Stolz, 2003).

1.1.1 Fate and Transport of Arsenic in the Environment

Soil

Arsenic naturally occurs in the environment, but the abundance of arsenic among soils widely varies depending on the mineral content of the soils and the bedrock from which it is derived, the parent rock (Mitchell, 2014; Smedley and Kinniburgh, 2002). Arsenic is present in more than 200 different mineral species, of which more than half are arsenates, a fifth are sulfosalts and sulfites, and the remaining fifth include elemental arsenic, arsenites, arsenides, sililates, and oxides. (Onishi, 1969). In nature, arsenic can most often occur in its sulfide form in minerals containing copper, lead, silver, cobalt, nickel, antimony, and iron (IARC, 2017). The major arsenic-containing minerals include realgar (As_4S_4), orpiment and arsenic trisulfide (As_2S_3), and arsenopyrites (FeAsS).

In the Earth's crust, arsenic can be found in concentrations ranging from 1.5-2 ppm (NRC, 2014). Arsenic in soils ranges from almost undetectable at 0.1 mg kg^{-1} to 40 mg kg^{-1}, the average concentration measuring around 5-6 mg kg-1 (Bowen, 1979). Elevated levels of arsenic deposition in soil can be attributed to the erosion of bedrock by geothermal waters (Bundschuh et al., 2013; Lord et al., 2012) and mining operations. Arsenic can also accumulate in the soils as dust from volcanic eruptions (Liu et al., 2013), burning of fossil fuels (Piver, 1983), waste from industrial practices (Woolson, 1983), and in the form of agricultural products from the application of fungicides, herbicides, rodenticides, insecticides (Blaurock-Blesch, 2013; Hathaway et al., 1991), fertilizers, cotton desiccants, soil sterilants and defoliants (IARC, 2017). Common arsenical

pesticides include organic arsenicals as ingredients such as monosodium methanearsonate (MSMA V) and dimethylarsinic acid (DMAs V), also known as cacodylic acid (Hughes et al., 2011). Although lead arsenate pesticides have not been in use in the last 50 years, their residues persist in the environment. This poses a significant public health concern for those living in modern residential areas of the United States, where the land was formerly utilized as orchards, which includes acres of land in Washington, Wisconsin, and New Jersey (Hood, 2006).

Water

Water can contain both organic and inorganic forms of arsenic. Aerobic surface waters such as lakes and pond waters can contain methylated forms of arsenic (i.e., MMA and DMA; (Welch *et al.*, 2000), but they mostly include inorganic forms of arsenic as they are highly soluble in water (ATSDR, 2016). Arsenic levels in rivers, however, are higher than concentrations found in lakes, possibly due to the adsorption by iron oxides (Welch *et al.*, 2000) and geothermal activity (Aggett and Kriegman, 1988). Concentrations of naturally-occurring arsenic in groundwater are generally below 10 µg will vary from region to region due to various factors such as geology and climate (Welch *et al.*, 2000). Wells that tap into deep sources of groundwater will contain predominantly arsenite forms (AsIII) (Barchowsky, 2020), which is a highly toxic species. High levels of arsenic in drinking water of many countries are primarily due to the contamination of groundwater, which can be a result of weathering of arsenic-rich ore deposits and acid mine leachate that can contain over 100 mg/L inorganic arsenic (Welch *et al.*, 2000). Due to high geothermal activity in the waters of Japan and New Zealand, the arsenic levels can range up to 6.4 mg /L (Nakahara, 1978) and up to 9.08 mg/L (Lord et al., 2012), respectively. In the United States, areas in Northeast, Northwest, Southwest, and Alaska with a high degree of geologic and tectonic activity have arsenic levels that range over 3 mg/L (ATSDR, 2016).

Air

Arsenic is emitted into the atmosphere through weathering of arsenic-containing minerals and ores, by volcanoes through eruptions, from the burning of vegetation (Chung et al., 2014), and as a byproduct of industrial and commercial processes (ATSDR, 2016). Anthropogenic activities account for an estimated 24000 tons of arsenic released into the atmosphere (IARC, 2017). Arsenic, for example, is released from the smelting process, which is a separation of metal from rock, of various metal ores that include gold, nickel, zinc, lead, and cobalt. Burning of fossil fuels that contain arsenic is another source for arsenical air emissions (ATSDR, 2016). When arsenic is emitted into the atmosphere, it is primarily in the form of arsenous acid (As2O3), where it can adhere to small particulate matter that is dispersed over distances by the wind. Eventually, the particles settle back onto the earth via wet or dry deposition. Microbial sources in sediments and soils can also release arsines into the air, where it is then oxidized. Once the arsenic is converted to non-volatile forms, it can deposit back to the soil or contaminate surface water sources such as rivers and lakes (ATSDR, 2016).

1.1.2 Routes of Human Exposure

Arsenic is ubiquitously distributed throughout the globe, either as natural geological deposition or as a residue byproduct of the utilization of natural resources. It is of no surprise, then, that the human population is exposed to arsenic primarily through air, food, and water. In the occupational setting, workers are mainly exposed to arsenic through inhalation and dermal absorption (Chung et al., 2014). But the vast majority of the global population is substantially exposed daily to arsenic through the consumption of drinking water or the consumption of dietary

sources, specifically rice and fish (Mitchell, 2014). Overall, there are many pathways through which arsenic may adversely affect human health.

Consumption of Arsenic from Contaminated Drinking Water

As a consequence of destructive anthropogenic activities, millions of people around the world suffer from toxic health effects that result from drinking water contaminated with high levels of arsenic (Blaurock-Blesch, 2013). In 2014, the WHO warned that about 200 million people worldwide are exposed to arsenic concentrations exceeding the threshold of 10 µg/L (Gugliemi, 2017). Chronic exposure to high arsenic concentrations in drinking water has been a significant concern in many regions of the world, most notably in Vietnam, Mexico, the United States of American, People's Republic of China, including Taiwan, and in some Central American and South American countries (Naujokas et al., 2013; IARC, 2017; Hunt et al., 2014), the worst affected being West Bengal (India) and Bangladesh (Iyer et al., 2016). These areas may have experienced contamination of arsenic in their groundwater designated for drinking and agricultural purposes. The higher concentrations of arsenic tend to be found in areas where the soil pH is high and near clay and sand younger than 10,000 years. The arsenic in older sediments are expected to have already been washed out to sea as these sediments may have already been exposed to a great deal of water over geological time (Gugliemi, 2017).

Beginning in 1928, tube wells were installed in the rural areas of Bangladesh to be utilized as safe sources of drinking water (Ahmad et al., 2018). The issue with this is that the groundwater, especially water sourced from bedrock wells, became contaminated with naturally occurring high levels of arsenic. When the geographical sediments are manipulated, the arsenic is released from being bound to sulfides, iron oxides, and organic matter and contaminates the groundwater (Welch *et al.*, 2000). In Pakistan, an estimated 1200 wells, which constitutes approximately 2/3 of the

wells throughout Pakistan, exceed the WHO-recommended threshold of arsenic of 10 µg/L (Podgorski *et al.,* 2017). Wells along the Indus River Valley, which spans northwest India and Pakistan, may contain extremely high levels of arsenic (> 200 µg/L). These wells provide water of high arsenic content to an estimated 60 million individuals (Podgorski *et al.,* 2017).

Millions of people were exposed to arsenic after many wells were drilled in Bangladesh and throughout the Indian subcontinent in the 1970s (Ahmad *et al.,* 2018; Podgorski *et al.*, 2017). Before the installation of the tube wells, people in rural areas were limited to surface water sources that include lakes, ponds, canals, and rivers. Most of these sources were heavily contaminated with biological hazards causing cholera and other diarrheal diseases. The groundwater located a few meters below the surface, however, was considered to be more sanitary. The tube wells were an attempt to avoid microbial contamination and incidences of diarrheal disease; they were to provide microbiologically safe water for the rural populations (Ahmad *et al.,* 2018).

By the 1990s, almost all of the total population of Bangladesh (97%) had access to drinking water through tube wells (Ahmad *et al.,* 2018). But by1993, the water that had low microbial content as determined to be contaminated with naturally-occurring arsenic. The WHO estimated that 35 to 77 million people in Bangladesh alone could be at risk of consuming drinking water with unsafe levels of arsenic (Ahmad *et al.,* 2018) as the country has about 6-11 million tube wells containing arsenic (Hawkesworth *et a*l., 2013) The concentrations of arsenic detected in the majority of the wells are within 0.10 – 0.30 mg/L; the highest concentration running as high as 4.7 mg/L (Ahmad *et al.*, 2018).

The concentrations of arsenic in drinking water in some private wells in the United States are as high as 5,000 µg/L, which is in the range of the highest concentrations reported in Bangladesh (USGS, 2017). In an attempt to address the long-term chronic effects of exposure to

low concentrations of inorganic arsenic in drinking water in the United States, the EPA revised the current Maximum Contaminant Level (MCL) for arsenic in drinking water from 50 µg/L to 10 µg/L in January 2001. The requirements for this final rule indicated that community water systems (CWSs), which are any public water system serving a minimum of 25 residents year-round, and non-transient, non-community water systems (NTNCWSs) such as churches, schools, and nursing homes, were required to meet compliance of the new arsenic MCL by January 2006 (EPA, 2001). Municipalities are mandated to test public drinking water sources for arsenic, but unregulated private wells remain a concern.

Consumption of Arsenic from Contaminated Foods

Among non-occupationally exposed individuals, food and drinking water are considered to be the primary sources for organic and inorganic arsenic exposures (Bernstam and Nriagu, 2000; Mantha et al., 2017). Humans run the risk of being exposed to both trivalent and pentavalent forms of arsenic from foods ingested daily (Oberoi *et al.*, 2019). Arsenic in food comes from uptake from the soil and irrigation water (Majumder & Banik, 2019; Meharg *et al.*, 2009; Nachman *et al.*, 2018; Punshon *et al.*, 2017). Soil is impacted by both anthropogenic and natural geological factors causing the content of arsenic in a given food to vary greatly (Carey *et al.*, 2012; Punshon *et al.*, 2017). However, genetics for plant uptake mechanisms also vary greatly, making generalized risk assessment a challenge (Nachman *et al.*, 2018; Punshon *et al.*, 2017).

Rice and grains are the most commonly ingested foods that contain arsenic. Rice is a staple food of Southeast Asia, but unfortunately, it is also a significant source of the highly toxic inorganic arsenic. While water contaminated with high levels of arsenic is used to irrigate paddy fields of areas in Bangladesh and West Bengal, rice grown in naturally or anthropogenically contaminated soils in other regions of the world, such as the southern parts of the United States

and France, contain much more arsenic (Meharg *et al.*, 2009). Rice is particularly vulnerable to contamination as arsenic is concentrated in the germ layer of the kernels (Carey *et al.*, 2012).

Rice straw is the leftover stalk of the rice plant that remains once the rice plant has been harvested. Similar to how the grains of rice can accumulate substantial concentrations of arsenic, arsenic is also able to accumulate throughout the rice straw, which is then used as feed for livestock such as cattle in Asian regions. The high levels of arsenic in this straw could directly threaten the health of humans who then consume milk from the arsenic-exposed cattle (Abedin et al., 2002). In arsenic-contaminated rural areas of the Ganga-Meghna-Brahmaputra (GMB), a grown cow will consume 30-40 liters of water per day and consume 10-12 kg of straw, which could give an idea of how much arsenic they ingest in arsenic-contaminated regions (Chakraborti et al., 2016).

In the United States, arsenic was also added to the animal feed directly for decades as an attempt to induce growth and prevent disease (Silbergeld & Nachman, 2008). Roxarsone, marketed by Pfizer as 3-Nitro®, for example, was an inorganic arsenical compound used mainly as a nutritional supplement for chickens. Roxarsone had been in use since 1944, intended not only for promoting growth but for killing intestinal parasites and for making the meat look pinker, most likely due to roxarsone and arsenic's angiogenic behavior (Basu et al., 2008). 3-Nitro was assumed not to affect people who consumed the exposed animals as the drug contained the organic form of arsenic, which is less toxic than inorganic arsenic. However, due to growing suspicions that the organic arsenic could convert into its more toxic counterpart, Pfizer voluntarily withdrew roxarsone from the US market once FDA scientists determined that levels of inorganic arsenic in the livers of chickens treated with roxarsone were higher than the levels in the livers of chickens not treated with the compound (Nigra et al., 2017). At that same time, two newer arsenic animal drugs, carbarsone and arsanilic acid were also voluntarily withdrawn from their approved status

for use in animal feeds. By the end of 2015, the last remaining arsenic-based animal drug on the US market, called Histostat (nitarsone), had been discontinued (FDA, 2019; Nigra et al., 2017).

Before the arsenical animal drugs were banned, terrestrial animals contained arsenic levels in the range of 7-30 ng As/g dry mass (Dabeka *et al.*, 1993). Still, they were not, and are not, the only organisms in danger of arsenic accumulation. For instance, feral marine fish may provide higher concentrations of arsenic when compared with freshwater organisms (Francesconi & Kuehnelt, 2004) and terrestrial organisms, such as cattle or poultry (Kuehnelt and Goessler, 2003; Sakurai et al., 2004). The arsenic levels found in marine fish may contain anywhere between 1µg As/g ww to more than ten µg As/g ww (species dependent) (Sele *et al.*, 2015; Sloth & Julshamn, 2008), whereas the arsenic levels found in freshwater fish is typically less than 1 µg As/g ww (Lawrence et al., 1986). More specifically, seawater ordinarily contains 0.001-0.008 mg/l of arsenic (Penrose *et al.*, 1977) so some shellfish can, therefore, contain levels of arsenic above 100 µg g^{-1} (Mandal & Suzuki, 2002) However, seafood generally contains organic forms of arsenic, arsenobetaine and arsenocholine, which pose no toxicity potential to humans (ATSDR, 2016). Arsenobetaine is considered to be the primary organoarsenic compound found in fish (Borak & Hosgood, 2007) as it constitutes more than 95% of the total arsenic present (Francesconi & Edmonds, 1996). Arsenobetaine is distributed differently throughout the bodies of fish of different species. In Atlantic salmon, for example, the highest concentration of arsenobetaine can be found in muscle tissue. In contrast, the most elevated levels measured in Atlantic cod are present in the muscle, liver, and gall bladder (Sele *et al.*, 2015).

Ingestion of Arsenic from Soil

Direct exposure to arsenic from soil is usually minimal in humans, but if such exposure were to occur, it would generally be via incidental ingestion, especially by children (Hughes *et al.*,

2011; Ngole-Jeme *et al.*, 2018). Young children have tendencies to play with dirt and in playgrounds and they also tend to place their hands in their mouths. Studies indicate that children living in areas near smelting activity displayed signs of elevated lead, cadmium, and arsenic due to soil contamination (Blaurock-Blesch, 2013). Another dietary source of arsenic is acquired through the ingestion of arsenic-containing clays and soils, such as in the deliberate practice of geophagia (Al-Rmalli *et al.*, 2010; Ngole-Jeme *et al.*, 2018), especially in pregnant women (Nyanza *et al.*, 2014). The bioaccessibilities of arsenic from mineral complexes in the soil are variable, but significant (Ngole-Jeme *et al.*, 2018). Heavy rainfall can induce downstream movement of arsenic from the mining-rich areas, which can then accumulate in the soil (Majumder & Banik, 2019) and nearby water sources.

Inhalation of Arsenic

The human exposure to arsenic through the air is relatively low. When compared to residential areas, the exposure to arsenic in airborne particulate matter is higher in areas with industrial activity (Chung *et al.*, 2014; Chung et al., 2014). The European Commission (2000) reported that level of arsenic in the air could "range 0-1 ng/m^3 in remote areas, 0.2-1.5 ng/m^3 in rural areas, 0.5-3ng/m^3 in urban areas, and up to about 50 ng/m^3 in the vicinity of industrial sites" (Hughes *et al.*, 2011).

According to the EPA, the general American public is exposed to an estimated 40-90 ng per day via inhalation (Pontius *et al.*, 1994). In the GMB Plain, people are directly exposed to high levels of arsenic through inhalation, which can then be absorbed by the respiratory tract. In the GMB Plain, cows ingest arsenic-contaminated water and straw and so their dung, utilized extensively for cooking purposes, contain a significant amount of arsenic. Cow dung cakes, which are used as a cheaper alternative to kerosene or coal, are readily available to the rural villages of

the region. The preparation of the cow dung cakes involves combining the dung with straw and more arsenic-containing water and drying the paddies in the sun before being used indoors in unventilated ovens. As the dung cakes are burnt as fuel, the arsenic is released in the smoke which is then inhaled. The villagers inhale 1859.2 ng of arsenic per day, of which a reasonably good amount (464.8 ng) could be absorbed by the respiratory tract (Pal *et al.*, 2007).

Occupationally, airborne arsenic exposures are significant in mining dusts and smelting. Arsenic is often a considerable workplace and environmental contaminant in areas where gold (Au) and Cu are smelted (Eisler, 2004; Enterline *et al.*, 1995).

1.1.3 Arsenic Metabolism

Inorganic arsenic is more rapidly absorbed by humans and is generally the more toxic species when compared to organic (methylated, dimethylated, or organo-sugars and lipids) arsenic (ATSDR, 2016). Overall, natural organic arsenical forms are considered to be the least toxic, whereas arsine gas has the highest toxicity (Barchowsky, 2020). The most common valance states of inorganic compounds include arsenite (AsIII or As^{3+}- trivalent form) and arsenate (AsV or As^{5+} – pentavalent form). Arsenite is considered a more toxicologically potent oxyanion than arsenate due to increased absorption and cellular uptake (Hughes *et al.*, 2011; McDermott *et al.*, 2010), as well the trivalent arsenicals' strong reactivity with compounds containing sulfhydryl groups (Cullen & Reimer, 1989; Styblo *et al.*, 2000).

Once inorganic arsenic is ingested, it is absorbed through aquaporins in the gastrointestinal tract (Z. Liu, 2010; Mukhopadhyay *et al.*, 2014; States, 2015) and undergoes reduction and methylation processes in the liver before the being eliminated most commonly through renal excretion in urine (Aposhian *et al.*, 2003; Hughes *et al.*, 2011). Inorganic arsenic is metabolized

by a sequential process involving a two-electron reduction of pentavalent arsenic, followed by oxidative methylation to pentavalent organic arsenic (Fig. 1). In this pathway, the pentavalent arsenic metabolite monomethylarsonic (MMA^{V}) is the result of the oxidative methylation of inorganic As^{3}, which is then reduced to monomethylarsonous acid (MMA^{III}), the trivalent methylated metabolite. Further oxidative reactions include methylation of MMA^{III} to form dimethylarsninic acid (DMA^{v}), which in turn is reduced to dimethylarsinous acid (DMA^{III}) (Aposhian *et al.*, 2003; Hughes *et al.*, 2011). The biotransformed metabolites are generally less toxic and more rapidly excreted than both forms of inorganic arsenicals (Aposhian *et al.*, 2003; Hughes *et al.*, 2011).

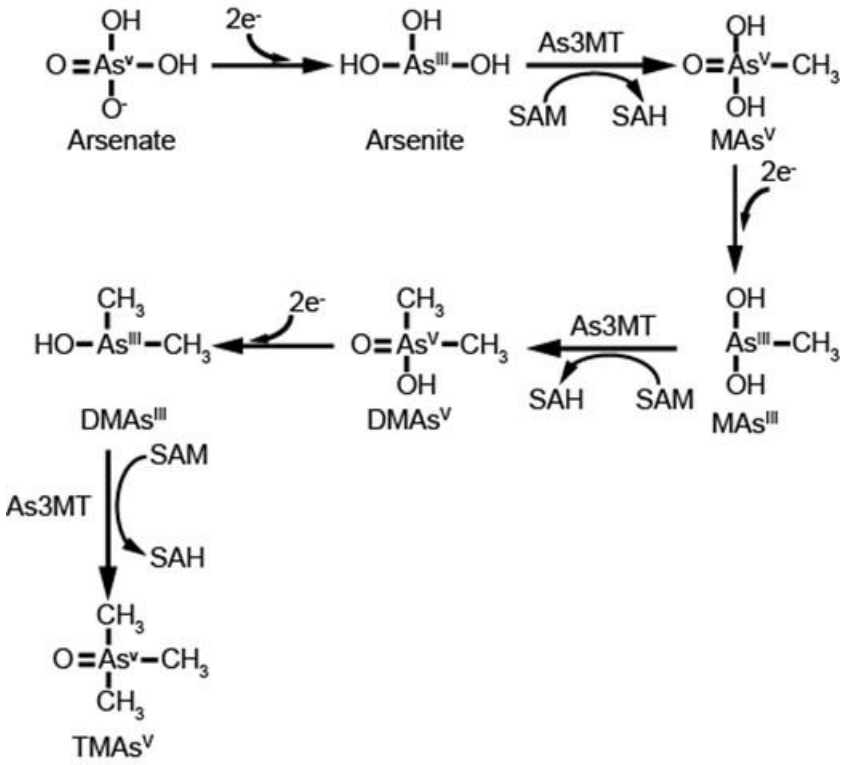

Figure 1: Oxidative methylation pathway for inorganic arsenic in mammals.

In addition to renal excretions, arsenic can be excreted through fecal matter, perspiration, milk, and bile (Aposhian *et al.*, 2003; Hughes *et al.*, 2011). Arsenic that is not excreted from the

body may accumulate in areas such as the skin, hair, nails, and muscles (Carter *et al.*, 2003; Hughes *et al.*, 2011). Inorganic arsenic has a particular affinity for hair and other keratin-rich tissues. In typical exposures, the average amount of arsenic present in hair is 0.8 µg/g, concentrations higher than 1.0 µg/g are indicative of poisoning or excess arsenic exposure (Arnold et al., 1990). The average amount of arsenic in nails is around 0.34 µg/g, but clippings can contain anywhere from 20-130 µg/g in cases of arsenic poisoning (ATSDR, 2016). Aside from nails, hair and teeth, analyses reveal that body tissues will contain less than 0.3 $\mu g/g^{-1}$ (ATSDR, 2016). When the liver has exceeded its methylating capacities during times of exposures to elevated levels of inorganic arsenic, the body will increase its retention of the arsenic in the soft tissues (ATSDR, 2016; Carter *et al.*, 2003). The level of arsenic retention will largely determine the resulting toxicity of arsenic.

1.2 Human Health Effects of Arsenic

Arsenic has had both a good and a bad reputation for centuries. Arsenical compounds have formerly been used for medicinal purposes by treating many diseases: malaria, ulcers, sleeping sickness, some forms of leukemia, syphilis, and skin conditions (Barchowsky, 2020; Hughes *et al.*, 2011). But the odorless and tasteless properties of arsenic have made it one of the most preferred and oldest poisons known to man – a "Poison of Kings." Arsenic, with its indiscernible nature, is considered to be highly toxic where one-tenth of a gram is acutely lethal in humans (Carter *et al.*, 2003; Hughes *et al.*, 2011). At specific points in history, arsenic was added to food or drink and causing arsenic poisoning symptoms – vomiting, diarrhea, and abdominal pain – that could have easily been mistaken for other common diseases of the time, such as cholera and pneumonia (ATSDR, 2016).

Chronic arsenic exposure became a modern global public health concern due to its toxicity and carcinogenic potential among hundreds of millions of individuals worldwide. The International Agency for Research on Cancer has classified inorganic arsenic as a Class 1 human carcinogen (IARC, 2017). Epidemiologic studies have associated arsenic exposure with an increased risk of cancers, including those of the stomach, liver, bladder, kidney, skin, and lung (Hughes *et al.*, 2011). In addition to the cancers, arsenic exposure has cardiovascular (Moon *et al.*, 2017), pulmonary (Sanchez *et al.*, 2018), neurological/cognitive (Tyler & Allan, 2014), hepatic (Das *et al.*, 2012), renal (Zheng *et al.*, 2015), gastrointestinal, reproductive, and immunologic toxicities (ATSDR, 2016; Engstrom *et al.*, 2013).

Cardiovascular disease and coronary artery disease, in particular, are the non-cancer diseases most strongly associated with environmental arsenic exposures (Moon *et al.*, 2017; Nigra *et al.*, 2016; NRC, 2014) and with the highest amount of mortality risk. Systematic review and meta-analysis of recent highly powered prospective epidemiological studies of the dose-dependent association of arsenic with coronary artery disease morbidity and mortality confirmed the significant risk of disease even at the current EPA maximum contaminant level (MCL) of 10 µg/L (Moon *et al.*, 2017). The dominant form of disease stems from the atherogenic potential of arsenic and its propensity to enhance both vessel disease and prolongation of the cardiac Q-T interval (Nigra *et al.*, 2016; Wu *et al.*, 2014). Mechanistically, arsenic-stimulated ROS and inflammatory cytokine expression promote endothelial cell dysfunction, proatherogenic monocyte and macrophage phenotypes, smooth muscle expansion, and vessel wall stiffening (Lemaire *et al.*, 2014; Wu *et al.*, 2014). The vascular endothelium is highly sensitive to arsenic exposures, with low-level exposure promoting cell proliferation, angiogenesis, vessel remodeling, and loss of vasodilator response (Soucy *et al.*, 2003; Soucy *et al.*, 2005; Straub *et al.*, 2008; Tseng *et al.*,

2005). In contrast, high-level As_2O_3 exposure in cancer therapies is antiangiogenic and causes endothelial cell death with loss of vessels (Roboz *et al.*, 2000; Soucy *et al.*, 2003; Soucy *et al.*, 2005). Atherosclerotic mouse models show increased atherosclerosis following even moderate arsenic exposures and indicate smooth muscle cell remodeling and aberrant macrophage lipid metabolism as the prime pathogenic targets (Lemaire *et al.*, 2011; Lemaire *et al.*, 2015). In addition, mouse models have also demonstrated that low to moderate levels (<100 μg/L) of exposure cause perivascular fibrosis and loss of perivascular matrix integrity, especially in the heart (Hays *et al.*, 2008; Soucy *et al.*, 2005). Blackfoot disease is a severe occlusive peripheral vascular disease associated with chronic, high-level arsenic exposure in drinking water, especially in endemic regions in Taiwan (Tseng *et al.*, 2005; C. H. Wang *et al.*, 2007). It is an arteriosclerosis manifested by acrocyanosis and Raynaud's phenomenon that may progress to endarteritis and gangrene of the lower extremities.

Chronic exposure to inorganic arsenic is associated with a range of nonmalignant respiratory symptoms, chronic obstructive pulmonary disease (COPD), and respiratory disease mortality (Parvez *et al.*, 2013; Sanchez *et al.*, 2016; Sanchez *et al.*, 2018). Susceptibility to the respiratory effects of arsenic is enhanced by in utero exposure (Rahman *et al.*, 2011; Ramsey *et al.*, 2013; Sanchez *et al.*, 2016) and co-exposure to tobacco smoke (Parvez *et al.*, 2013). Also, arsenic exposure is associated with increased respiratory tract infections (Farzan *et al.*, 2016; Rahman *et al.*, 2011) and chronic lung infections, including pulmonary tuberculosis (A. H. Smith *et al.*, 2011). Prospective epidemiologic studies indicate that arsenic causes chronic loss of lung defenses, such as secretion of CC16 protein from airway cells (Parvez *et al.*, 2010). Rodent studies suggest that low to moderate levels of in utero arsenic exposure decreases immune gene expression and promote inflammatory protein expression (Kozul, Hampton, *et al.*, 2009; Ramsey *et al.*, 2013)

that may make mice more susceptible to airway infections (Kozul, Ely, *et al.*, 2009). In keeping with reduced lung defenses, as in the heart, arsenic compromises lung matrix, wound repair, and barrier function (Hays *et al.*, 2008; Lantz & Hays, 2006; Petrick *et al.*, 2009; Sherwood *et al.*, 2013).

Lung cancer accounts for the majority of arsenic-related cancer deaths, with in utero exposures increasing the risk of lung cancer later in life (ATSDR, 2016; IARC, 2017). Based upon a meta-analysis, Begum et al. estimated that about 4.51 additional lung cancer cases per 100,000 people for a maximum contamination level of 10 µg/L of arsenic in drinking water and enhanced risk with co-exposure to tobacco smoke. It is likely that arsenic is not the primary carcinogen, but synergizes with the carcinogenic potential of tobacco smoke constituents. Arsenic is unique among the metals and chemical carcinogens since arsenic-promoted lung cancer is independent of the route of exposure with oral ingestion and inhalation giving equivalent increases in the risk of disease (A. H. Smith *et al.*, 2009). Metabolism of arsenic is also a critical component of lung cancer risk as higher percentages of MMA(III) are associated with higher risk (Kuo *et al.*, 2017).

Chronic arsenic exposures, especially higher-level exposures, are associated with metabolic diseases. Arsenic is predominantly metabolized in the liver and chronic exposure is associated with liver disease. Arsenic associated liver disease manifests initially as jaundice, abdominal pain, and hepatomegaly (Mazumder & Dasgupta, 2011). Liver injury may progress to cirrhosis and ascites, as well as to hepatocellular carcinoma (IARC, 2017; J. Liu *et al.*, 2008; Straif *et al.*, 2009). However, liver cancer is poorly associated with arsenic exposure (Straif *et al.*, 2009). Arsenic exposure and arsenic metabolites are associated with diabetes (Grau-Perez *et al.*, 2017; Kuo *et al.*, 2015; Kuo *et al.*, 2017). It is not clear, however, whether low-level arsenic is directly diabetogenic or enhances diabetes in diabetic individuals (Grau-Perez *et al.*, 2017; Maull *et al.*,

2012). Interestingly, a lower percentage of MMA is associated with the diabetogenic effects of arsenic (Grau-Perez *et al.*, 2017), and again in utero exposure may enhance disease in later life (reviewed in (Young *et al.*, 2018). Cell and animal studies have shown that arsenic directly impairs pancreatic beta cells and insulin release (Fu *et al.*, 2010; Martin *et al.*, 2017).

It is not surprising that the organs most susceptible to the effects of arsenic are those involved in the absorption, excretion, or accumulation processes of arsenic. The primary form of arsenic present in drinking water is the inorganic form of arsenic (Mantha *et al.*, 2017; Naujokas *et al.*, 2013), predominantly AsIII (Ahmad *et al.*, 2018), rendering it highly incidental for developing kidney and bladder cancers. The unique kinetics of arsenic and metabolites significantly increase the risk for transitional cell carcinoma of the bladder (Cohen *et al.*, 2013; IARC, 2017; Tokar *et al.*, 2011). The metabolites are readily excreted into the urine, and enhanced concentration promotes cyclic epithelial cell death and regeneration that ultimately results in cellular transformation (Cohen *et al.*, 2013). However, the metabolism of inorganic arsenic is not required to produce carcinogenic responses in the bladder epithelium, as hyperplasia and transformation is increased in mice lacking As3MT, relative to wild-type controls (Chen *et al.*, 2011). Arsenic also forms intracellular cytoplasmic granules in the bladder epithelium (Yokohira et al., 2011). Bladder cancer risk is up to six-fold higher in arsenic-exposed females relative to males, and the association of arsenic exposure with bladder cancer risk is greatly enhanced in smokers or ever-smokers (Ferreccio *et al.*, 2013). It appears that most renal cancers associated with arsenic exposure are transitional cell carcinomas and are thought to arise from the urothelial bladder cells (IARC, 2017).

The neurotoxicity of arsenic is well recognized, especially in the clinical signs of severe polyneuropathy from poisonings (ATSDR, 2016). Occupational exposures, as in copper smelters

or repeated exposures to high levels of arsenic, produce peripheral neuropathy (ATSDR, 2016; Sinczuk-Walczak *et al.*, 2010). This neuropathy usually begins with sensory changes, such as numbness in the hands and feet, but later may develop into a painful "pins and needles" sensation. Both sensory and motor nerves can be affected, and muscle tenderness often develops, followed by weakness, progressing from proximal to distal muscle groups. Sensorimotor dysfunction is pronounced in exposed children (Parvez *et al.*, 2011), suggesting that in utero or early development exposures increase susceptibility. Arsenic exposures, especially in utero and during early developmental exposures, also impair central cognition and memory behaviors (Tyler & Allan, 2014; Wasserman *et al.*, 2014; Wasserman *et al.*, 2011). A large cross-sectional study in Maine, USA, found that drinking water levels greater than 5 µg/L were associated with cognitive deficits in children (Wasserman *et al.*, 2014). This suggests that the 10 µg/L U.S. drinking water standard may not be protective for all health effects of arsenic. Studies in mice confirm that arsenic exposure during development impairs neural stem cell function, as well as epigenetically reduces cognition and enhances depression (Cronican *et al.*, 2013; Tyler & Allan, 2014). Interestingly, studies in mice suggest that the impact of low (50 µg/L) in utero and perinatal exposures on cognition may be less in females that respond to arsenic with more significant antioxidant adaptation (Allan *et al.*, 2015).

Arsenic, especially with chronic early-life exposure, is immunotoxic and potentially increases the risk of infections and inflammatory-like diseases during childhood and in adulthood (ATSDR, 2016; Ferrario *et al.*, 2016). This immunosuppressive potential is prevalent in respiratory tract infections (Kozul, Ely, *et al.*, 2009; Rahman *et al.*, 2011; Ramsey *et al.*, 2013; Steinmaus *et al.*, 2016), and enhancement of a global inflammatory state may underlie the etiology of arsenic-promoted neural, cardiovascular, metabolic, and cancer disease. For example, arsenic exposures

alter transcriptional programming in circulating macrophages to enhance atherogenesis that underlies arsenic-promoted cardiovascular disease (Lemaire *et al.*, 2011). In addition to increasing inflammation, arsenic may increase allergy and autoimmune diseases (Ferrario *et al.*, 2016). Further impacts on the circulation include hematologic consequences of chronic exposure to arsenic that interferes with heme synthesis and increases urinary porphyrin excretion, which is a biomarker for arsenic exposure (Ng *et al.*, 2005).

The skin is also a primary target of arsenic toxicity, and skin lesions are often diagnostic of high-level arsenic exposure. In humans, chronic exposure induces a series of characteristic changes in skin epithelium, perhaps due to preferential uptake into sulfhydryl-rich keratin structures (Kitchin & Wallace, 2008). Diffuse or spotted hyperpigmentation and, alternatively, hypopigmentation can first appear between 6 months and three years with chronic arsenic exposure (ATSDR, 2016). The characteristic hyperpigmented spots or palmar-plantar hyperkeratosis usually follows the initial appearance of arsenic-induced pigmentation changes within years (ATSDR, 2016). Susceptibility to skin lesions increases with deficiencies in folic acid and vitamin B due to impaired methylation of ingested arsenic (Ahsan *et al.*, 2007; Gamble *et al.*, 2006). Arsenic-induced skin cancers include basal cell and squamous cell carcinomas (IARC, 2017). Skin cancer that presents as in situ squamous carcinoma that is often indistinguishable from Bowen's disease is common with protracted high-level arsenical exposure (ATSDR, 2016; IARC, 2017). The basal cell cancers are usually only locally invasive, but squamous cell carcinomas may have distant metastases. The skin cancers often occur in areas of the body not exposed to sunlight (e.g., on palms of hands and soles of feet) and usually occur as multiple primary malignant lesions.

1.3 Skeletal Muscle and the Muscle Stem Cell Niche as Targets of Arsenic Toxicity

1.3.1 Overview of Skeletal Muscle

Skeletal muscle tissue represents the most abundant type of tissue in the human body, accounting for approximately 40% of the average human body weight (Birbrair, Zhang, Wang, *et al.*, 2014). Skeletal muscle is a highly organized network of connective tissue, neurovascular structures, and various types of resident cells. Its primary functions are to support soft tissues, maintain posture and position, generate movement, and contribute to metabolic homeostasis. Adult skeletal muscle in mammals is a stable tissue under normal circumstances; it possesses the extraordinary ability to repair itself and regenerate new muscle fibers if any fibers are lost to damage through injury or pathological conditions such as muscular dystrophy (Tedesco *et al.*, 2010).

1.3.2 Satellite Cells and Myogenesis

Postnatal skeletal muscle repair is a highly orchestrated process involving the integrated activation of several molecular and cellular responses. The adult satellite cells, the primary skeletal muscle resident stem cell, are central to the repair program is responsible for myofiber regeneration and growth (Tedesco *et al.*, 2010). These stem cells usually reside in a quiescent state until activated by growth signals or damage (Kuang et al., 2007; Relaix et al., 2005). The signals trigger asymmetrical division, either for self-renewal or to differentiate and generate a population of myoblasts that fuse, forming nascent myotubes, which then mature into myofiber replacements (Siegel et al. 2011; Zammit et al. 2004).

Satellite cells are classically defined by their anatomical location, being positioned between the basal lamina and sarcolemma of muscle fibers. Satellite cells have also been distinguished by the presence of a canonical paired box transcription factor 7 (Pax7) (Seale et al., 2000; Tedesco et al., 2010) and various surface receptors including α7 integrin, CD29/B1 integrin, CXCR4 (Maesner et al., 2016), CD34 (Beauchamp et al., 2000), c-met (Cornelison and Wold, 1997), CD56/NCAM (Seale et al., 2000), caveolin-1 (Gnocchi et al., 2009), M-cadherin (Irintchev et al., 1994, Cornelison and Wold, 1997), and syndecans 3 and 4 (Cornelison et al., 2001). None of these surface markers, however, are specific or exclusive to identifying satellite stem cells. As satellite cells proliferate before they undergo myogenic differentiation, they begin to lose their expression of Pax7 and begin to express four myogenic regulatory transcription factors (MRFs) that help promote myogenesis, including Myf5, MyoD, myogenin, and MRF4 (Hernandez-Hernandez et al., 2017). While the activation of satellite cells is marked by the rapid onset of MyoD and Myf5 expression, myogenin marks the commitment to differentiate into myoblasts. Activated myoblasts also express begin desmin, an intermediate filament (IF) protein that commonly found along the entirety of immature and mature muscle fibers. Data from knockout studies of desmin in mice indicate that desmin is essential for the structure and function of skeletal muscle. The mice lacking desmin present with abnormal neuromuscular junction morphology and adipocyte accumulation (Agbulut et al., 2001). However, the upregulation of desmin is not exclusively specific to myoblasts, as it is also expressed by other progenitor cells in regenerating muscle (e.g., myogenic pericytes, refs).

1.3.3 The Extracellular Matrix Composition and Communication

Skeletal muscle is comprised of living tissue and a noncellular component that provides the structural integrity of the overall muscle niche known as the extracellular matrix (ECM). Although the ECM comprises only about 1 to 9% of skeletal muscle's cross-sectional area (Light and Champion, 1984), it is a principal component of muscle health and metabolic activity. Resident muscle cells recognize and bind to growth factors and cell-surface receptors embedded within the ECM, which is continuously subjected to remodeling. These interactions trigger signaling cascades capable of mitigating transcriptional changes of various ECM-related genes which can then influence cell adhesion, migration, proliferation, and differentiation (Grounds, 1987; Yanagishita, 1993). When the microenvironment of the ECM endures drastic change, the consequences can be manifested in clinical dysfunction of the muscle. Fibrosis, for example, is an indication of an injury or disease state of skeletal muscle. Fibrosis is an induced pathological syndrome that results from an excess accumulation of ECM-associated components. The ECM, therefore, relies on a delicate balance of roles played by the resident cells and their molecular programs, all of which maintain the intricate workings that allow the muscle to develop, function, thrive, and regenerate.

The ECM is comprised of water, polysaccharides (Frantz et al. 2010), metalloproteinases, and fibrous proteins, such as elastin, laminin, and various collagens (Alberts et al., 2007). Fibrous collagen is the most abundant structural protein in adult interstitial ECM (Rumian et al., 2007) and each layer of muscle ECM is mostly comprised of collagen types 1 and III (Kjaer, 2004; Light and Champion, 1984), which are secreted and organized primarily by CTF and possibly pericytes residing in the interstitial space (Frantz et al., 2010; Archile-Contreras et al., 2010; Gillies and Lieber, 2011). Collagen IV, which is not typically found in fibrillar ECM (Gillies and Lieber, 2011; Grounds et al., 2005; Yurchenco and Patton, 2009), can be found concentrated in the

basement membrane of mature skeletal muscle (Kjaer, 2004) and may to serve as an intermediary between myofibers and the fibrillar ECM (Voermans et al., 2008). Overall, collagens help to regulate cell adhesion, provide tensile strength to the muscle, support migration and chemotaxis of resident cells, and direct tissue development (Rozario and Desimone, 2010)

Although no one cell surface marker can indisputably identify a fibroblast, Tcf4 (transcription factor7-like 2, Tcf7L2) (Mathew et al., 2011) and αSMA (alpha-smooth muscle actin (Tomasek et al. 2002) have been identified as common cell markers for CTF. Studies where Tcf4-expressing cells were ablated in mice showed impaired muscle regeneration with premature satellite cell differentiation, followed by a depletion of the early pool of satellite cells, and smaller regenerated myofibers (Murphy et al., 2011). These results suggest that during regeneration, CTF may not only regulate satellite cell expansion, but they may also regulate the myogenic differentiation program of the satellite cells.

1.3.4 Muscle Progenitors and Skeletal Muscle Management

Satellite cells typically reside on the surface of myofibers beneath the basal laminin α2. Laminin α2 is a component that helps anchor myofibers in the ECM, provides membrane stability, and plays a role in the regeneration of muscle fibers (Uezumi et al., 2014). The primary cell source for laminin α2 in skeletal muscle is fibroblast activation protein-α (FAP)-expressing stromal cells (Roberts et al., 2013). FAP+ stromal cells in muscle also express PDGFRα (Platelet-Derived Growth Factor Receptor Alpha), Sca-1 (Stem cells antigen-1), and CD90 (Roberts et al., 2013), which are cell surface markers shared by mesenchymal progenitors. Moreover, with the FAP+ cell population localized in the interstitial spaces of muscle along where the mesenchymal progenitors

reside, these FAP+ stromal cells may also be closely related to, if not the same as, mesenchymal progenitors (Tedesco *et al.*, 2017).

Mesenchymal progenitor cells express markers that overlap with various types of side populations within skeletal muscle, and these overlapping traits make it challenging to decipher the origin and function between all the populations (Tedesco *et al.*, 2017). One particular subset of mesenchymal progenitors embodies a plasticity in that they differentiate into bipotent cells with either fibrogenic or adipogenetic traits. These are the mesenchymal progenitor cells that are Lin−Sca-1+CD34+, and that also express PDGFRα and α-SMA, have the capability of generating either fibroblasts or adipocytes in vitro, and thus are aptly referred to as fibro/adipogenic progenitors (FAPs). (Joe *et al.*, 2010; Tedesco *et al.*, 2017; Uezumi *et al.*, 2014)).

Quiescent FAPs are generally abundant in healthy muscle, but their numbers rapidly increase in response to acute muscle damage as they participate in the muscle regeneration process. Under normal conditions, the skeletal muscle will include resident cells positive for Tcf4 and PDGFR-α. Following denervation and chronic damage, however, the muscle may experience an increase of cells co-labeled with the two markers, indicating a rise in the total number of FAPs during the regeneration process (Contreras *et al.*, 2016). This study also revealed an increase in the production of collagen I and the chondroitin sulfate proteoglycan, Neural/glial antigen 2 (NG2), within the injured muscle (Contreras *et al.*, 2016). If a muscle is in a chronic state of injury and damage, the muscle tissue can undergo fatty degeneration and be replaced by a combination of white fatty tissue and fibrous tissue (Lipton & Schultz, 1979). The accumulation of intramuscular fat, termed myosteatosis, can be observed in the etiology of metabolic diseases, obesity and sarcopenia (Correa-de-Araujo *et al.*, 2017; Prado *et al.*, 2018) where the formation of fat cells could be attributed to the expression of PDGFRα (Wren *et al.*, 2008). Only PDGFRα+

cells were efficient in undergoing adipogenic differentiation both *in vivo* and *in vitro* (Uezumi *et al.*, 2010). This infiltration of fat and fibrosis can undoubtedly diminish the muscle's function, potentially limiting the success of regenerative capacities by altering the muscle environment.

We observed that flow-sorted FAPs that were $CD31^{-}CD45^{-}Sca\text{-}1^{+}\alpha7\text{-}integrin^{-}$ from young and old mice also express αSMA and NG2, a chondroitin sulfate proteoglycan. NG2 is a reported marker of pericytes (Birbrair, Zhang, Wang, *et al.*, 2014; Moyle *et al.*, 2019) that pericytes may be distinct from FAPs and/or CTF as they reside in different locations (Moyle *et al.*, 2019; Tedesco *et al.*, 2017). However, when the muscle is injured, pericytes migrate to the interstitial ECM (Birbrair, Zhang, Wang, *et al.*, 2013) and the distinctions between the progenitor populations are not as straightforward. NG2 was not initially known to be expressed in FAPs but these cells are likely a subset of pericytes that can also be $Sca\text{-}1^{+}NG2^{+}PDGFR\alpha^{+}$ (Birbrair, Zhang, Wang, *et al.*, 2013). Pericytes are a heterogeneous cell population with distinct subsets. In skeletal muscle, there are two classes of pericytes: Type I ($PDGFR\alpha^{+}Nestin^{-}NG2^{+}$) and Type II ($PDGFR\alpha^{-}Nestin^{+}NG2^{+}$) (Birbrair, Zhang, Wang, *et al.*, 2014). Type I is considered to be non-myogenic as they express αSMA and produce collagen 1 (Birbrair, Zhang, Wang, *et al.*, 2014). In contrast, type 2 are myogenic, in that these cells express desmin and can form multinucleated myofibers. Type-1 pericytes contribute to fibrosis and fat accumulation (Birbrair, Zhang, Wang*et al.*, 2013), whereas the type-2 is likely to contribute to muscle, as well as neural tissue regeneration after injury (Birbrair, Zhang, Wang, *et al.*, 2014; Santos *et al.*, 2019). Conclusively, by identifying the full capacities and origins of various cell populations residing within the skeletal muscle niche, such as the myogenic and non-myogenic pericytes, we may be better suited for developing targeted therapies for a spectrum of skeletal muscle disorders.

1.3.5 Skeletal Muscle Dysfunction in Cardiometabolic Disease and Epidemiology of Arsenic in Muscle Morbidity

Loss of lean body mass and muscle quality are increasingly recognized as a major risk, if not etiological, factors for cardiovascular, lung, and metabolic disease (Correa-de-Araujo *et al.*, 2017; Prado *et al.*, 2018). Loss of muscle quality is critically important to overall well-being as highlighted by the fact that skeletal muscle comprises 40-50% of total body mass and that myosteatosis, increased skeletal muscle intra- and inter-myofiber adiposity, is associated with increased risk of all-cause and cardiovascular disease mortality (Carobbio *et al.*, 2011; Goodpaster *et al.*, 2000; Miljkovic & Zmuda, 2010; Prado *et al.*, 2018). Increased myosteatosis may be one of the earliest clinical signs of insulin resistance and impaired metabolism (Goodpaster *et al.*, 2000; Sell *et al.*, 2006; Vigouroux *et al.*, 2011). As the importance of muscle quality decline and myosteatosis in etiology and promotion of disease has only recently increased, there is a limited number of epidemiological studies to implicate their full impact on disease. This knowledge gap is magnified when considering environmental factors, such as arsenic exposure, in promoting deleterious change in muscle quality. However, epidemiological studies are indicating that 7.5-10% of the hundreds of millions of arsenic-exposed individuals present with sensorimotor deficits, muscle atrophy, and weakness, as well as impaired gait (Chakraborti *et al.*, 2003; Mukherjee *et al.*, 2003; Parvez *et al.*, 2011). Populations exposed to very high arsenic levels demonstrate rates of muscle morbidity in the range of 35-85% (Chakraborti *et al.*, 2003; Mukherjee *et al.*, 2003). Thus, it is plausible that significant environmental exposure may surreptitiously contribute to myosteatosis, muscle quality decline, weakness, and disability in large populations, ultimately leading to enhanced risk of cardiovascular and metabolic diseases.

1.3.6 Arsenic Effects on Skeletal Muscle and Muscle Progenitor Cells

Our findings indicate that low to moderate arsenic exposure directly affects skeletal muscle metabolism and composition (Ambrosio *et al.*, 2014; Garciafigueroa *et al.*, 2013) and disrupts MuSC function to impair muscle quality and regeneration (Cheikhi *et al.*, 2020; Cheikhi *et al.*, 2018; Zhang *et al.*, 2016). We found that low (10 µg/L) to moderate (100 µg/L) arsenic exposures in mice caused both dose and time-dependent increased ectopic fat deposition in skeletal muscle (Garciafigueroa *et al.*, 2013), as well as dysfunctional bioenergetics, physical properties, and regenerative capacity (Ambrosio *et al.*, 2014; Zhang *et al.*, 2016). In keeping with our observations in mice and human cells, others have shown that developmental arsenic exposures result in more fibrotic and adipogenic muscle tissue, especially following muscle injury (Szymkowicz *et al.*, 2018). Mechanistically, the arsenic-induces an epigenetic memory that impairs the transcriptional program in muscle progenitor cells to disrupt differentiation and misdirect cell fate (Bain *et al.*, 2016; Cheikhi *et al.*, 2020; Cheikhi *et al.*, 2019; Hong & Bain, 2012; Zhang *et al.*, 2016). Disrupting the between the resident cell population in the progenitor cell niche and modifying their molecular contributions to the ECM microenvironment compromises the overall ECM structure and intercellular communication that is mediated by the ECM constituents (Engler *et al.*, 2006; L. Smith *et al.*, 2017; Stearns-Reider *et al.*, 2017; Williams *et al.*, 2015; Zhang *et al.*, 2016). We found that when human muscle stem cells (hMSC) are cultured on decellularized ECM constructs derived from arsenic-exposed skeletal muscle, there was an increase in fibrogenic conversion of the hMuSC, when compared to the hMuSC cultured on control ECM constructs (Zhang et al., 2016). These data confirmed that the myofiber-producing MPCs are receptive to signals from the skeletal muscle ECM and suggested that arsenic can impart ECM change that misdirects MPC differentiation. Furthermore, CTF isolated from arsenic exposed mice (CTF^{ars}) retain elevated NF-

κB-driven ECM gene transcript levels, relative to CTF isolated from control mice (CTF^{ctr}) (Zhang et al., 2016) that may promote ECM remodeling and dysfunctional ECM to cell communications. However, much is unknown regarding the mechanisms underlying miscommunication in the ECM that lead to arsenic promotion of fibro-adipogenic programs and impaired muscle maintenance and metabolism.

1.4 Hypothesis and Aims

As indicated, arsenic exposures are a critical global public health hazard that many millions of individuals worldwide. Arsenic poses a significant risk to health by promoting cardiometabolic diseases; however, the etiology of arsenic disease promotion is poorly understood. Our previous work indicated that skeletal muscle progenitor cells and their anatomical niche are targets of arsenic that may promote muscle dysfunction and compromise metabolism in a manner consistent with increased cardiometabolic disease risk. Previous studies revealed that arsenic exposures disrupt the regulation of proteins essential for producing vital ECM components and signaling mechanisms responding to the ECM, a complex meshwork that supports skeletal muscle integrity and function (Bain *et al.*, 2016; Hays *et al.*, 2008; Petrick *et al.*, 2009; Zhang *et al.*, 2016). The ECM provides a niche for resident cells such as muscle stem cells, endothelial cells, pericytes, macrophages, and CTFs, as well as the mechanical structure needed for functional muscle contractions. Disrupting the molecular dynamics within the ECM microenvironment and its interaction with these cell populations is sufficient to compromise the homeostatic balance of the skeletal muscle and overall physiological function.

We previously found that muscle isolated from arsenic-exposed mice displayed misshapen mitochondria with damaged cristae (Ambrosio *et al.*, 2014). CTF isolated from these mice also retained disrupted mitochondrial morphology, as well as a phenotype with elevated expression of specific ECM-modulating proteins (Zhang *et al.*, 2016). Further studies demonstrated that when hMPC were seeded onto decellularized ECM constructs derived from arsenic-exposed gastrocnemius muscle, there was increased fibrogenic conversion of the MPCs, as compared to MPCs seeded onto control ECM constructs (Zhang *et al.*, 2016). Overall muscle maintenance, including repair and regeneration, involves tightly regulated bi-directional communication between muscle niche components, such as the ECM, the CTF that elaborate the ECM, and the myofiber producing MPCs. Thus, arsenic-induced changes in CTF mitochondria and matricellular regulator expression may directly impair tissue regeneration by signaling for aberrant MPC responses by maladaptive ECM constituents.

This dissertation research investigated two aims that explored the central hypothesis that arsenic targets the skeletal muscle niche by disrupting the highly orchestrated balance between resident CTF and myomatrix composition. Furthermore, we explored the arsenic-induced stimulation of CTF-mediated matrix alterations that result in MPC lineage misdirection and, ultimately, a decline in healthy myogenesis. We also examined novel interventions to rescue the dysfunctional arsenic-exposed CTF phenotype and thereby restore healthier elaboration of ECM constituents and the overall integrity of regenerating muscle. Specifically, we investigated the aims of 1: demonstrating that a mitochondrial protectant can revert the phenotype of As(III)-exposed CTFs to restore impaired MPC differentiation and muscle regenerative capacity and 2: determining if an arsenic-induced ECM orchestrates Notch-dependent MPC misdirection that drives the memory for arsenic-induced myogenic impairment.

2.0 Chapter 2: A Historic Perspective of Anthropogenic and Occupational Arsenic Exposures

2.1 The Deadly Color of Fashion: A Historical Account of Green Arsenical Pigments

Manuscript to be submitted to lay press.

Green is typically the color associated with luck, paradise, and lush vegetation - but the color has not always had such a positive reputation. Green has also been used to denote the color of jealousy, greed, decay, poison, and other unfavorable facets of nature. These portrayals are not without merit, however. Throughout the 19th century, variations of a deadly green pigment was ubiquitously found in home décor and fashions even as they were notoriously accompanied with detrimental health effects.

Scheele's Green – The Dawn of a Greener Era

In the Middle Ages, wearing green-colored garments was often indicative of belonging to a higher class or having an elevated status as the green dyes were expensive to produce. At the time, green dyes were made of components found in the natural environment, such as "buckthorn berries, the juice of nettles and leeks, and many other plants and trees (Parute, 2018)." These plant-based dyes were typically not long-lasting and somewhat challenging to prepare until the invention of a new vivid green pigment. In 1775, German-Swedish chemist Carl Wilhelm Scheele "almost accidentally" discovered a brilliant yellowish-green compound. This single pigment became known as Scheele's green or by other descriptive names that included mineral green, mountain green, and Swedish green (Sharples 1876).

Scheele's green was formed from a precipitate produced as a result of combining copper sulfate with sodium arsenic (Zerr and Rubencamp, 1908), a known carcinogen in today's world. Scheele never made a quantitative analysis but the formula most commonly accepted for Scheele's green is CuHAsO3, having the following composition: Arsenic trioxide (As2O3) 52.83%, Cupric oxide (CuO) 42.37%, and Water (H2O) 4.80% (Sharples 1876). The copper arsenite compound (also known as cupric hydrogen arsenite or acidic copper arsenite) is not soluble in water (US Bureau of Labor Statistics, 1917) like a typical dye would be. Still, it could be dissolved in acids or ground into a fine green-colored powder.

Scheele suspected his brilliant pigment of having a poisonous disposition and felt that people should be aware of its properties (Kelleger, 2018). Scheele stated that "the water with which the color is lixiviated contains a little arsenic, and must not be thrown out in a place to which cattle have access (Sharples, 1876)." Manufacturers, enticed by the pigment's cheap cost, were unreceptive to its potential toxicity and continued with production. The color then grew extremely popular because it yielded a brilliantly saturated green with no tinge of gray or brown undertones. Earlier dyes tended to lean towards one of its compounding contributors, as green was typically attained by overlaying a blue dye with a yellow dye, or the other way around (Meier, 2014). In addition, the new green pigment was longer-lasting and less expensive to produce when compared to the existing traditional dyes, therefore making it highly profitable for manufacturers to produce (Kelleger, 2018).

Scheele's green was used to color home furnishings and interior décor, such as carpeting, lampshades, curtains, and wallpaper (Hawksley, 2016; Wright, 2017). The arsenical pigment was also utilized to color fabrics used in making waistcoats, shirts, shoes, boot linings, stockings, socks, gloves, silk handkerchiefs, dresses, ball gowns, hats, and trousers. It was also used to stain

artificial flowers, fruits, leaves, and feathers that had been included in headdresses and other fancy hair adornments (Hawksley, 2016; Whorton, 2010). The fact that some of these decorative components were found to contain up to a grain and a half of arsenic apiece (Whorton, 2010) did not deter consumers as arsenic was ubiquitously used for domestic purposes. Arsenic had already been included in facial creams and soaps marketed to improve complexion and to enhance the luminosity of the skin - which was yielded by destroying red blood cells beneath the skin (Hawksley, 2016). Although arsenic has long been known to be poisonous, the use of the odorless, tasteless, and colorless toxic metalloid was not perceived as a danger as long as it was not ingested.

As implausible as it may seem, arsenic within apparel was able to make its way into the human body. The wearing of green garments led to various arsenic exposure-related disabilities and deaths. Scheele's green pigment powder was often loosely applied to fabric (Laricheva, 2015) used in gloves and purses, and often stained the wearer's hands when the pigment was not adequately sealed (Whorton, 2010; Wright, 2017). Those who wore any green-colored garment next to their skin could have also experienced a rash or a more severe dermal irritation that produced open sores on the hands, face, and other parts of the body (Kelleger, 2018; Whorton, 2010).

The toxic dust coating green eveningwear also could shed into the air as a woman danced around at a ball, allowing for the arsenic to be inhaled by other attendants (Whorton, 2010). When a London physician named George Rees analyzed a sample of muslin tinted with the Scheele's green pigment that would be included in a ball gown, it was found to contain more than sixty grains of Scheele's green per square yard. Considering that a dress at that time required about twenty yards of fabric, one could easily be worn containing more than 1,000

grains of arsenic (Whorton, 2010). To make matters worse, the Massachusetts State Board of Health, Lunacy and Charity reported in 1884 “that a dress made from arsenic-green tarlatan might shed twenty to thirty grains of poisonous pigment in just one hour of dancing (Hawksley, 2016).” To put this into greater perspective, a British newspaper later reported that four to five grains of arsenic was sufficient to kill a human being (Hawksley, 2018).

Paris Green – Brighter Hues Come with Deadlier Expenditures

In an attempt to improve Scheele’s Green, a bolder, more stable (albeit extremely toxic) green pigment, was developed in 1814 by two industrialists at the Wilhelm Dye and White Lead Company located in Schweinfurt, Germany (Vic, 2010; Zerr and Rubencamp, 1908). This new pigment, (copper aceto-arsenite, cupric aceto arsenite), was known as Schweinfurt green, emerald green, Vienna green (Vic, 2010), English green, meadow green, parrot green, and most popularly as Paris green (US Bureau of Labor Statistics, 1917; Sharples, 1876). This hue shown more brilliant than any existing pigment, even as the quality of lighting at the time was improving from candlelight to gas (Wright, 2017). The brighter form of lighting provided an avenue for the social and fashionably elite to showcase their bolder shades of jewel-toned hues, ignoring the circulating rumors implicating all the health risks involved in creating their extravagant fashions.

The associated dangers of Paris green were many as the inorganic compound also served as a notably potent rodenticide and insecticide (US Bureau of Labor Statistics, 1917). Acute exposure to Paris green induced symptoms such as headaches, dizziness, rapid heart rate, dermatitis, eye and respiratory tract irritation, diarrhea, vomiting, and dehydration. Chronic exposures led to more severe side effects, including acute hemolysis, convulsions and seizures, and even death.

Occupational Exposure in the Fashion Industry

For the general modern population, arsenic exposure through dermal absorption tends to be reasonably negligible when compared to people who work with arsenic in an industrial setting. For these types of workers, inhalation and dermal absorption of arsenic are typically the major routes in which they may be chronically exposed to the dangerous element (Chung *et al.*, 2014). Because the finely ground arsenical pigment was not well-contained during processing, the workers inhaled a considerable amount of the pigment dust on a daily basis. Floors in these facilities were infrequently cleaned, which allowed for workers to track the green dust from room to room, including the area where they would take their meal breaks. Additionally, with the workers changing into their street clothes before leaving the factories, the toxic dust likely made its way into their homes (US Bureau of Labor Statistics, 1917).

No matter the route in which arsenic enters the body, whether workers inhaled air containing the pigment dust, accidentally absorbed it from ulcers on their skin, or ingested it from eating with their green-tinted hands, the arsenic would eventually make its way to the lungs. When arsenic deposits in the lung, it is distributed throughout the body via the bloodstream where it can reduce the number of blood cells, resulting in an arsenic-related anemic condition (ATSDR, 2016). It was this same hematological response, however, that attributed the use of arsenic (arsenic trioxide) in the modern-day treatment of acute promyelocytic leukemia (Antman, 2001).

Nevertheless, the toll that arsenic was taking on the green pigment-handling factory workers was growing considerably in the 19th century. The general public was slowly becoming more aware of the deadly effects attributed to the arsenicals as headlines were beginning to recount heart-wrenching stories of deaths brought about by the occupational exposure to the

toxic substances. One such tragic story involved the death of a 19-year-old artificial flower maker named Matilda Scheurer in November of 1861. The artificial flowers Matilda produced were included in adorned headdresses of the time. Matilda, along with thousands of other young and poor workers like her, was exposed to the arsenic pigment as she worked to dust the green powder onto the fabric that would be used to procure the flower's green leaves. Not only was her skin exposed to the pigment, but she inhaled the fugitive pigment particles throughout her 14-to 16-hour workdays (Whorton, 2010).

Absorption of the arsenic within the pigment dust may have occurred through dermal absorption and inhalation, but arsenical poisonings could also be due to accidental ingestion. The green pigment would become trapped under the fingernails, which would have easily been ingested during the point of eating or biting of the nails. In the case of Matilda, her autopsy reported her fingernails had been prominently stained green and the whites of her eyes had also turned green. The arsenic was found to be present in her stomach, lungs, and liver (Mathews, 2015).

Despite these devastating stories of factory workers being circulated among the major newspapers of the time, consumers still sought after the most vivid of pigments that science could create. The trend of using the arsenical pigments and dyes continued until the end of the century when chemists were able to formulate less toxic compounds that eventually superseded several of the existing arsenical-containing pigments. Following the First World War, women's fashions also became "more streamlined" and strayed from the "elaborate decorations of the late Victorian period (Behlen and Fenner, 2016), meaning that the demand for the arsenic-laden artificial flowers and other adornments was no longer vast. This would mark the end of a major

public health crisis, in which chemistry and industry advancements invigorated consumerism and the fashion industry… at deadly costs.

3.0 Chapter 3: Arsenic Stimulates Myoblast Mitochondrial EGFR to Impair Myogenesis

This is a truncated version of a publication in Toxicological Sciences (Cheikhi *et al.*, 2020)

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FUNDING: NIH NIA Grant AG052978-01 (FA), the Pittsburgh Claude D. Pepper Older Americans Independence Center P30 AG024827 (AC), NIEHS Grant R01ES023696 (FA and AB) and R01ES025529 (FA and AB) supported this work. We acknowledge the NIH-supported microscopy resources in the Center for Biologic Imaging (NIH grant #1S10OD019973-01). Finally, flow cytometry was performed in the University of Pittsburgh Unified Flow Core (NIH grant 1S10OD019942-01).

Abstract

Arsenic exposure impairs muscle metabolism, maintenance, progenitor cell differentiation, and regeneration following acute injury. Low to moderate arsenic exposures target muscle fiber and progenitor cell mitochondria to epigenetically decrease muscle quality and regeneration. However, the mechanisms for how low levels of arsenic signal for prolonged mitochondrial dysfunction are not known. In this study, arsenic attenuated murine C2C12 myoblasts differentiation and resulted in abnormal undifferentiated myoblast proliferation. Arsenic prolonged ligand-independent phosphorylation of mitochondrially localized epidermal growth

factor receptor (EGFR), a major driver of proliferation. Treating cells with a selective EGFR kinase inhibitor, AG-1478, prevented arsenic inhibition of myoblast differentiation. AG-1478 decreased arsenic-induced colocalization of pY^{845}EGFR with mitochondrial cytochrome c oxidase subunit II (MTCO2), as well as arsenic-enhanced mitochondrial membrane potential, reactive oxygen species generation, and cell cycling. All of the arsenic effects on mitochondrial signaling and cell fate were mitigated or reversed by addition of mitochondrially-targeted agents that restored mitochondrial integrity and function. Thus, arsenic-driven pathogenesis in skeletal muscle requires sustained mitochondrial EGFR activation that promotes progenitor cell cycling and proliferation at the detriment of proper differentiation. Collectively, these findings suggest that the arsenic-activated mitochondrial EGFR pathway drives pathogenic signaling for impaired myoblast metabolism and function.

4.0 Introduction

Arsenic exposure poses a significant risk of cardiovascular and metabolic (cardiometabolic) diseases in hundreds of millions of individuals worldwide (Kuo *et al.*, 2017; Moon *et al.*, 2017; Sung *et al.*, 2015). Despite strong epidemiological evidence of these associations, the etiology for arsenic-promoted cardiometabolic diseases is unclear. Over the past decade, skeletal muscle metabolic dysfunction and decline of muscle composition have become a primary focus in establishing the major underlying risks for insulin resistance and type 2 diabetes (T2D) (Goodpaster *et al.*, 2003; Granados *et al.*, 2019; Miljkovic *et al.*, 2013), as well as cardiovascular disease and all-cause mortality (I. Miljkovic *et al.*, 2015; Reinders *et al.*, 2016). We previously reported that arsenic exposures increase ectopic skeletal muscle adiposity (Garciafigueroa et al., 2013), an earlier indication of insulin resistance and impaired metabolism than elevated serum free fatty acids or glucose (Goodpaster *et al.*, 2000; Sell *et al.*, 2006; Vigouroux *et al.*, 2011). Arsenic also degrades muscle maintenance and regeneration (Ambrosio *et al.*, 2014; Zhang *et al.*, 2016). The fundamental mechanism for the effects of low to moderate arsenic exposures (10-100 ppb in drinking water) leading to muscle dysfunction and quality decline appears to be disruption of muscle and stem cell mitochondrial function and mitochondrial control of epigenetic regulatory programs (Ambrosio *et al.*, 2014; Cheikhi *et al.*, 2019). Indeed, arsenic reportedly increases DNMT3a-dependent DNA methylation of myogenic genes to impair muscle progenitor cell differentiation (Cheikhi *et al.*, 2019; Hong & Bain, 2012; Steffens *et al.*, 2011). However, it remains unclear how low levels of arsenic, which are stoichiometrically unlikely to affect mitochondrial respiration directly, signal for sustained mitochondrial dysfunction.

Low (nM) levels of arsenic activate receptor-initiated signal amplification cascades, which promote pathogenic phenotypic change and transformation in target cells (Andrew *et al.*, 2009; Garciafigueroa *et al.*, 2013; Simeonova & Luster, 2002; Straub *et al.*, 2009). The epidermal growth factor receptor (EGFR) has long been a focus of investigation of receptor-mediated arsenic actions, especially those contributing to carcinogenesis (Andrew *et al.*, 2009; Germolec *et al.*, 1998; Simeonova & Luster, 2002; Simeonova *et al.*, 2002) and stimulation of progenitor cell proliferation at the expense of differentiation (Patterson & Rice, 2007; Reznikova *et al.*, 2010). Arsenic and other stressors stimulate non-canonical (ligand-independent) EGFR activation where proliferative signals are sustained as the receptor is not degraded upon ligand binding (Andrew *et al.*, 2009; Simeonova *et al.*, 2002; Tan *et al.*, 2016). In cancer cells, activated EGFR scaffolds mitochondrial remodeling enzymes, disrupts respiration, increases reactive oxygen species (ROS) generation, and enhances cell growth and motility (Bollu *et al.*, 2014; Demory *et al.*, 2009; Li *et al.*, 2017; Yao *et al.*, 2010). While the role of sustained mitochondrial EGFR activity has not been explored in muscle progenitor cells or skeletal muscle regeneration, it is clear that mitochondrial dysfunction impedes myogenic differentiation and muscle maintenance (Sahu *et al.*, 2018; Tsitkanou *et al.*, 2016; Wagatsuma & Sakuma, 2013). Dysfunctional mitochondrial metabolism also underlies development of myosteatosis (intramuscular adipose tissue) (Gumucio *et al.*, 2019), a pathogenic mechanism in the etiology of cardiometabolic diseases (Miljkovic *et al.*, 2013; I. Miljkovic *et al.*, 2015; Reinders *et al.*, 2016).

We previously reported that arsenic, in low nM concentrations, targets muscle and progenitor cell mitochondria to disrupt muscle metabolism, maintenance, and regeneration (Ambrosio *et al.*, 2014; Cheikhi *et al.*, 2019). However, the mechanisms through which low nM levels of arsenic initiate and sustain abnormal mitochondrial morphology and function, as well as

alter muscle progenitor cell fate decisions, are unknown. Thus, we investigated the hypothesis that arsenic requires receptor-mediated signal amplification to disrupt mitochondrial function and retain myoblasts in an activated, proliferative state, which ultimately impairs myogenic differentiation.

4.1 Materials and Methods

Cell Culture. C2C12 myoblasts were plated at 10,000 cells per cm^2 on glass coverslips and cultured until 80% confluent in growth medium (GM, Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO2. Myogenic differentiation was induced with differentiation medium (DM, Dulbecco's modified Eagle's medium supplemented with 2% horse serum and 1% penicillin/streptomycin) in the presence or absence of sodium arsenite (2-500 ηM, Fisher Scientific) and AG-1478 (1 μM, Fisher Scientific). At the end of the differentiation period, the cultures were fixed and imaged to quantify myotube formation relative to undifferentiated reserve cell RC proliferation (RC) (Nagata *et al.*, 2014; Yoshida *et al.*, 1998). In separate experiments, C2C12 cultured in non-coated 25 cm^2 flasks were differentiated in the absence or presence of 20 ηM arsenic for 4 days, and then mononucleated, undifferentiated RC were collected by mild trypsinization and separated from myotubes by filtering through a 40-μm strainer. The RC were either rinsed and fixed for flow cytometry or replated in arsenic-free GM on glass coverslips and allowed to proliferate for 3 days in the absence or presence of SS-31 or XJB-5-131 before fixing and immunofluorescent imaging.

Quantitative flow and imaging flow cytometry analyses. To quantify receptor protein expression and phosphorylation, RC were harvested at the fourth day of differentiation, washed with phosphate buffered saline (PBS) and fixed in 1.5% paraformaldehyde in PBS. The cells were permeabilized with 0.05% Triton X-100 in PBS for 15 minutes at room temperature, washed with PBS plus 0.5% BSA (PBB), and then incubated with primary antibody in PBB for two hours at 37 °C. For flow cytometric analysis of EGFR phosphorylation, cells were immunostained with rabbit anti-pY^{845}EGFR (1:100, Cell Signaling # 2231) and mouse anti-EGFR (1:100, containing mouse anti-EGFR (1:100, BD Laboratories #610017). After three washes with PBB, the samples were incubated for 30 minutes at room temperature with the secondary conjugated antibodies, Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:500, ThermoFisher Scientific) or Alexa Flour 488-goat anti-mouse IgG (1:500, ThermoFischer Scientific). The labelled cells were then washed three times with PBB, and fluorescence quantified by flow cytometry using a Biosciences FACSCanto flow cytometer.

Quantitative immunofluorescence imaging. Control and arsenic-primed RC were plated on coverslips in 24 well-plates or eight-well chamber slides. After three days in culture, the cells were washed three times with HBSS, fixed with 2% paraformaldehyde for 10 minutes at room temperature, and permeabilized with 0.1% Triton X-100 made in PBS solution for 15 minutes. The cells were then washed with PBS followed by PBS with 0.5% BSA (PBB). Cells were then blocked with 5% donkey serum (Millipore, S30-100KC) diluted in PPB for 60 minutes, and then washed again three times with PBB before adding primary antibodies for 60 minutes. Primary antibodies recognized: Cyclin D1, (1:100, R&D Systems AF4196), pY^{845}EGFR (1:150, Cell Signaling Technology 2231), and MTCO2 (1:200, ThermoFisher Scientific 12C4F12). Cell monolayers were washed with PBB and then species specific, fluorescent conjugated secondary antibodies

were added for 60 minutes. These antibodies included: Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (1:2000, Invitrogen, A21206), Alexa Fluor 647-conjugated donkey anti-goat IgG (H+L) (1:700, Jackson ImmunoResearch Laboratories 705-605-003); and Cy3-Conjugated donkey anti-mouse (1:800, Jackson ImmunoResearch Laboratories 715-165-151). All samples were additionally stained with 4',6-diamidino-2-phenylindole (DAPI, Biolegend) for one minute. Slides were washed with PBS and coverslips mounted with Fluoromount-G (eBioscience 004958-02).

Z stack images were acquired by confocal microscopy using a Nikon A1 Confocal Laser Microscope with NIS-Elements C Software (Center for Biologic Imaging, Pittsburgh, Pennsylvania). Fluorescent intensities and Colocalization measurements were determined using Nikon Elements AR (Nikon Elements) and Imaris software. Statistical analyses of fluorescent signals were performed using GraphPad Prism v8.2 software.

Mitochondrial membrane potential. At the end of the differentiation protocol, RC were re-seeded in a 96 well black culture plate at 50,000 cells/well and incubated at 37°C and 5% CO_2 in a humidified chamber. After 6 hours of equilibration, mitochondrial membrane potential was measured. Alternatively, RC were replated in a 96 well black culture plate at 10,000 cells/well and cultured for 4 days with or without 100 ηM of mitochondrial targeting SS-31 (Campbell *et al.*, 2019; Mitchell *et al.*, 2020; Szeto & Liu, 2018). At the end of the incubation periods, the culture medium was discarded, the cells were rinsed with PBS, and then incubated in assay medium (140 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2 and 20 mM HEPES, pH = 7.4, mOsm = 300) containing an excess of JC-1 (1 μM). After 90 minutes of equilibration at 37 °C and 5% CO_2, the ratio of the fluorescence of JC-1 aggregates formed as mitochondrial membrane potential ($\Delta\Psi m$) increases (ex 353 nm, em 595 nm) to JC-1 monomer (ex 485, em 535) was then measured every 10 minutes over 3 hours.

Measurement of mitochondrial superoxide. Cells were stained with 250 ηM of MitoSOX red (Ex: 488 nm/Em: 575 nm, cat #: M36008, ThermoFisher) for 15 min at 37 °C in the dark. The cells were then collected by trypsinization and washed with Hank's balanced salts solution (HBSS), fixed in 1.5% paraformaldehyde for 10 min, washed to remove any residual paraformaldehyde, and resuspended in 0.5 ml of PBS. Relative MitoSOX fluorescence intensity per cell was then quantified by flow cytometry.

4.2 Results

Arsenic stimulates EGFR to attenuate myogenesis

The most common exposures to arsenic come from drinking contaminated water, and humans drinking water containing 50 ppb arsenic have a blood concentration of approximately 90 ηM inorganic arsenic (Hall *et al.*, 2006). Comparatively lower levels should distribute in skeletal muscle tissue. Thus, we used 2-500 ηM concentrations to examine arenite effects on C2C12 myogenic differentiation. We confirmed the observations of Bain *et al.* (Hong & Bain, 2012; Steffens *et al.*, 2011) that the point of departure for impairing myoblast fusion into myofibers and myofiber length is between 20 ηM and 50 ηM arsenite (Figures 2A-C). We previously found that arsenic stimulates proliferation of undifferentiated, mononucleated RC (Cheikhi *et al.*, 2019). As seen in (Figures 2D-E) this proliferation decreased the relative proportion of multinucleated myotubes. To examine the role of EGFR in arsenite-stimulated RC proliferation, we added the selective EGFR tyrosine kinase inhibitor, AG-1478, with arsenite at the start of the differentiation protocol. The ratio of RC to differentiated myotubes in the cultures receiving AG-1478 with arsenic was the same as in control cultures (Figure 2). Taken together, these findings confirm that

exposure to arsenic promotes RC proliferation and aberrant retention of "stemness" (Cheikhi *et al.*, 2019), and implicate sustained EGFR activity in arsenic-impaired differentiation.

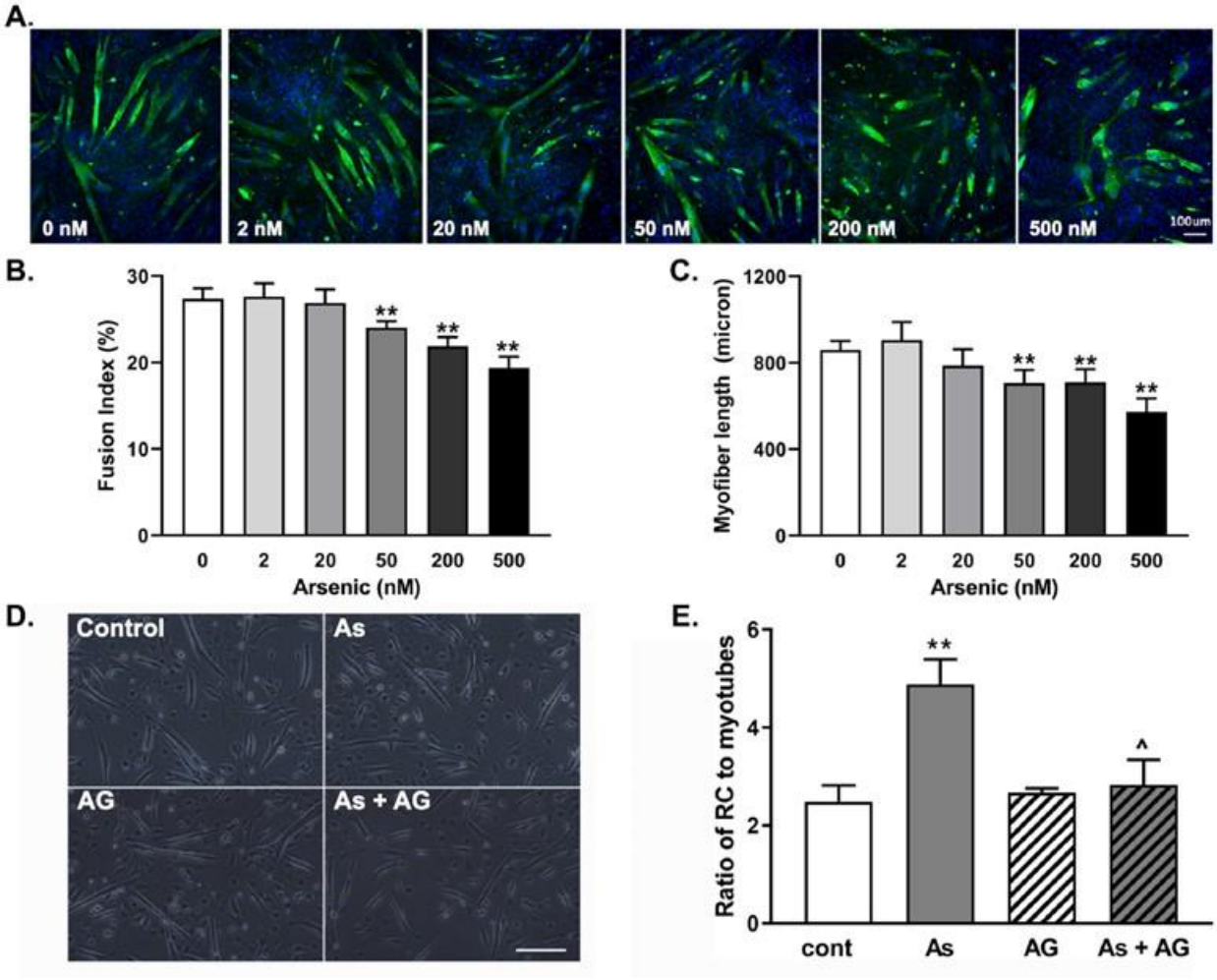

Figure 2: Arsenic impairs myoblasts differentiation efficiency through an EGFR dependent mechanism. **(A-C)** C2C12 cultures were exposed to the indicated range of arsenite concentrations during the myogenic differentiation protocol. The cultures were fixed and myotubes were immunostained antibody to MHC (green) and nuclei were stained DAPI (A). A fusion index was calculated (B) and tube length measured (C). Significant differences from control (0 ηM As) were determined using one-way ANOVA with Dunnett's test and difference at $p<0.01$ is designated by ** (n=6). (D) Cultures were differentiated in the absence or presence of 20 ηM arsenite (As) and/or 1 μM AG-1478 (AG) for 4 days, and then fixed and brightfield images were captured (scale bar=50 μm). The multinucleated myotubes and mononuclear RC were enumerated and the ratio of RC to myotubes calculated. The graph in (E) presents the mean + SEM of the RC/myotube ratio and significant differences between groups was calculated using ANOVA followed by Tukey's multiple comparison (**=$p<0.01$ from control, ^=$p<0.05$ from As).

Arsenic stimulates prolonged EGFR activation and colocalization with mitochondrial regulatory proteins.

Given the protective effects of AG-1478, we next sought to investigate whether arsenic stimulated noncanonical EGFR activation by quantifying EGFR phosphorylated on tyrosine 845 (pY^{845}EGFR) in RC that were re-plated after the differentiation protocol and cultured in arsenic-free growth medium for 6 hours. Src family kinase phosphorylation of EGFR tyrosine 845 is a hallmark of oxidant-mediated, noncanonical EGFR activation, as well as activated mitochondrial EGFR (Boerner *et al.*, 2004; Bollu *et al.*, 2014; Demory *et al.*, 2009; Lambertini & Byun, 2016). As seen in Figure 3A, RC from C2C12 cultures exposed to arsenic during the differentiation protocol and replated in the absence of arsenic retained elevated pY^{845}EGFR, relative to RC from the arsenic-free differentiation protocol. Flow cytometric analyses of the RC confirmed that arsenic exposure during the 4 days of differentiation increased pY^{845}EGFR without affecting EGFR expression (Figure 3A). However, the flow cytometric analysis indicated that only a portion of the RC from the arsenic-exposed cell population retained activated EGFR.

Activated mitochondrial pY^{845}EGFR binds to and phosphorylates the MTCO2 subunit of complex IV in the respiratory chain (Boerner *et al.*, 2004; Demory *et al.*, 2009). Quantitative immunofluorescent imaging demonstrated that arsenic increased colocalization of pY^{845}EGFR with MTCO2 (Figures 3B-C) in the replated RC progeny, and adding AG-1478 with arsenic during differentiation prevented this sustained colocalization (Figures 3B-C).

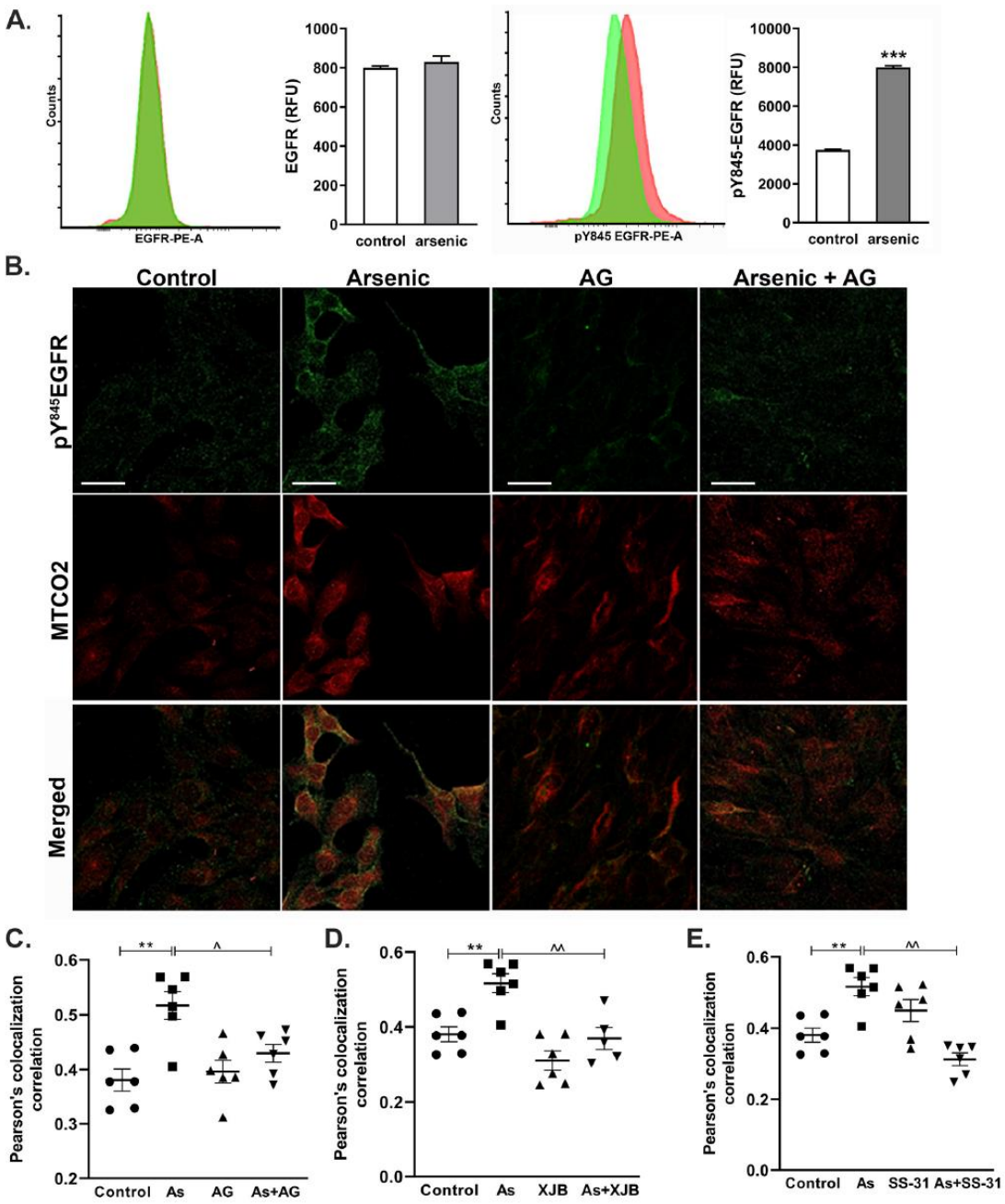

Figure 3: Sustained EFGR activation and mitochondrial localization in arsenic-primed RC.
(A) Protein levels of total EGFR and pY845EGFR were compared by flow cytometry in control RC (green) and arsenic-primed RC (red) re-plated for 6 hours in the absence of arsenic. The bar charts report the mean + SEM of fluorescent intensity in cells from a combined three separate experiments. *** $p<0.001$ by two-tailed Student's t-test. **(B)** Replated RC were immunostained with antibodies to pY845EGFR (green) and MTCO2 (red) and imaged with confocal microscopy (scale bar=50 microns). Arsenic (As, 20ηM) and AG-1478 (AG, 1μM) were added only in the differentiation phase of the experiment. **(C)** Pearson's colocalization coefficients for pY845EGFR and MTCO2 were calculated and the graph shows mean +/- SEM for six separate cell images from each group. **(D-E)** Pearson's colocalization coefficients for pY845EGFR and MTCO2 were calculated six separate cell images of control or arsenic-primed RC, as well as control and arsenic-primed RC that received either XJB-5-131 (XJB, 100 ηM) or SS-31 (100 ηM) after being replated and cultured for 4 days. Statistical differences were determined by one-way ANOVA, followed by Tukey's post-hoc test (**$p<0.1$ different from control and ^^$p<0.01$ different from arsenic).

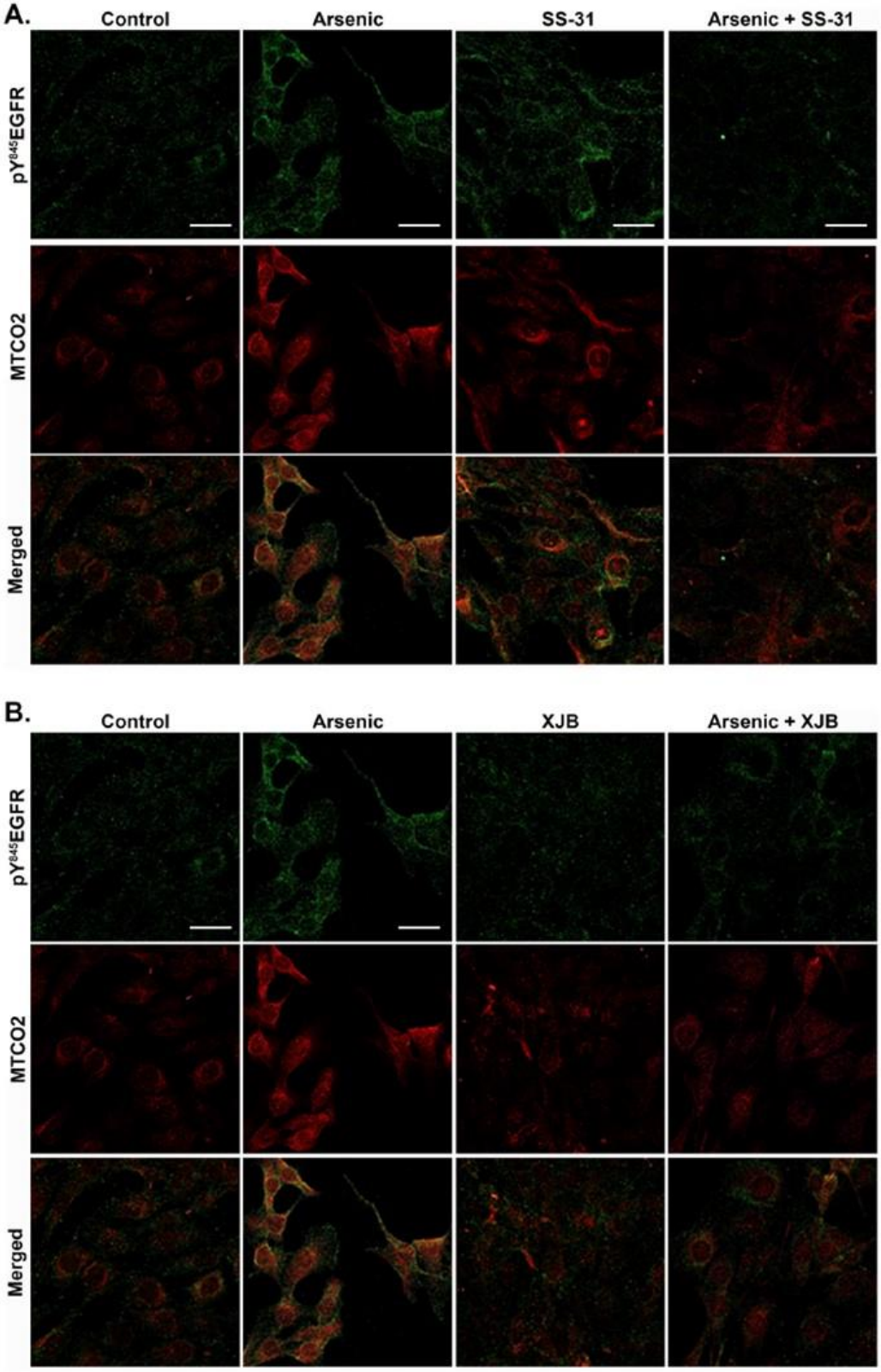

Figure 4: Images for graphs 3D-E.

At the level of mitochondrial function, low-level arsenic exposure led to a sustained excess of mitochondrial superoxide production in RC progeny, as well as morphological change, over multiple divisional generations in arsenic-free growth medium; an indication of altered epigenetic memory (Cheikhi *et al.*, 2019). Since elevated ROS is a non-canonical activator of EGFR, we confirmed a role of mitochondrial ROS and/or disrupted respiration in maintaining the pY^{845}EGFR/MTCO2 colocalization by reversing arsenic effects with two mitochondrially targeted compounds that have separate mechanisms of reducing ROS. Control and arsenic-primed RC progeny (re-plated in arsenic-free medium) were cultured for 4 days with either XJB-5-131, a mitochondrial targeting catalytic electron scavenger (Javadov *et al.*, 2015), or SS-31, a mitochondrial targeting peptide that preserves the integrity of the mitochondrial membrane phospholipid cardiolipin (Campbell *et al.*, 2019; Mitchell *et al.*, 2020; Szeto & Liu, 2018). Addition of either compound to arsenic-primed RC progeny reversed the arsenic-promoted pY^{845}EGFR colocalization with MTCO2 to a level comparable to the control RC progeny (Figures 3D-E and 4). Note that despite removing arsenite at the time of replating, pY^{845}EGFR remained elevated in that arsenic-primed RC.

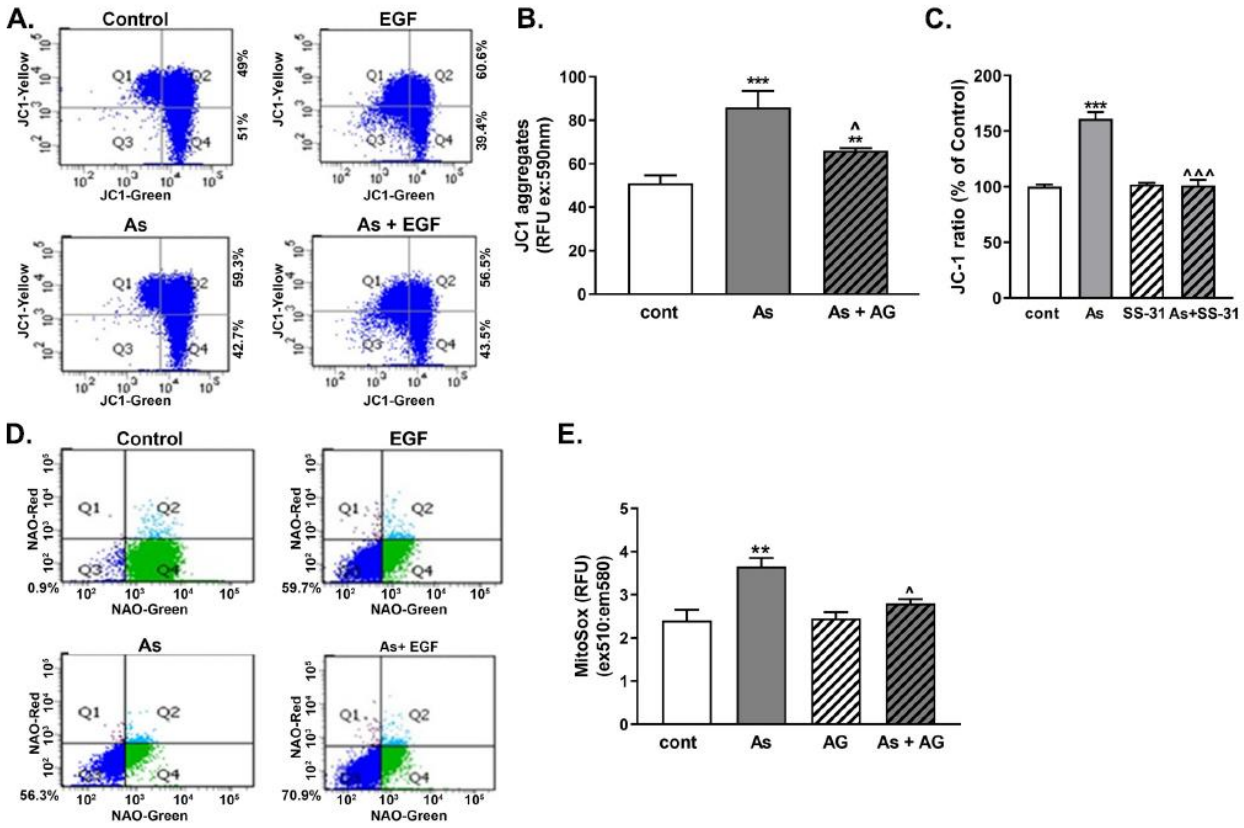

Figure 5: Dysfunctional mitochondrial effects of arsenic and EGF.
(A) Flow cytometric analysis of mitochondrial membrane potential using JC-1 ratiometric dye in arsenic (20nM) or EGF (100 ηM) exposed RC, relative to control. Percentages show the relative abundance of cells in quadrant 2 (JC-1 aggregates) and quadrant 4 (JC-1 monomers). **(B)** Quantification of JC-1 aggregates that indicate more positive membrane potential. AG-1478 (1 μM) was added to cells simultaneously with arsenic in the differentiation protocol and JC-1 fluorescence was measured in undifferentiated RC. **(C)** SS-31 (100 ηM) was added to RC replated in arsenic-free medium and JC-1 fluorescence was measure after 3 days in culture. **(D)** Flow cytometric analysis of cardiolipin oxidation (loss of NAO fluorescence) in RC treated with EGF or arsenic in the differentiation protocol. The percentage of the cell population in quadrant 3 (decreased NAO staining) is given. **(E)** Mitochondrial superoxide was measured in undifferentiated control RC and RC exposed to arsenic and AG-1478 during the differentiation protocol. All experiments were repeated three times and group comparisons were made using ANOVA followed by Tukey's post-hoc test for significance (**p<0.01, ***p<0.001 relative to control, ^p,0.05, ^^^p<0.001 relative to arsenic).

Mitochondrial dysfunction from arsenic-stimulated mitochondrial pY^{845}EGFR

Phosphorylation of MTCO2 by activated pY^{845}EGFR causes mitochondrial morphological change and increased ROS generation as cytochrome C oxidase becomes dysfunctional (Demory *et al.*, 2009). We previously observed that arsenic exposure during the differentiation protocol promotes mitochondrial remodeling, increased mitochondrial mass, increased ΔΨm, and increased

mitochondrial ROS in the replated RC progeny (Cheikhi *et al.*, 2019). Flow cytometric analysis with JC-1 dye demonstrated that treating cells with EGF caused a similar ΔΨm increase and that adding EGF with arsenic resulted in no more of a ΔΨm increase than adding either agent alone (Figure 5A). Treating the arsenic-exposed cells with AG-1478 or SS-31 during differentiation prevented the increased ΔΨm in the arsenic-primed replated RC progeny (Figures 5B-C). Dysfunctional cytochrome c oxidase promotes cardiolipin oxidation and remodeling of the inner mitochondrial membranes (Mitchell *et al.*, 2020; Szeto & Liu, 2018). Addition of either EGF or arsenic decreased the amount of reduced cardiolipin (10-nonyl acridine orange (NAO) staining) in the RC progeny (Figure 5D). Addition of EGF and arsenic together was again less than additive in oxidizing cardiolipin, suggesting that they act through the same mechanism. This mechanism is likely driven by activating mitochondrial EGFR, since AG-1478 attenuated mitochondrial ROS generation in the arsenic-primed progeny (Figure 5E).

Arsenic-enhanced RC stemness and proliferation requires activated EGFR and mitochondrial dysfunction.

Arsenic exposure increases the "stem-like character" (i.e. increased CD34 and CD133 expression) and proliferation of RC progeny, and this proliferative response was associated with RC populations with sustained cyclin D1 levels (Cheikhi *et al.*, 2019). AG-1478 has been shown to inhibit EGFR-induced cyclin D1 upregulation and cell cycle progression (W. Liu *et al.*, 2014; Zhu & Bunn, 2001). Adding AG-1478 with arsenic during differentiation also decreased arsenic-increased nuclear cyclin D1 levels in replated RC progeny (Figures 6A-B). Nuclear cyclin D1 levels in the arsenic-primed RC progeny were also returned to control levels by adding either XJB-5-131 or SS-31 to the re-plated RC progeny (Figures 6C-D, 7). Taken together, these data suggest that the adverse effects of arsenic on myogenesis may be sustained through noncanonical

mitochondrial EGFR activation, which is known to dysregulate cyclin D1-dependent myogenic cell cycle progression (Owusu-Ansah *et al.*, 2008).

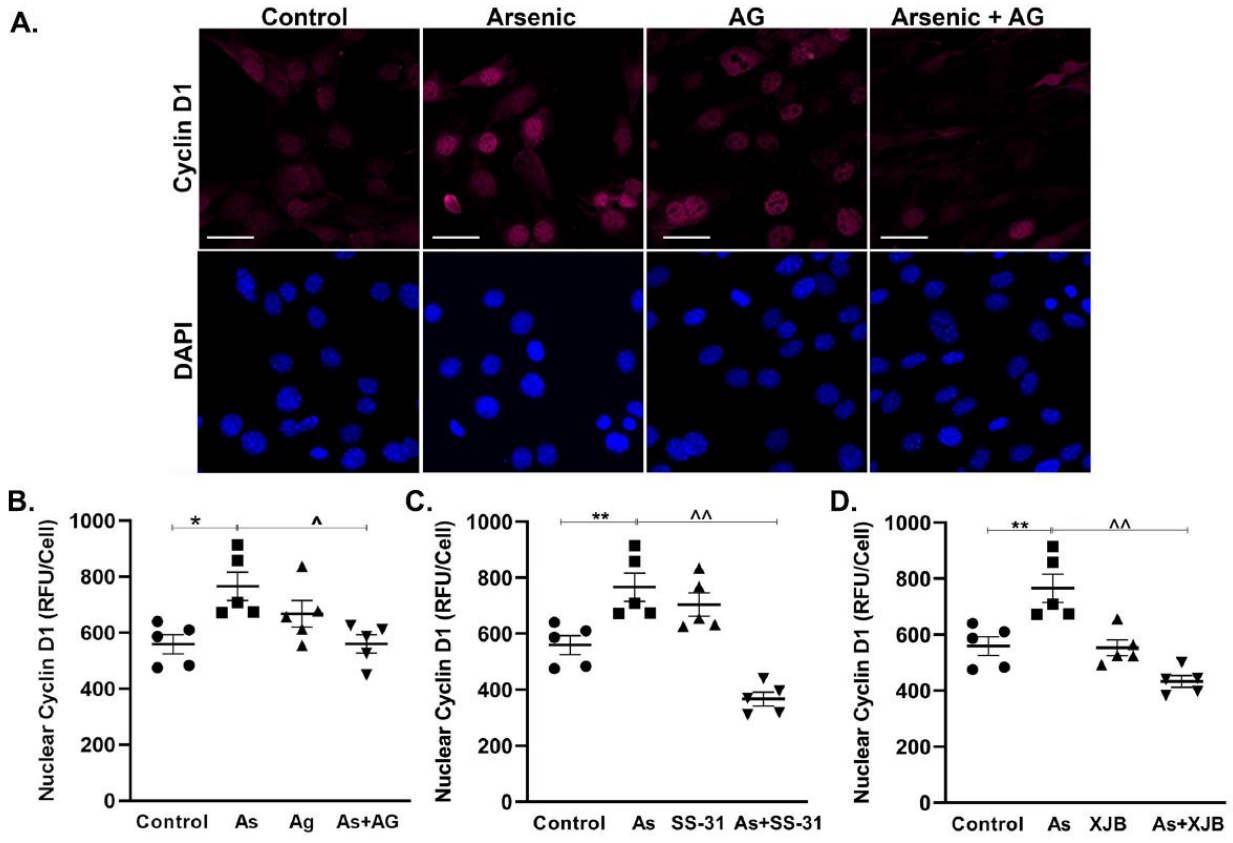

Figure 6: Arsenic effects on EGFR and mitochondrial regulation of nuclear cyclinD1.
(A) Representative images of cyclin D1 immunofluorescence in undifferentiated RC replated in arsenic-medium. AG-1478 was added simultaneously with arsenic in the differentiation protocol. (B-D) Quantitative comparison of nuclear cyclin D1 levels in replated RC. Groups of cells in C and D were treated with SS-31 or XJB-5-131 after replating and all groups in B-D were fixed and analyzed after three days in arsenic-free culture. Representative images of cells from C and D are provided in Supplemental Figure 2. All experiments were repeated three times and the data are presented as mean +/- SEM of relative fluorescence per cell captured in five separate images. Group comparisons were made using ANOVA followed by Tukey's post-hoc test for significance (*$p<0.05$, **$p<0.01$ relative to control, ^$p<0.05$, ^^$p<0.01$ relative to arsenic).

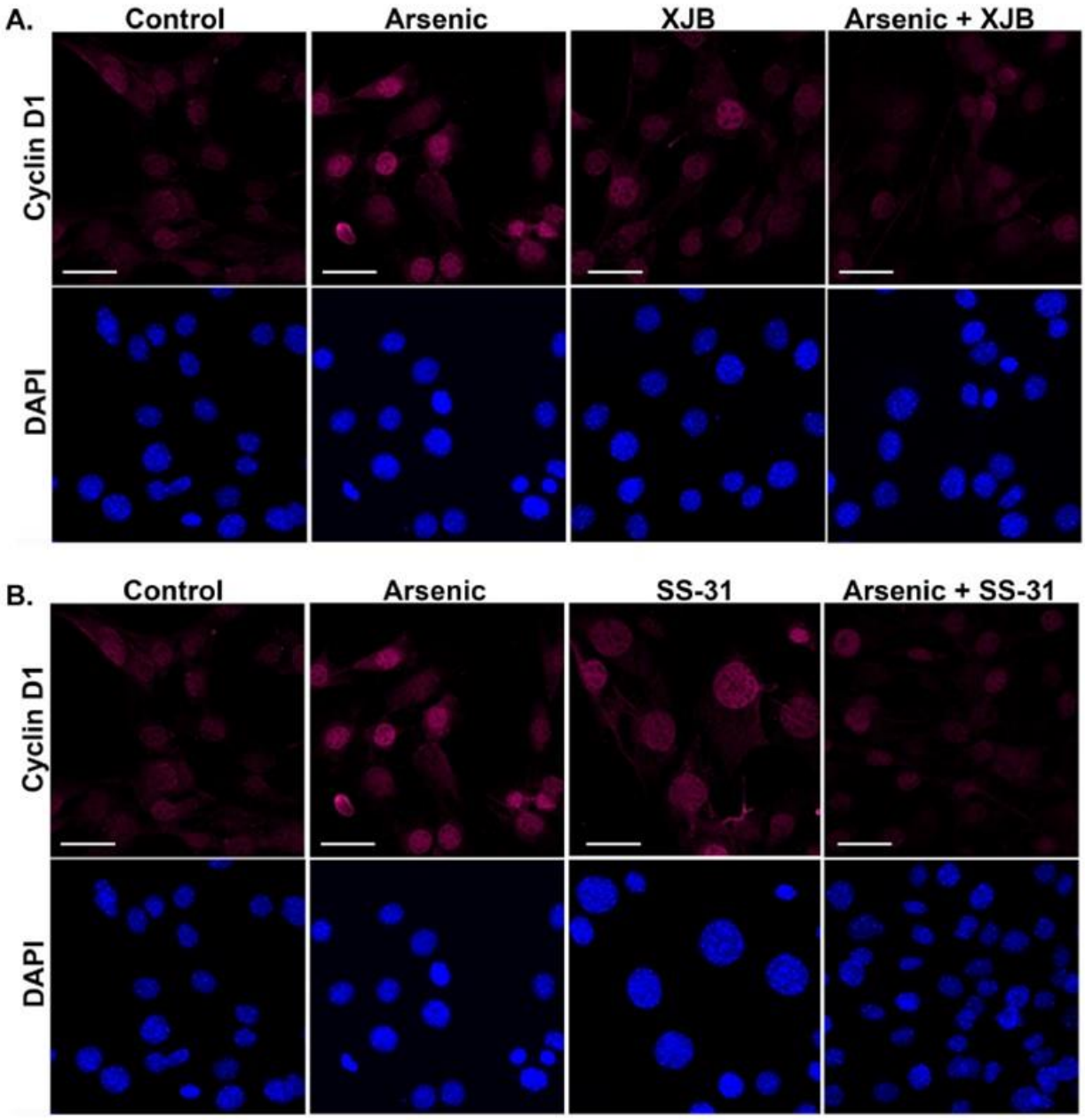

Figure 7: Images for Grafts 6 C-D.

4.3 Discussion

Skeletal muscle homeostasis and regeneration is critically dependent on the balance between muscle stem cell self-renewal and commitment to differentiation. Here, we demonstrate that arsenic exposure disrupts this critical balance to impair myogenesis, and identify mitochondrial EGFR activation as an upstream mechanism for arsenic dysregulation of RC fate. Indeed, low-level arsenic stimulation of mitochondrial pY^{845}EGFR was associated with remodeling of the mitochondrial membranes, increased $\Delta\Psi m$, increased mtROS, and proliferative signaling at the expense of myogenic differentiation. This mechanism for low-level arsenical effects on mitochondrial function and cell fate varies greatly from mechanisms through which much higher levels of arsenic inhibit mitochondrial ATP generation and promote cell death.

Skeletal muscle maintenance and regeneration depend on resident stem cells (satellite cells, MuSC), that are normally found in a quiescent state (Lipton & Schultz, 1979; Schultz, 1989). In response to injury, MuSCs activate, enter a state of transient amplification, and begin to express muscle regulatory transcription factors (MRFs) (Charge & Rudnicki, 2004; Hawke & Garry, 2001). The commitment to terminal differentiation occurs with expression of the MRF, myogenin, and degradation of the transcriptional repressor of differentiation, Pax7 (Ciciliot & Schiaffino, 2010; Olguin *et al.*, 2007). *In vivo*, EGFR is activated in adult MuSCs and RCs as they exit quiescence in the early stage of myogenic differentiation (Golding *et al.*, 2007; Nagata *et al.*, 2014) and this response is recapitulated in differentiating myoblasts *in vitro* (Andrechek *et al.*, 2002; Ford *et al.*, 2003). However, prolonged EGFR activity inhibits myogenic differentiation as the activated MuSCs are locked in a proliferative state (Golding *et al.*, 2007; Leroy *et al.*, 2013). While most attention has focused on ligand-dependent canonical action of plasma membrane EGFR, noncanonical, ligand-independent EGFR signaling in different cellular organelles is now

recognized to be a primary driver of pathogenic cell proliferation and proliferative diseases (Demory *et al.*, 2009; Tan *et al.*, 2016).

Murine C2C12 myoblasts provide a useful model for studying the consequence of adverse EGFR activation in quiescent MuSCs (Charville *et al.*, 2015; Golding *et al.*, 2007; Leroy *et al.*, 2013; Nagata *et al.*, 2014; Yoshida *et al.*, 1998). C2C12 RC display key characteristics that are shared by MuSC *in vivo*, including quiescence, activation, self-renewal and generation of myotubes (Yoshida *et al.*, 1998). The C2C12 differentiation model was shown to be extremely sensitive to arsenic and arsenic-induced epigenetic repression of myotube differentiation (Hong & Bain, 2012; Steffens *et al.*, 2011). These previous studies found that ηM arsenic suppressed MRF activation with increased DNMT3a-dependent methylation of the *Myod* promoter. We found that ηM arsenic induces C2C12 *Dnmt3a* through mitochondrially-regulated epigenetic changes, and that this epigenetic regulation enhances C2C12 proliferation over myogenic differentiation (Cheikhi *et al.*, 2019). The current findings suggest that low, ηM levels of arsenic promote signal amplification through mitochondrial EGFR and its downstream pathways that disrupt respiratory chain activity, promote mitochondrial remodeling, and alter mitochondrial to nuclear communication.

In addition to increasing DNMT3a repression of MRF expression, arsenic may suppress differentiation through EGFR and mitochondrial-dependent temporal dysregulation of cyclin D1 (Figure 5). We previously found that arsenic-induced temporal dysregulation of cyclin D1 oscillatory behavior impedes adipogenic stem cell differentiation (Beezhold *et al.*, 2016). In the current studies, arsenic-increased nuclear cyclin D1 levels were attenuated by AG-1478 or reversed by adding XJB-5-131 or SS-31 to arsenic-primed RC. Sustained nuclear cyclin D1 affects self-renewal and differentiation in MPC by preventing MyoD binding to DNA (Wei & Paterson,

2001). Growth factor-stimulated nuclear cyclin D1 bound to cyclin-dependent kinase 4 (cdk4) is essential for allowing MPCs to choose to remain in cell cycle or exit to terminal differentiation (Wei & Paterson, 2001). Thus, by sustaining mitochondrial EGFR activity and its consequent effects on mitochondrial function, arsenic likely prevents the fall in cyclin D1 in G0 of cell cycle that allows MyoD and other MRF complexes to transactivate differentiation.

The full health impacts of arsenic effects on muscle mitochondrial function and impaired regeneration are unknown. There have been only a few epidemiological studies focused specifically on arsenic-impaired muscle function. Parvez *et al*. found that even low to moderate level arsenic exposures (less than 100 ppb) cause strength and motor deficits in children (Parvez *et al.*, 2011). Others found rates of muscle morbidity, atrophy, and weakness in the range of 35-85% in populations exposed to very high arsenic levels (Chakraborti *et al.*, 2003; Mukherjee *et al.*, 2003). The effects of arsenic on sensory motor coupling and neural maintenance of muscle are a large component of these morbidities. We found direct effects of arsenic on muscle strength, muscle regeneration and muscle compositional change, as well as on isolated muscle cells that are likely independent of neural input (Ambrosio *et al.*, 2014; Cheikhi *et al.*, 2019; Garciafigueroa *et al.*, 2013; Zhang *et al.*, 2016). Our findings of low-level arsenic-induced compromise of muscle mitochondrial function, bioenergetics, and compositional change towards ectopic adiposity (myosteatosis) may explain an underlying mechanism for arsenic-promoted diseases beyond skeletal muscle defects.

Loss of muscle quality is critically important to overall well-being as highlighted by the fact that skeletal muscle comprises 40-50% of total body metabolism, and that myosteatosis is associated with increased risk of all-cause and cardiovascular disease mortality (Carobbio *et al.*, 2011; Goodpaster *et al.*, 2000; Miljkovic & Zmuda, 2010; Prado *et al.*, 2018), as well as diabetes

(Miljkovic *et al.*, 2013). Thus, continued findings of direct effects on low-level arsenic on muscle metabolism, function, and composition warrant further population level investigation of the role of these environmentally-derived pathologies in the etiology of arsenic promoted diseases. The finding that mitochondrial protectants can mitigate and reverse arsenic-promoted pathogenic determination of progenitor cell fate has broad implications for developing mitochondrially directed strategies to treat disease in arsenic-exposed populations.

5.0 Chapter 4: Arsenic Directs Stem Cell Fate by Imparting Notch Signaling into the Extracellular Matrix Niche

This article is currently in review at Toxicological Sciences.

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FUNDING: NIEHS grants R01ES023696 (FA and AB) and R01ES025529 (FA and AB) supported this work. We acknowledge the NIH-supported microscopy resources in the Center for Biologic Imaging (NIH grant #1S10OD019973-01).

Abstract

Compromise of skeletal muscle metabolism and composition may underlie the etiology of cardiovascular and metabolic disease risk from environmental arsenic exposures. We reported that arsenic impairs muscle maintenance and regeneration by inducing maladaptive mitochondrial phenotypes in muscle stem cells (MuSC), connective tissue fibroblasts (CTF), and myofibers. We also found that arsenic imparts a dysfunctional memory in the extracellular matrix (ECM) that disrupts the MuSC niche and is sufficient to favor the expansion and differentiation of fibrogenic MuSC subpopulations. To investigate the signaling mechanisms involved in imparting a dysfunctional ECM, we isolated skeletal muscle tissue and CTF from mice exposed to 0 or 100 μg/L arsenic in their drinking water for five weeks. ECM elaborated by arsenic-exposed CTF

decreased myogenesis and increased fibrogenic/adipogenic MuSC subpopulations and differentiation. However, treating arsenic-exposed mice with SS-31, a mitochondrially targeted peptide that repairs the respiratory chain, reversed the arsenic-promoted CTF phenotype to one that elaborated an ECM supporting normal myogenic differentiation. SS-31 treatment also reversed arsenic-induced Notch1 expression, resulting in an improved muscle regeneration after injury. We found that persistent arsenic-induced CTF Notch1 expression caused the elaboration of dysfunctional ECM with increased expression of the Notch ligand DLL4. This DLL4 in the ECM was responsible for misdirecting MuSC myogenic differentiation. These data indicate that arsenic impairs muscle maintenance and regenerative capacity by targeting CTF mitochondria and mitochondrially-directed expression of dysfunctional regulators in the stem cell niche. Therapies that restore muscle cell mitochondria may effectively treat arsenic-induced skeletal muscle dysfunction and compositional decline.

5.1 Introduction

The World Health Organization estimates that disease burden is increased in more than 200 million people worldwide who are exposed to arsenic concentrations in drinking water exceeding the threshold of 10 µg/L (Bailey *et al.*, 2016). Chronic arsenic exposure increases the risk of developing a number of cancers and non-cancer diseases that include cardiovascular and metabolic diseases (Kuo *et al.*, 2017; Moon *et al.*, 2017). In addition, arsenic exposures contribute to adverse health outcomes that include skeletal muscle weakness, mobility dysfunction, and impaired muscle metabolism (Mazumder and Dasgupta, 2011; Parvez *et al.*, 2011).

Skeletal muscle tissue accounts for 40-50% of body weight in lean individuals and approximately 50% of metabolism. Loss of lean body mass and muscle quality are increasingly recognized as significant risks, if not etiological factors for cardiovascular, lung, and metabolic disease (Correa-de-Araujo *et al.*, 2017; Prado *et al.*, 2018). Muscle quality is critically important to overall well-being, as increased fibrosis and adiposity (fibro-adipogenesis, myosteatosis) are associated with an elevated risk of all-cause and cardiovascular disease mortality (Miljkovic-Gacic *et al.*, 2005; Miljkovic and Zmuda, 2010; Santanasto *et al.*, 2017). Increased inter- and intra-myocellular fat is an early sign of insulin resistance and impaired metabolism (Goodpaster *et al.*, 2000; Sell *et al.*, 2006; Vigouroux *et al.*, 2011). As awareness of the importance of muscle quality decline and myosteatosis in the etiology and promotion of disease has only recently increased (Aleixo *et al.*, 2020; Nachit and Leclercq, 2019; Prado *et al.*, 2018), few studies have investigated environmental influences on muscle decline and myosteatosis. Nonetheless, a limited number of epidemiological (Parvez *et al.*, 2011) and animal studies (Ambrosio *et al.*, 2014; Garciafigueroa *et al.*, 2013; Yen *et al.*, 2010; Zhang *et al.*, 2016) found that environmental arsenic exposure impairs skeletal muscle function and promotes ectopic fat deposition, dysfunctional metabolism, dysfunctional myogenesis, and aberrant progenitor cell differentiation.

Skeletal muscle is composed of highly metabolic, insulin-sensitive fibers, neural and vascular structures, elaborate networks of connective tissue and extracellular matrix (ECM), and multiple stem/progenitor cell populations (Moyle *et al.*, 2019; Tedesco *et al.*, 2010; Tedesco *et al.*, 2017). While the primary role of the ECM is to provide structural support required for motor function, it is also an essential repository for cellular communications and helps to regulate metabolic functions (Hays *et al.*, 2008; Zhang *et al.*, 2016). The ECM is predominantly generated by connective tissue fibroblasts (CTF) and vascular-associated cells, and it provides the regulatory

niche for resident stem cells (Gillies and Lieber, 2011). Disrupting the interactions within these resident cell populations and modifying their molecular contributions to the ECM microenvironment compromises the balance within the skeletal muscle niche, including its overall structure and metabolism (Engler *et al.*, 2006; Smith *et al.*, 2017; Stearns-Reider *et al.*, 2017; Williams *et al.*, 2015; Zhang *et al.*, 2016).

We have previously shown that human muscle stem cells (hMuSC) cultured on decellularized ECM constructs derived from arsenic-exposed skeletal muscle undergo a fibrogenic conversion, unlike hMuSC cultured on control ECM constructs (Zhang *et al.*, 2016). These data confirm that hMuSC are receptive to signaling from the skeletal muscle ECM and suggest that arsenic exposure imparts changes within the ECM that misdirect hMuSC differentiation. Furthermore, CTF isolated from arsenic-exposed mice (CTF^{ars}) retain elevated NF-κB-driven ECM gene transcript levels, relative to CTF isolated from control mice (CTF^{ctr}) (Zhang *et al.*, 2016), and these levels may promote ECM remodeling and dysfunctional ECM to cell communications. We also found that arsenic targets mitochondrial morphology and dynamics to disrupt muscle metabolism, mitochondrial oxidant generation, and epigenetic memory in muscle progenitor cells (Ambrosio *et al.*, 2014; Cheikhi *et al.*, 2020; Cheikhi *et al.*, 2019). Thus, in the current study, we investigated whether arsenic effects on CTF mitochondria underlie dysfunctional elaboration of a stress memory into the ECM. Given that signaling from this memory may also direct MuSC fate and impair muscle maintenance and regenerative capacity, we also investigated the signaling mechanisms involved in imparting the CTF^{ars} ECM memory that reduces myogenesis and drives fibro-adipogenic differentiation of hMuSC.

6.0 Materials and Methods

Animal exposures. Six-week-old male C57Bl/6J mice (Jackson Laboratory) were exposed to 0 or 100 sodium meta-arsenite ($NaAsO_2$; Fisher Scientific) in their drinking water for five weeks, at which time mice were euthanized and hind limb muscles collected. Arsenite was used as it is the most environmentally-relevant toxic form of inorganic arsenic in drinking water. We found that this exposure causes pathological tissue and ECM remodeling in multiple organs of the mouse without causing any signs of overt lethality or changes in weight gain (Ambrosio *et al.*, 2014; Garciafigueroa *et al.*, 2013; Soucy *et al.*, 2005; Straub *et al.*, 2008; Zhang *et al.*, 2016). Fresh arsenite-containing water was provided every 2-3 days to ensure that there is little arsenite oxidation to pentavalent arsenate. All studies were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. As shown in the scheme in Figure 3A, in experiments evaluating SS-31 for reversing arsenic effects, arsenic exposure was stopped after five weeks, and the mice received daily i.p. injections of SS-31 (1 mg/kg in saline) or saline for one week. Bilateral tibialis anterior (TA) muscles were then injured with 20 µl of 1.2% (w/v) barium chloride ($BaCl_2$). The mice were allowed to recover for two weeks, and, after euthanizing, TA muscles were evaluated for tissue regeneration, gastrocnemius muscles were collected for gene transcript and ChIP analysis, and CTF were isolated from the remaining hind limb muscles.

CTF isolation. For each isolation, CTF were isolated from the hind limb muscles of two mice, essentially as described (Goetsch *et al.*, 2015). After euthanizing the mice, hind limb muscles were removed and finely minced in Hank's balanced salt solution (HBSS). The minced pieces were collected by centrifugation at 900g for five minutes, resuspended in Collagenase XI (2 mg/ml in HBSS C7657 Sigma Aldrich), and digested for 60 minutes at 37^{o}C with shaking

every 10 minutes. The suspension was centrifuged for five minutes at 900g, and the supernatant was discarded. The pellet was resuspended in dispase II (2.4 U/ml in HBSS, ThermoFisher 17105041) and digested for 45 minutes at 37^{o}C with shaking every 10 minutes. The suspension was again centrifuged for five minutes at 900g, the supernatant was discarded, and the pellet was resuspended in 0.1% Trypsin. After a 30 minute incubation followed by centrifugation, the resulting cell pellet was resuspended in growth medium (DMEM (4.5 g glucose/ml) supplemented with 10% fetal bovine serum (Hyclone), 10% horse serum (Hyclone), 1% penicillin-streptomycin antibiotics and 0.5% chicken essential extract). The suspension was filtered through a 70 micron strainer and the cells in the filtrate were seeded in collagen-coated six-well plates. Non-adherent cells were removed after three hours and the adherent CTFs were cultured in fresh growth medium. Greater than 95% of the adhered CTF^{ctr} and CTF^{ars} stained positive for the fibroblast marker, α-smooth muscle actin (αSMA).

CTF ECM elaboration, decellularization, and myoblast differentiation. Primary CTF were seeded onto collagen-coated eight-well glass chamber slides (10,000 cells/well) and allowed to elaborate an ECM for 2-3 days. The ECM was decellularized by incubating the wells with HBSS at room temperature for 10 minutes and then in distilled water for one hour. The slides were then rinsed three times with HBSS until all the cells and cell ghosts were removed. Human skeletal muscle stem cells (hMuSC, ScienCell #3510) were seeded onto the decellularized ECM construct at 10,000 cells/well and differentiated for two days by culturing in differentiation medium (DMEM (4.5 g/L glucose) supplemented with 2% Horse Serum (Hyclone) and 1% penicillin-streptomycin). At the end of the differentiation period, the cultures were fixed and prepared for immunofluorescent imaging.

To examine the effect of inhibiting CTF^{ctr} and CTF^{ars} Notch on hMuSC differentiation on the elaborated ECM, DAPT (1 μM) was added to CTF one day after seeding and for an additional two days of ECM elaboration. DAPT was removed by decellularizing and rinsing the ECM prior to seeding hMuSC. To demonstrate the role of ECM DLL4, the elaborated ECM was coated with antibody (1 μg/ml) for three hours at 37°C. The antibody solution was rinsed away prior to seeding hMuSC for differentiation.

Quantitative immunofluorescence imaging. CTF^{ctr} and CTF^{ars} were seeded on coated eight-well chamber glass slides and cultured until near confluence. hMuSC were seeded on ECM elaborated onto the glass slides and cultured in differentiation medium for two days. At the end of the experimental period for both cell types, the cells were then fixed with 2% paraformaldehyde for 15 minutes at room temperature, washed three times with phosphate-buffered saline (PBS), and permeabilized with 0.1% Triton X-100 in PBS for 15 minutes at room temperature. Slides were then washed three times with PBS and five times with PBS + 0.5% BSA (PBB). Slides were subsequently blocked with 5% natural donkey serum (Millipore S30-100KC) diluted in PBB for 60 minutes, washed five times with PBB, and then incubated with primary antibodies (Supplemental Table 1: List of antibodies and dilutions) for 60 min at room temperature. After five washes with PBB, the slides were incubated with fluorophore-conjugated secondary antibodies (Supplemental Table 1) for 60 minutes, washed five times with PPB, three times with PBS, and stained with 4',6-diamidino-2-phenylindole (DAPI; BioLegend 422801) for one minute. After mounting coverslips with Fluromount-G (eBiosciences 004958-02), the slides were imaged using a Nikon A1 confocal microscope (University of Pittsburgh Center for Biologic Imaging). Multiple fields were imaged per culture. The mean fluorescent intensity of each protein (desmin,

NICD, PDGFRα, and CD34) was quantified using Nikon Elements AR software (Nikon). Intensity of MyoD within cell nuclei was calculated and averaged per field of view.

Transmission electron microscopy. CTF grown in coated 12-well plastic plates were fixed in 2.5% glutaraldehyde in 100 mM PBS (8 gm/l NaCl, 0.2 gm/l KCl, 1.15 gm/l Na_2HPO_4.7H2O, 0.2 gm/l KH_2PO_4, pH 7.4) overnight at 4ºC. Monolayers were washed three times with PBS and then post-fixed in aqueous 1% osmium tetroxide, 1% Fe_6CN_3 for 1 hr. After three washes with PBS, the cells were dehydrated through a 30-100% ethanol series and then several changes of Polybed 812 embedding resin (Polysciences, Warrington, PA). Cultures were embedded in by inverting Polybed 812-filled BEEM capsules on top of the cells. Blocks were cured overnight at 37ºC and then cured for two days at 65ºC. Monolayers were pulled off the coverslips and re-embedded for cross section. Ultrathin cross sections (60 nm) of the cells were obtained on a Reichert Ultracut E microtome, post-stained in 4% uranyl acetate for 10 minutes and in 1% lead citrate for seven minutes. Sections were imaged using a JEM 1011 TEM (JEOL, Peabody, MA) at 80kV. Images were taken using a side-mount AMT 2k digital camera (Advanced Microscopy Techniques, Danvers, MA).

Second Harmonics Generation imaging (SHG) of whole mount muscles. SHG imaging of intact TA muscles was performed as we previously described. Briefly, after two weeks of recovery from $BaCl_2$ injury, TAs were excised, placed in 2% paraformaldehyde for two hours, and then in Scaleview solution (Olympus) for at least one week. Scaleview solution was replenished every three days. The samples were rinsed and stored in PBS at 4°C until imaged. All imaging was performed with an Olympus multi-photon microscope (Model FV1000, ASW software, Tokyo, Japan). Prior to imaging, samples were placed in hanging drop slides in PBS and a coverslip was placed over the well and in contact with the muscle. The muscle was oriented with the superior

border aligned parallel to the superior edge of the slide and the dorsal surface of the muscle against the coverslip. Images were taken of the medial and lateral muscle bellies at the site of injury to a depth of 100μm, with a step thickness of 2μm and a scan pixel count of 1024x1024.

Western analysis. Western analyses for changes in OXPHOS complex protein abundance were performed, as previously described (Barchowsky *et al.*, 1999; Klei *et al.*, 2013). Briefly, CTF cultures were lysed in RIPA buffer containing protease, kinase, and phosphates inhibitors, and homogenized by sonication. After centrifugation at 10,000 g for 5 minutes at 4^{o}C, protein content of the supernatant was determined by BCA assay (ThermoFisher) and the remainder of the supernatant was mixed with 6x Laemmli sample loading buffer. Equal amounts of protein were separated by PAGE and transferred to PDVF membranes (Immobilon-IF, EMD Millipore Corporation, Billerica, MA). After blocking, the membranes were incubated with Total OXPHOS Rodent WB Antibody Cocktail (Abcam #ab110413) and antibody to VDAC (Abcam # ab15895), rinsed, and then incubated with IRDye® 800CW Donkey anti-Mouse IgG or IRDye® 680LT Donkey anti-Rabbit IgG (Li-COR Biosciences), respectively. Digital infrared imaging and quantification of the protein bands was performed using a LI-COR Odyssey Clx imager and Image Studio v5.2 software.

***Gene transcript and ChIP analysis*.** Gastrocnemius muscles were snap frozen in liquid nitrogen and RNA was isolated from pulverized tissue powder using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Germantown, MD). Isolated RNA was reverse transcribed using iScript™ gDNA Clear cDNA Synthesis Kit (BIO-RAD, Hercules, CA). Notch1 mRNA levels were quantified by SYBR Green-based real-time PCR (qPCR) using SsoAdvanced™ Universal SYBR® Green Supermix (BIO-RAD, Hercules, CA). Transcript levels were normalized to the expression level of Rpl44, and the fold changes of *Notch1* relative to

universal mouse reference RNA (Qiagen) were calculated using 2-ΔΔ Ct method. Each sample was measured in duplicate. Primers sequence: *Rpl44* (Forward: 5'-AGATGAGGCAGAGGTCCAA-3' and Reverse: 5'-GTTGTAAGAAAGGCGGTCA-3') and *Notch1* (Forward:5'- ACAGTGCAACCCCCTGTATG-3', Reverse: 5'-TCTAGGCCATCCCACTCACA- 3'). For ChIP analysis, pulverized tissue powder was fixed with 1% formaldehyde for 15 minutes, followed by quenching with a 125mM glycine solution. Crosslinked proteins were pelleted and washed with PBS containing protease inhibitor cocktail. Chromatin isolation and immunoprecipitation was performed using ChIP-IT kit (Active Motif, Carlsbad, CA), following the manufacturer's instructions.1-2 µg of ChIP-validated antibodies against RNA Polymerase II (A2032, Epigentek, Farmingdale, NY), or H2A.Zac (ab18262, Abcam, Cambridge, MA) were used for immunoprecipitation of each sample. 1% of non-immunoprecipitated chromatin was used as input. Following reverse crosslinking and elution of chromatin, DNA from each sample was purified with QIAquick PCR purification kit (Qiagen). Ten ng of purified DNA was used for PCR of the 5' promoter region of *Notch1* (-107 to +90 nt) using the primers: forward: 5'- CACAAGGGGGTAAGGGTTC-3' and Reverse: 5'-GGCTCGTTCCTTCACTGC-3'. The percent of immunoprecipitated DNA relative to the input was calculated.

Statistical analysis. Data in graphs are expressed as mean ± SEM. Significance between groups was determined by Student's t-tests or one-way ANOVA followed by either Dunnett's or Tukey's tests for multiple comparisons. *A priori,* differences were considered to be significantly different at $p<0.05$. Statistical analysis and graphing were performed using Graphpad Prizm v8.3.1 software.

6.1 Results

ECM elaborated from arsenic-exposed CTF impairs myogenic differentiation. We previously found that environmentally relevant exposure to arsenic (100 µg/L: 2-5 weeks) in drinking water impairs skeletal muscle maintenance and injury repair (Ambrosio *et al.*, 2014; Zhang *et al.*, 2016). 100 µg/L in drinking water is not an uncommon human exposure and is approximately the EC90 we found for arsenic promoted remodeling of mouse liver vasculature in this mouse model (Ambrosio *et al.*, 2014; Garciafigueroa *et al.*, 2013; Soucy *et al.*, 2005; Straub *et al.*, 2008; Straub *et al.*, 2007; Zhang *et al.*, 2016). While the exact translation of this exposure to the human equivalent is difficult, allometric scaling (FDA, 2005) would estimate this to be a human exposure of approximately 20 µg/L, or twice the WHO/EPA safe drinking water level.

CTF^{ars} retained aberrant expression of ECM proteins when cultured in the absence of arsenic, and arsenic exposure caused decellularized ECM scaffolds to direct naïve heterogenic hMuSC populations toward fibrogenic instead of myogenic differentiation (Zhang *et al.*, 2016). To test the functional consequence of the aberrant ECM elaborated by the CTF^{ars}, we seeded CTF isolated from the hind limb muscles of control or arsenic-exposed (100 µg/L: 5 weeks) mice and allowed them to elaborate matrix for 2-3 days. The cells were then removed, and hMuSC not exposed to arsenic were cultured in myogenic differentiation medium on the elaborated matrix for two days. As seen in Figures 1A-C, myogenic differentiation, as determined by MyoD and desmin expression, was reduced in hMuSC seeded on ECM elaborated by CTF^{ars} when compared to hMuSC seeded onto ECM from CTF^{ctr}. In contrast, the hMuSC seeded on the CTF^{ars} ECM had an increased number of cells expressing both the proliferation marker CD34 (Figs. 1D-E) and fibro-adipogenic marker PDGFRα (Fig. 1F-G).

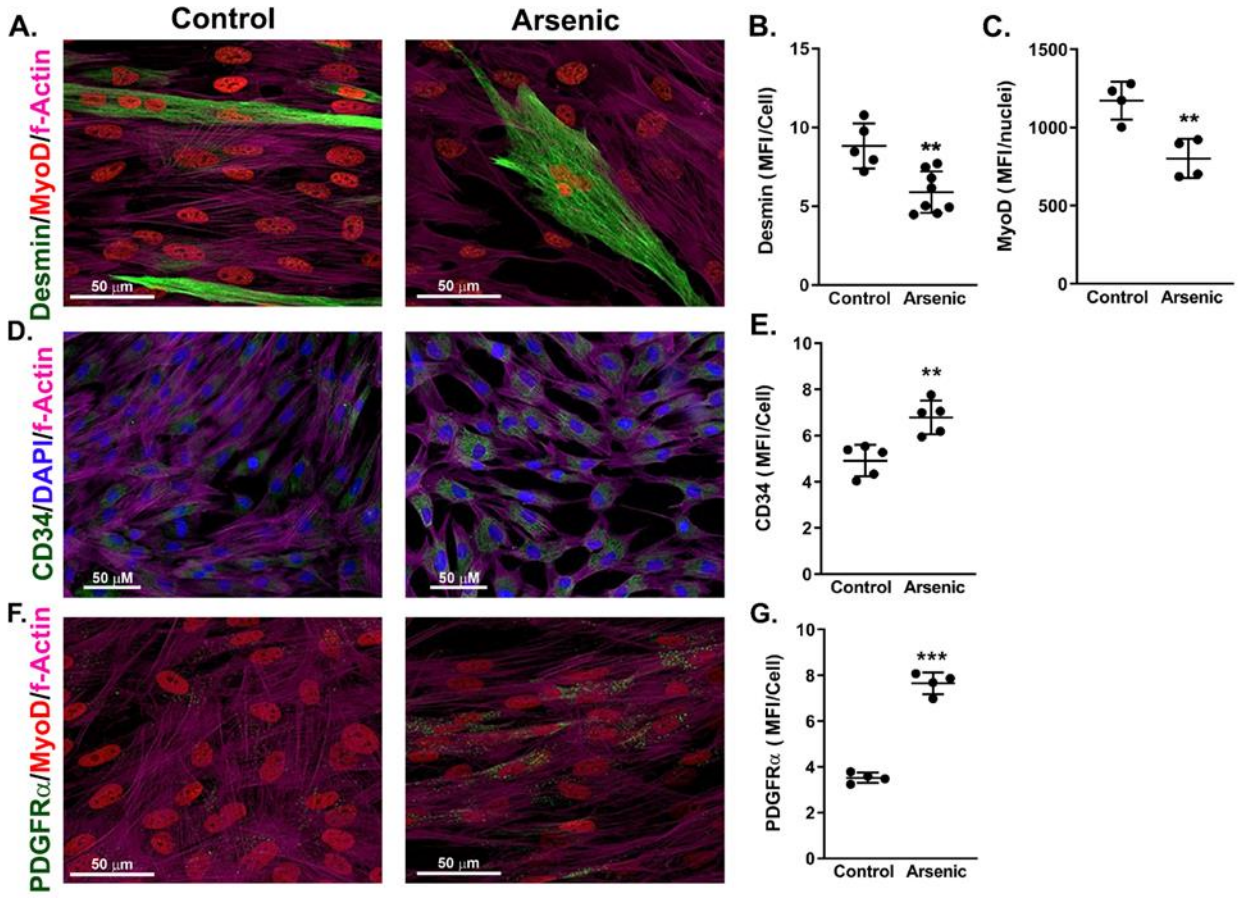

Figure 8: ECM from CTF^{ars} inhibits myogenesis and promotes fibro-adipogenic differentiation.
Hind limb muscle CTF were isolated from control mice (CTF^{ctr}) and mice exposed for five weeks to 100 µg/L arsenic in drinking water (CTF^{ars}). The CTF were seeded in the absence of arsenic and allowed to elaborate matrix for three days. The cells were removed, and unexposed hMuSC were cultured on the elaborated ECM in differentiation medium for two days. Cells were fixed and immunostained for the indicated proteins, as well as stained for nuclei (DAPI) and actin filaments (phalloidin). Immunofluorescence in 40x or 60x magnification confocal images was quantified and the data are presented as mean and SEM of the mean fluorescence intensity (MFI) per cell or nuclei captured in individual microscopic fields (images from three replicate cultures representative of cultures isolated from at least four mice per treatment). Statistical differences were determined by unpaired t-tests (**p<0.01, ***p<0.001).

Arsenic targets CTF mitochondria to impair myogenesis and muscle regeneration. The fact that the CTF^{ars} elaborated a pathogenic ECM even when cultured in the absence of arsenic suggests that the cells retained an epigenetic-driven memory of the arsenic stress, as we found in MuSC isolated from arsenic exposed mice and in myogenic cell lines exposed to arsenic in culture (Ambrosio *et al.*, 2014; Cheikhi *et al.*, 2019). Specifically, we showed that the regulation of this memory was directed by dysfunctional mitochondria (Cheikhi *et al.*, 2019). The CTF^{ars} possessed the same elongated mitochondrial networks with disrupted cristae (Figure 2A) that we found in arsenic exposed muscle tissue, MuSC, and myocytes (Ambrosio *et al.*, 2014; Cheikhi *et al.*, 2019). Despite the apparent increase in large fused mitochondria, there was an approximately 25% loss of respiratory Complex I and Complex II proteins, as well as the voltage-dependent anion channel (VDAC) protein in CTF^{ars} relative to other respiratory complex proteins and relative to levels in control CTF (Figure 2B, Table 1).

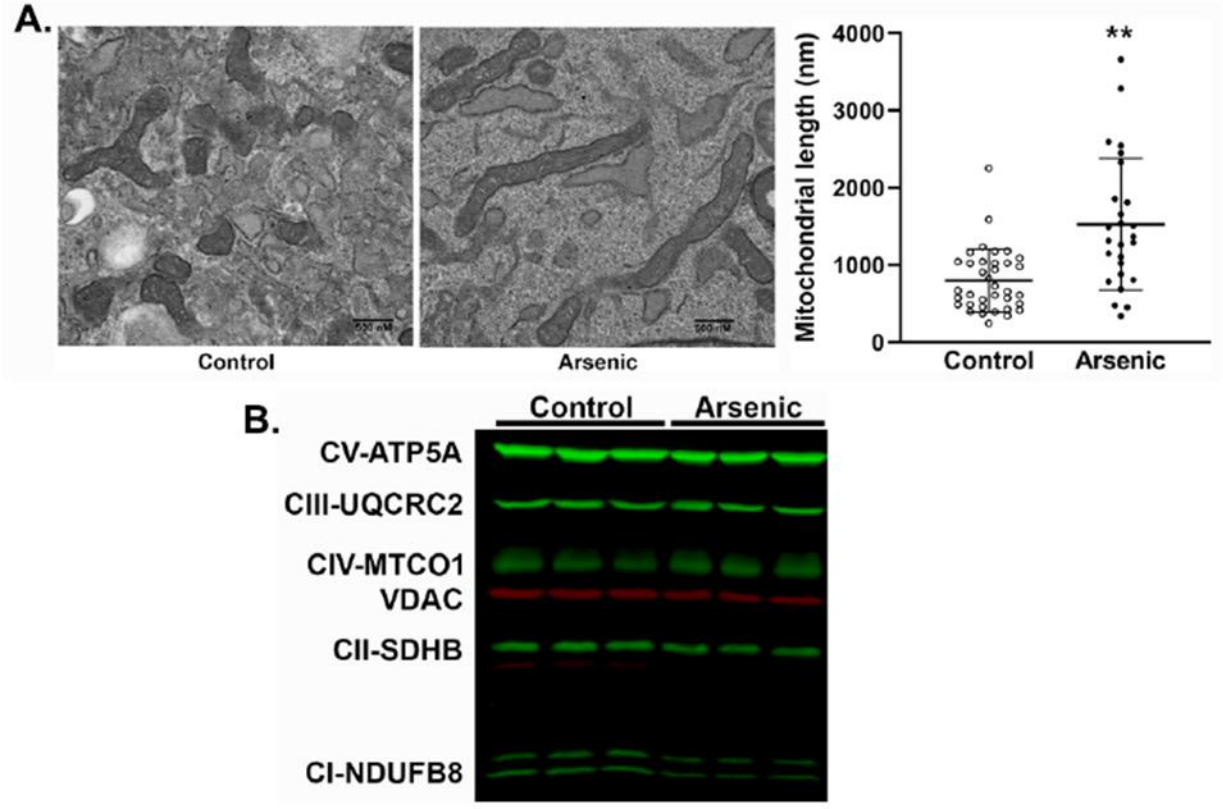

Figure 9: Arsenic effects on CTF mitochondrial morphology and respiratory complexes.
A. CTF^{ctr} and CTF^{ars} isolated from two mice per treatment were cultured in the absence of arsenic for three days and then fixed and processed for imaging at 25,000x magnification by transmission electron microscopy. The length of mitochondria in 3-4 separate images was measured using Image J software (dots are individual mitochondria). Statistical significance between groups was determined by unpaired t-test (**$p<0.01$). **B.** CTF^{ctr} and CTF^{ars} from two mice per treatment were amplified in culture and total protein was probed for expression of mitochondrial respiratory complexes by immunoblotting (second blot presented in Figure S2). The abundance of respiratory complex proteins (green) and VDAC (red) are given in Table 1.

Table 1: Abundance of CTF mitochondrial channel and respiratory proteins.

Protein	Control	Arsenic	Arsenic / Control
Complex V (ATP5A)	4320 ± 480.6	3477 ± 430.3	0.80 +/- 0.08
Complex IV (MTCO1)	1385 ± 74.0	1285 ± 51.8	0.89 +/- 0.06
Complex III (UQCRC2)	1144 ± 64.7	983.0 ± 66.8	0.86 +/- 0.04
Complex II (SDHB)	1044 ± 25.0	789.0 ± 36.2***	0.75 +/- 0.03^
Complex I (NDUFB8)	374.2 ± 16.7	271.7 ± 4.8***	0.73 +/- 0.03^
VDAC	41717 ± 1831	31567 ± 725.1***	0.75 +/- 0.02^

Data are mean ± SEM of relative Infrared band intensity or fold intensity relative to control
*** designates significant difference ($p<0.001$) between cells from control and arsenic mice (n=6) as determined by an unpaired t-test.
^ designates significant difference ($p<0.05$) from the fold control abundance of Complex IV (n=6) as determined by one-way ANOVA followed by Dunnett's multiple comparison test.

SS-31 is a mitochondrially-targeted peptide that binds cardiolipin and cardiolipin-associated proteins to preserve or restore respiratory chain function (Campbell *et al.*, 2019; Szeto and Liu, 2018). We have demonstrated that SS-31, and other mitochondrial protectant compounds, reverse arsenic effects on myocyte mitochondrial function and epigenetic regulation (Cheikhi *et al.*, 2020; Cheikhi *et al.*, 2019). To test whether repairing the respiratory chain could revert the CTF^{ars} phenotype *in vivo*, mice were treated with SS-31 for one week after stopping arsenic exposure. The mice then received bilateral injections of $BaCl_2$ in their TA muscles to produce a defined myofiber injury and were allowed to recover for two weeks (Figure 3A: experimental paradigm). As seen in Figures 3B-D, SS-31 treatments reversed the effects of the arsenic exposure as $CTF^{ars/SS\text{-}31}$ elaborated a matrix with the same myogenic instruction as that elaborated by CTF^{ctr}. It should be noted that ECM from $CTF^{ctr/SS\text{-}31}$ had a negative effect that was equivalent to ECM from CTF^{ars}. Regardless, the SS-31 treatment only slightly impaired *in vivo* regeneration of injured TA myofibers (number of centrally nucleated regenerating fibers and myotube fusion index), but completely reversed arsenic-inhibited repair (Fig. 3E-G).

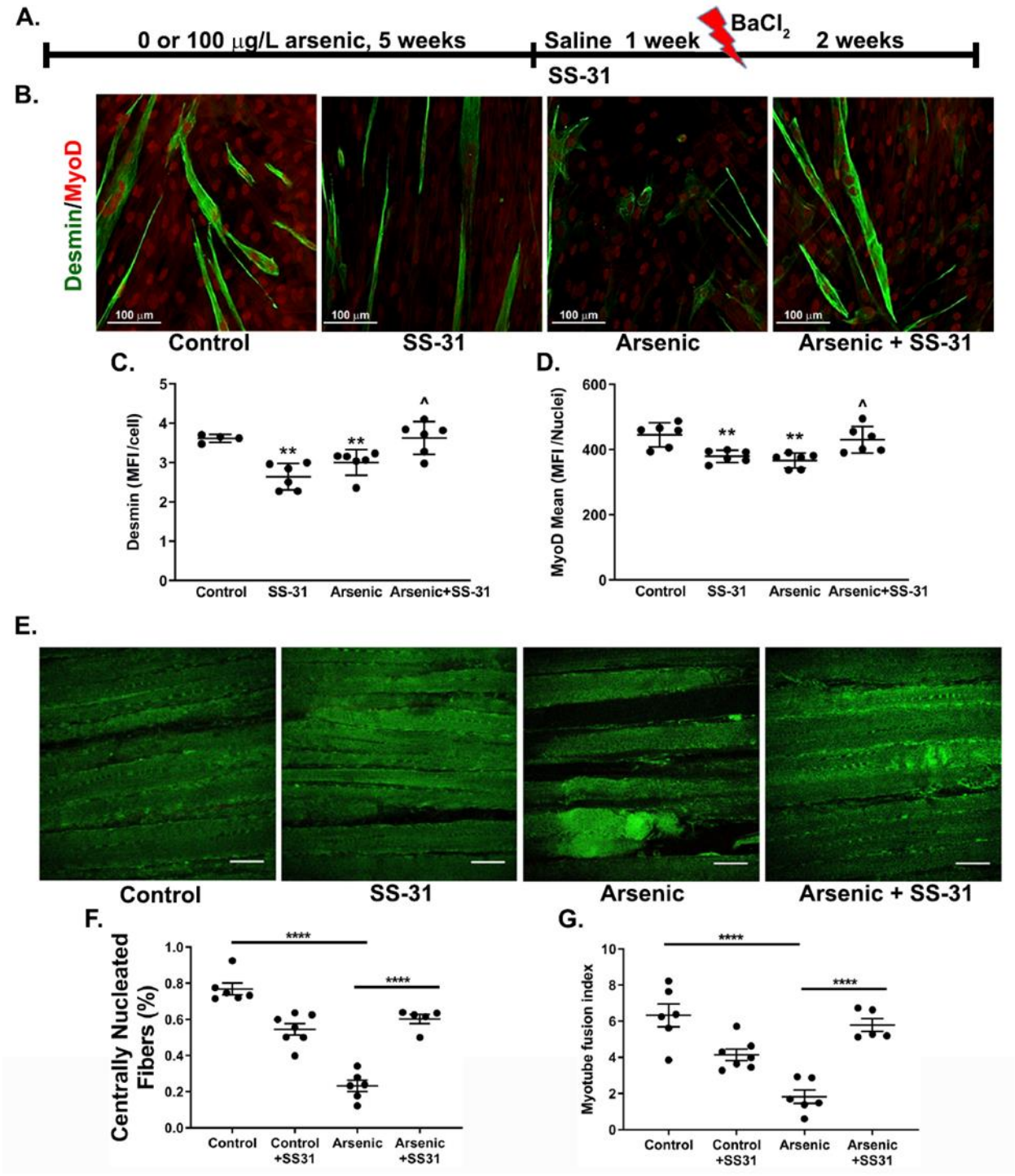

Figure 10: Mitochondrial SS-31 treatment reverses arsenic effects on ECM and regeneration.
A. Experimental scheme and timeline for mouse exposure, SS-31 intervention and TA injury. **B.** Myofiber differentiation (**C.** desmin positive structures and **D.** nuclear MyoD expression) of unexposed hMuSC seeded on ECM elaborated from CTF isolated from control, arsenic, and SS-31 ($CTF^{SS\text{-}31}$, $CTF^{ars/SS\text{-}31}$) mice (six replicate cultures from four mice in each treatment). **E.** Whole TAs were isolated and cleared for SHG imaging of intact muscle. **F-G.** Regenerating centrally nucleated fibers and muscle fiber fusion index were quantified and compared for statistical differences. Mean ± SEM values from six mice in each treatment group were compared by one-way ANOVA followed

by Tukey's ad hoc multiple comparison. In **C-D** ** designates statistical significance from hMuSC seeded on CTF^{ctr} ECM ($p<0.01$) and ^ designates difference from cells seeded on CTF^{ars} ECM ($p<0.05$). In **F-G**, **** designates difference from control or arsenic ($p<0.0001$).

Arsenic-stimulated Notch signaling mediates dysfunctional repair and ECM elaboration. Notch proteins are master regulators of cell fate. Continuous Notch signaling in muscle inhibits myogenesis but promotes fibro-adipogenic differentiation of muscle progenitor cells (Brack *et al.*, 2008; Buas and Kadesch, 2010; Kitzmann *et al.*, 2006; Marinkovic *et al.*, 2019; Pasut *et al.*, 2016; Verma *et al.*, 2018). In screening for regulatory gene changes in the uninjured gastrocnemius muscles of the groups of mice shown in Figure 4, we found that arsenic induced a sustained increase in Notch1 expression and that SS-31 treatment returned *Notch1* mRNA to control expression levels (Figure 4A). *Notch1* expression remained elevated three weeks after arsenic was removed from the drinking water, suggesting prolonged epigenetic regulatory change. Indeed, ChIP analysis of the *Notch1* promoter revealed that the transcriptional start site was held in an open, RNA polymerase Pol II-bound conformation (Fig. 4B), perhaps driven by the increased binding of the active epigenetic mark, acetylated H2A.Z (Fig. 4C), in muscles from the arsenic-exposed mice. As we previously reported (Cheikhi *et al.*, 2019), this epigenetic memory was driven by arsenic-promoted mitochondrial dysfunction, since SS-31 treatment reversed arsenic-induced *Notch1* mRNA expression and the binding of active epigenetic marks on the *Notch1* promoter.

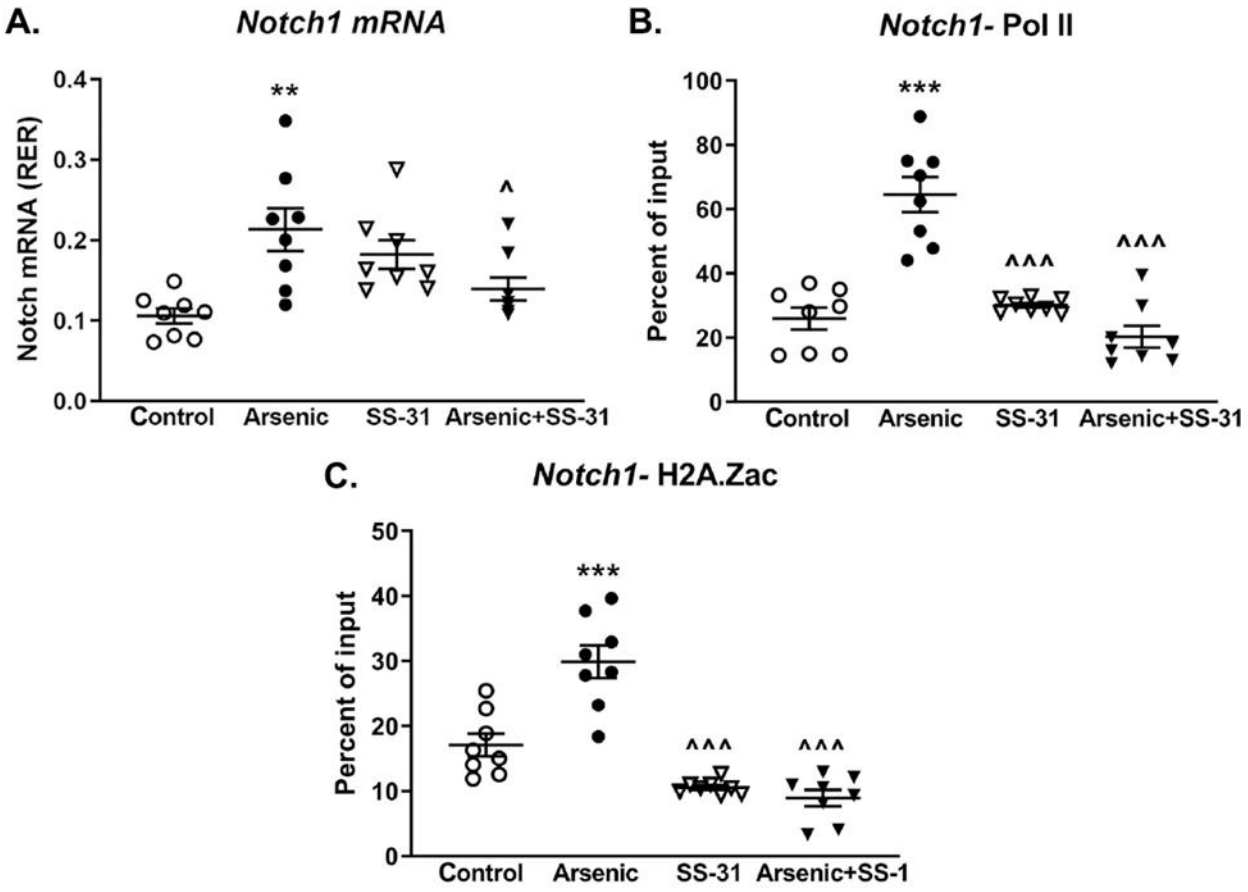

Figure 11: SS-31 reversal of arsenic-induced Notch1 expression in vivo.
A. Quantitative PCR measure of Notch1 mRNA levels in uninjured gastrocnemius muscle from the mice in Figure 3 (n=8 mice). **B-C**. ChIP analysis of Pol II or H2A.Zac binding at the Notch1 promoter. Means and + SD of the treatment groups were compared with one- way ANOVA followed by Tukey's ad hoc multiple comparison. Significant differences from control are indicated by ** ($p<0.01$) or *** ($p<0.001$), and from arsenic by ^ ($p<0.05$) or ^^^ ($p<0.001$); n=8 mice in each treatment group.

To demonstrate that arsenic-induced CTF *Notch1* expression and functionally increased Notch1 signaling, CTF^{ctr} and CTF^{ars} were seeded in arsenic-free culture and compared for activated Notch1 (NICD) protein expression and expression of the NICD-driven Notch ligand, DLL4. Quantitative immunofluorescence analysis demonstrated that CTF^{ars} retained elevated NICD levels *ex vivo*, relative to control CTF (Fig. 5A-B). The CTF^{ars} also retained increased expression of DLL4 (Fig. 5A; C). In contrast, CTF^{ars} isolated from mice that received one week of SS-31 treatment had NICD and DLL4 levels that were essentially equal to CTF^{ctr} (Fig. 5A-C). In addition, treating CTF^{ars} with the γ-secretase inhibitor, DAPT, *ex vivo* reduced both NICD and DLL4 expression. These data suggest arsenic exposure creates an epigenetic memory for enhanced Notch signaling in CTFs and that DLL4 expression was downstream of NICD transactivation.

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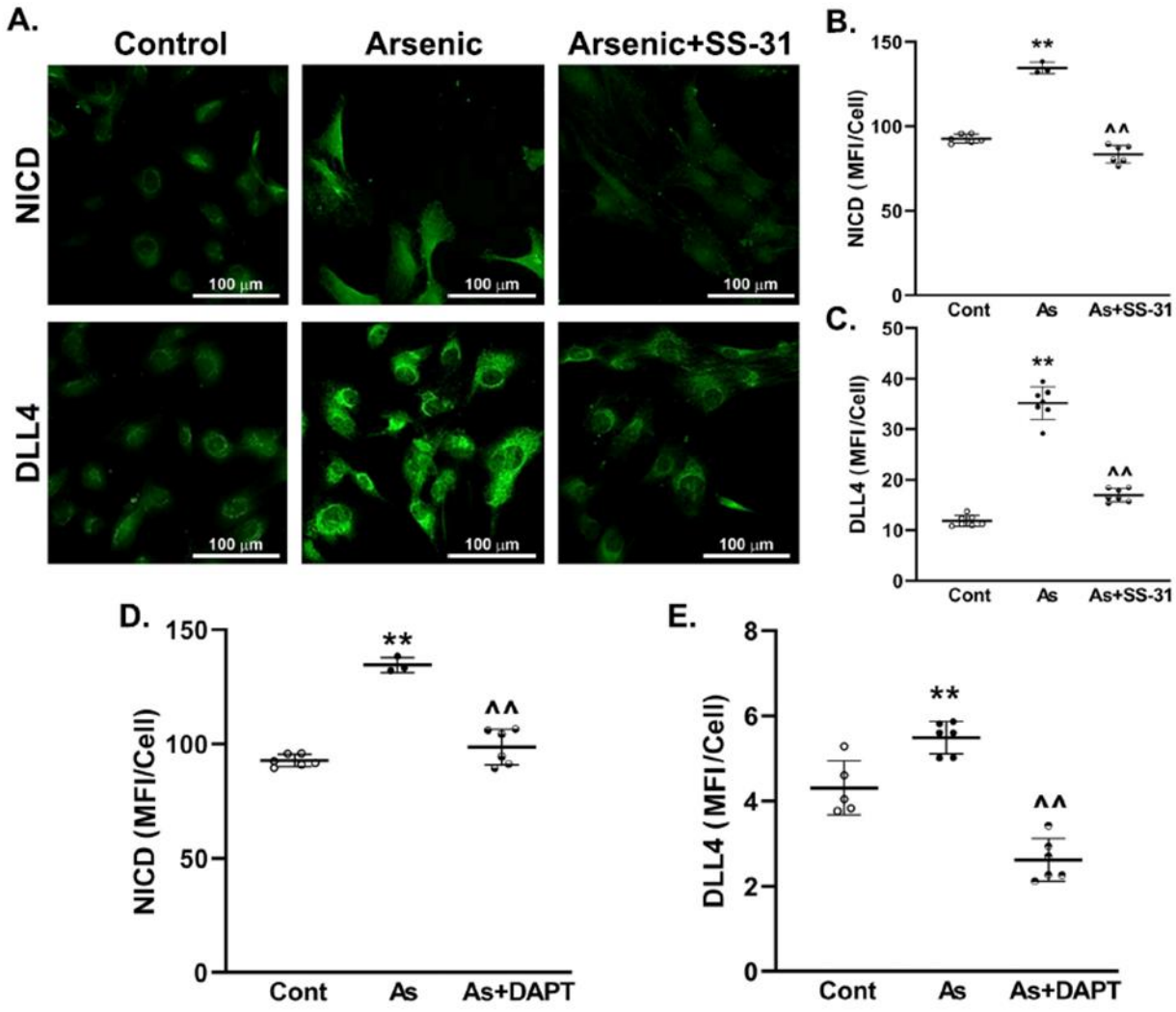

Figure 12: SS-31 reversal of arsenic-induced CTF Notch1 activity.
A-C. CTF were isolated from hind limb muscles (excluding TA and gastrocnemius) from the mice in Figure 3 and cultured for 24 hr before fixing and quantitative immunofluorescence analysis of activated Notch (NICD) and DLL4 protein expression. D-E. Groups of CTF^{ars} were cultured *ex vivo* with 1 μM DAPT and abundance of NICD and DLL4 was compared to CTF^{ctr} and CTF^{ars} (note that values for CTF^{ctr} and CTFars NICD in **B** and **D** are from the same cultures, while data in **C** and **D** are from separate cultures). Mean ± SEM of the MFI/cell from the groups (cont = control, As = arsenic) were compared using one-way ANOVA and Tukey's ad hoc multiple comparison. Significant differences from control are indicated by ** ($p<0.01$) or and from arsenic by ^^ ($p<0.01$).

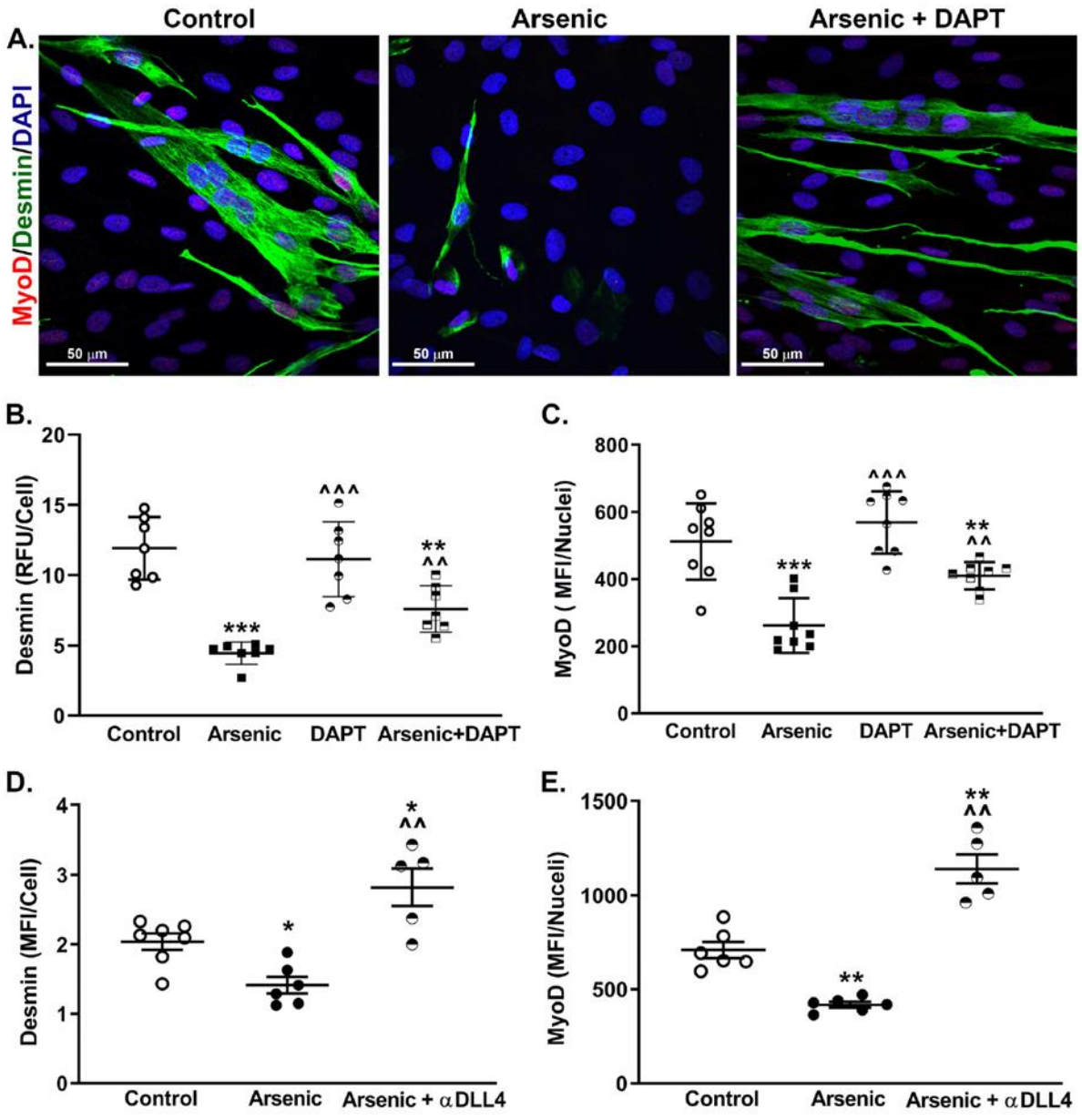

Figure 13: Notch-induced ECM DLL4 impairs hMPC myogenic differentiation.
A-C. CTF^{ctr} and CTF^{ars} were seeded and cultured for one day before adding 1 μM DAPT to the designated groups. On culture day 3, cells were removed and hMuSC were seeded on elaborated ECM and cultured in differentiation medium for two days before fixing and quantifying desmin and nuclear MyoD expression. **D-E**. CTF^{ctr} and CTF^{ars} were cultured for three days to elaborate ECM. The cells were removed and the ECM was incubated with an antibody that blocks DLL4/Notch binding. After rinsing to remove unbound antibody, hMuSC were seeded and cultured in differentiation medium for two days before fixing and quantifying desmin and nuclear MyoD expression. Mean ± SEM expression from the groups were compared using one-way ANOVA and Tukey's ad hoc multiple comparison. Significant differences from CTF^{ctr} ECM are indicated by * ($p<0.05$), ** ($p<0.01$), or *** ($p<0.001$) and from CTF^{ars} ECM by ^^ ($p<0.01$) or ^^^ ($p<0.001$).

To investigate the functional importance of Notch1 and NICD-induced DLL4 in arsenic-inhibited myogenesis, CTF^{ctr} and CTF^{ars} were again seeded to elaborate a matrix. After one day, DAPT was added to groups of the CTF^{ctr} and CTF^{ars} cultures, and decellularized ECM was prepared on culture day 3. hMuSC were then seeded on the ECM for two days in differentiation media. ECM elaborated by CTF^{ars} cultures inhibited the generation of desmin positive fibers myofibers and decreased hMuSC nuclear MyoD levels (Fig. 6A-C). However, treating the CTF^{ars} with DAPT attenuated the effect of the CTF^{ars} elaborated on hMuSC differentiation. To demonstrate that the effect of DAPT resulted from decreased DLL4 elaboration into the ECM, decellularized ECM from CTF^{ctr} or CTF^{ars} was incubated with an antibody that blocks DLL4 binding to Notch. Unbound antibody was rinsed from the ECM and hMuSC were seeded in differentiation medium. Blocking ECM DLL4 increased hMuSC myogenic differentiation to levels greater than control and more than reversed the effect of the CTF^{ars} phenotype (Figs. 6D-E). Antibody blocking of DLL4 may have been more effective than DAPT since the antibody would block both basally expressed and NICD-induced DLL4.

6.2 Discussion

Collectively, the data indicate that low-to-moderate arsenic exposure induces functional ECM-associated changes in the MuSC niche. These findings may help explain muscle weakness and metabolic dysfunction experienced by individuals in arsenic-endemic areas. Furthermore, the data implicate arsenic effects on mitochondria in creating prolonged epigenetic signaling for dysfunctional ECM and dysregulated MuSC fate. The study demonstrates that muscle tissue and CTF isolated from mice weeks (1.5-2 human year equivalents) after arsenic exposure was

terminated retained elevated Notch1 expression and signaling that misdirected populations of MuSC away from myogenesis and towards fibro-adipogenesis. The CTF^{ars} phenotype was also accompanied by atypical mitochondrial morphology. Intervention with SS-31 reversed arsenic-inhibited tissue regeneration, epigenetic induction of Notch1, and functional impairment from the ECM elaborated by CTF^{ars}. Together, these data implicate aberrant Notch1 signaling as the mechanism for the arsenic-promoted myomatrix remodeling that impairs muscle metabolism, maintenance, and regeneration.

Notch signaling is a key regulator of MuSC quiescence, self-renewal activity, and stem cell fate determinations that promote muscle pathologies (Bjornson *et al.*, 2012; Gerli *et al.*, 2019; Kitzmann *et al.*, 2006). Notch activity represses skeletal muscle myogenesis by preserving MuSC in a proliferative stem cell-like state (e.g., increased CD34 expression, Figure 1) while concomitantly reducing the number of CD34-positive reserve cells that fuse to make myotubes (Cappellari *et al.*, 2013; Gerli *et al.*, 2019; Kitzmann *et al.*, 2006). Notch promotes asymmetric MuSC division and divergent fates of daughter cells (Conboy and Rando, 2002). Over-activation of Notch signaling in skeletal muscle impaired stem cell support of muscle regeneration (Brack *et al.*, 2008) and/or promoted differentiation of subpopulations of MuSC into brown adipocytes (Pasut *et al.*, 2016) or pericytes (Cappellari *et al.*, 2013). While increased DLL4 signaling is sufficient for directing MuSC subpopulations towards pericyte differentiation, maximal trans-differentiation occurs with co-stimulation by PDGF-BB, a platelet-derived growth factor that activates PDGFRα (Cappellari *et al.*, 2013). Pericytes can be derived from multiple lineages and, as a result, express heterogenic phenotypes even within the same tissues (Dias Moura Prazeres *et al.*, 2017). The data in Figure 1 suggest that as CTF^{ars}-elaborated ECM reduces hMuSC myogenic differentiation, it enhances a population of cells expressing PDGFRα and αSMA, which are

indicative of type 1 pericytes (Birbrair *et al.*, 2013). This is significant since type 1 pericytes are more prone to adipogenic differentiation relative to their myogenic type 2 counterparts (Birbrair *et al.*, 2013) and may underlie the increase in perivascular ectopic adipose tissue that we reported in skeletal muscle of arsenic exposed mice (Garciafigueroa *et al.*, 2013).

Notch signaling is initiated when the membrane-bound ligands of the Delta-like (DLL1, DLL3, DLL4) and the Jagged (JAG1 and JAG2) families engage and transactivate Notch receptors on neighboring cells (Buas and Kadesch, 2010; Wakabayashi *et al.*, 2015). Notch signaling is essential for MuSC quiescence, health, and expansion of myogenic progenitors in postnatal myogenesis and regeneration (Bjornson *et al.*, 2012; Brack *et al.*, 2008; Conboy *et al.*, 2003). However, myogenic differentiation requires a temporal switch from Notch signaling to cell-cell transactivation of wnt/β-catenin signaling. Sustained NICD or DLL4 signaling, such as observed from the CTF^{ars}-elaborated ECM, prevents this transition and directs the fibro-adipogenic and pericytes differentiation of MuSC populations. (Cappellari *et al.*, 2013; Moyle *et al.*, 2019; Pasut *et al.*, 2016). The novel observation that CTF^{ars}-elaborated DLL4 is essentially a memory of arsenic-promoted stress imparted into the ECM and that it continues to direct pathogenic tissue remodeling, even after arsenic has been removed, is significant. It has important implications for the impacts of chronic arsenic exposures on muscle maintenance and regenerative capacity. It is also consistent with our previous demonstration that decellularized muscle ECM scaffolds isolated from arsenic-exposed mice caused hMuSC to be redirected to a fibrogenic determination rather than their normal myogenic fate (Zhang *et al.*, 2016). It appears that, without intervention such as SS-31 administration, the pathogenic ECM and epigenetic memory of arsenic exposures persists. However, it remains unclear whether or when these

memories may ultimately be spontaneously erased. The data in Fig. 3-4 suggest that this reversal could occur years after human arsenic exposure is terminated.

The successful *in vivo* reversal of arsenic-inhibited tissue regeneration and Notch signaling resulting from SS-31 administration confirms our previous findings that arsenic targets mitochondria to impart the persistent epigenetic memory of stress (Ambrosio *et al.*, 2014; Cheikhi *et al.*, 2019). Arsenic-promoted mitochondrial respiratory chain dysfunction and possibly mitochondrial oxidants appear to be upstream of epigenetic control of genes regulating DNA and chromatin methylation and acetylation (Cheikhi *et al.*, 2019; Cronican *et al.*, 2013) and Notch1/DLL4 signaling in skeletal muscle (Figure 4). Mitochondrial dynamics and ROS generation have been placed upstream of Notch expression and signaling in a range of stem cells with implications on their homeostasis and fate (Paul *et al.*, 2014; Wakabayashi *et al.*, 2015). However, Notch signaling has also been shown to affect mitochondrial function, as it inhibits mitochondrial complex 1 by repressing expression of multiple *Nduf* subunit transcripts (Lee and Long, 2018). The arsenic-promoted decrease in mitochondrial complexes 1 and 2 (Fig. 2B, Table1) would be consistent with a reciprocal feed-forward dysregulation of mitochondrial signaling and Notch activation. The memory of this dysregulation may be manifest in the enhanced binding of epigenetic marks in the *Notch1* promoter, such as the increased acetylation and DNA binding of H2A.Zac that may be promoted by decreased deacetylase substrate NAD^+ (decreased complex 1) or increased acetylase substrate acetyl-CoA (dysfunctional complex 2)(Shaughnessy *et al.*, 2014; Wallace and Fan, 2010; Weinhouse, 2017).

The axis of oxidative stress leading to Notch expression is strongly linked through activation of the Nrf2 (nuclear factor, erythroid factor 2, type 2) transcription factor, as the *Notch1* promoter contains Nrf2 responsive ARE cis-elements (Wakabayashi *et al.*, 2015). However, this

is not a linear relationship as the NICD affects Nrf2 expression and both transcription factors can elicit feedback mechanisms on the respective signaling pathways (Wakabayashi *et al.*, 2015). It is important to note that Nrf2 appears to be essential for the regenerative response of muscle, as well as lung tissue, and that induction of Notch may be a portion of this essentiality (Paul *et al.*, 2014; Shelar *et al.*, 2016; Wakabayashi *et al.*, 2015). However, Nrf2 is also important for limiting the increased flux of ROS that is critical for initiating Notch signaling, stem cell self-renewal and tissue regeneration, as excessive ROS impairs these temporal transitions (Paul *et al.*, 2014; Shelar *et al.*, 2016). Arsenical effects on mitochondria are very effective in activating myocyte antioxidant responses (Cheikhi *et al.*, 2019), and the data in Figures 5 and 6 suggest that chronic activation may be linked to loss of temporal *Notch1* regulation both through cell-autonomous and ECM signaling. However, the data in Figure 4 also suggest that mitochondrial driven epigenetic regulation may contribute to or bypass the Nrf2 axis of *Notch1* regulation.

In summary, the present study demonstrated that arsenic exposure impairs muscle regeneration by eliciting chronic mitochondrial-driven activation of Notch signaling at the level of fibroblast elaboration of ECM bound NICD-induced DLL4. This DLL4 is a memory of arsenic-promoted stress imparted into the ECM that pathogenically misdirects the fate of the heterogenous MuSC populations. Remarkably, the memory of arsenic exposure is held both in mitochondrial-driven epigenetic regulation and cell elaborated transactivating ligands. As Notch signaling is activated in resident MuSC populations of the skeletal muscle niche, subpopulations are disproportionally activated to proliferate and overwhelmingly advance non-myogenic/adipogenic cells that decrease regenerative integrity and contribute to decline of muscle metabolism and tissue integrity.

7.0 Chapter 5: Future Directions and Conclusions

7.1 Introduction

Skeletal muscle ECM is a dynamic tissue that provides both structural integrity and cellular integration. Although the ECM comprises only approximately 1% to 9% of skeletal muscle cross-sectional area (Light & Champion, 1984), it is a principal element of muscle health and metabolic activity. Resident muscle cells recognize and bind to growth factors and structural ligands embedded within the ECM, which is continuously subjected to remodeling (Frantz *et al.*, 2010; Rozario & DeSimone, 2010). Fibrous collagen is the most abundant structural protein in adult interstitial ECM (Rumian *et al.*, 2007), where it provides the operational framework for muscle and adipose tissue (Flint & Pickering, 1984). Each layer of muscle tissue is primarily comprised of type 1 and type III collagen fibrils secreted and organized mainly by connective tissue fibroblasts (CTFs) (Frantz *et al.*, 2010; Kjaer, 2004; Light & Champion, 1984). Overall, collagens help to regulate cell adhesion, provide tensile strength, support migration and chemotaxis of resident cells, and direct tissue development (Rozario & DeSimone, 2010).

In skeletal muscle, there must be order to the production of the ECM constituents, otherwise, remodeling of the ECM can affect the muscle's regenerative capacity and overall composition (Frantz *et al.*, 2010; Rozario & DeSimone, 2010; Zhang *et al.*, 2016). Typically, a muscle can be dominated by either a Type I muscle fiber, generally considered to be a slow-twitch oxidative muscle fiber or a Type II fiber that is a fast-twitch glycolytic muscle fiber (Zimmerman *et al.*, 1993). Slow-twitch muscle, such as the soleus muscle, has a higher proportion of type I

collagen than type III collagen. In comparison, fast muscles will contain more type III collagen, as seen in the gastrocnemius muscles (Miller *et al.*, 2001; Zimmerman *et al.*, 1993).

Muscle fiber composition is not permanently determined, as external stimuli can promote changes within the ECM to influence muscle fiber direction (Rozario & DeSimone, 2010). The type I muscles tend to have much more mitochondria and, therefore, contain a much higher phospholipid content (Blackard *et al.*, 1997; Costa *et al.*, 2008). Type II muscle fibers are more associated with cancer and diabetes, as they are not as insulin-sensitive as the type I fibers (Clore *et al.*, 2000; Y. Wang & Pessin, 2013). Type II muscles are more affected by age, as the aging muscle experiences a switch from a dominance of fast-twitch fibers to those of slow-twitch (N. Miljkovic *et al.*, 2015). The oxidative type I muscles are smaller and so the loss of muscle mass in aging can be attributed to the smaller muscle fiber and not necessarily muscle fiber loss (Nilwik *et al.*, 2013). Furthermore, age-related decline in muscle strength may be due to the accumulation of intramuscular adipose tissue. It is plausible that ECM remodeling underlies the cues for these fiber and compositional cell-type changes.

In addition to aging, environmental factors, such as arsenic exposure, may cause pathogenic ECM remodeling that misdirects muscle composition (Zhang *et al.*, 2016). As we found in Chapter 4, arsenic can activate CTF Notch1 signaling to increase their expression and ECM deposition of Notch DLL4 ligand. This DLL4 deposition hindered hMPCs from proper myogenic differentiation on the ECM, and instead redirected their differentiation to cell phenotype expressing PDGFRα (Figure 8). Others have shown that dermal fibroblasts from individuals with systemic sclerosis (SSc) produced collagen in excess amounts, resulting in one of the disease's prevailing features of fibrotic tissue (Dees *et al.*, 2011). Fibroblasts from these patients also presented high levels of Notch1 activation (Dees *et al.*, 2011). The data suggest that Notch-1

activates the CTF to produce excess collagen proteins into the ECM. Inhibition of Notch-1 with the γ-secretase inhibitor, DAPT, reduced the basal col1a1 and col1a2 mRNA production in these SSc fibroblasts, as well as their release of collagen protein (Dees *et al.*, 2011).

To further investigate the hypothesis that arsenic-induces an ECM memory for myogenic impairment both through induction of Notch-dependent transcriptional programs and dependent on Notch signaling in the ECM, we examined arsenic-induced changes in ECM composition and niche cell population dynamics. By understanding the relationships between ECM-associated cell populations, we anticipate discovering new strategies for restoring normal elaboration of ECM constituents and intercellular communication that support healthy muscle maintenance and regenerative capacity.

7.2 Results of Preliminary Studies and Discussion

Disruptive ECM remodeling and altered ECM constituent (e.g., collagens, laminins, elastins) expression in multiple organs have been consistent findings in arsenic-exposed animal studies (Hays *et al.*, 2008; Petrick *et al.*, 2009; Soucy *et al.*, 2005; Straub *et al.*, 2007; Szymkowicz *et al.*, 2018; Zhang *et al.*, 2016). However, the direction of arsenic-induced ECM matrix constituent changes has been mixed. It may be dependent on the organ, localization of the ECM within the organ, life stage of the exposure (i.e., in utero versus young adult), or species examined. We previously found that arsenic-exposures changed the collagen orientation of the skeletal muscle matrix (Zhang *et al.*, 2016), increased transcript for collagens I and II in CTF isolated from arsenic-exposed mice (Zhang *et al.*, 2016), and increased perivascular adiposity (Garciafigueroa *et al.*, 2013). To confirm these changes at the protein level *in vivo* and to begin characterizing the

cells responsible for ECM and adiposity changes, we compared the abundance of collagen and markers of adiposity in TA muscles from control and arsenic-exposed mice., Arsenic increased interstitial collagen I and III expression in the uninjured muscle (Figure 14A and C-D). Interstitial PDGFRα, an adipose tissue marker, was increased in the arsenic-exposed muscle, but staining for fat with Lipidtox dye only trended towards an increase. PDGFRα is one marker for type 1 pericytes that have been shown to generate both the fibrotic and adipogenic remodeling in muscle (Birbrair, Zhang, Files, *et al.*, 2014; Birbrair *et al.*, 2013). In addition, we found an increased abundance of the pericyte marker, NG2, in both perivascular and interstitial locations (Figure 14B).

To determine whether similar changes in ECM constituent expression and fibro-adipose tissue remodeling occurred in regenerating muscle, we quantified collagen, PDGFRα, NG2, and lipids in TA muscles that were acutely injected with 20 nM arsenite 24 hours before receiving $BaCl_2$ injury. The muscles were excised and sectioned after a two week recovery. The fibro-adipogenic tissue remodeling was more severe in this exposure paradigm (Fig 15), relative to the environmentally-relevant sub-chronic drinking water exposures (Zhang *et al.*, 2016). Nonetheless, the images and quantification in Figure 16 reveal highly significant increases in PDGFRα, NG2, and lipids in the regenerating tissue. Surprisingly, collagen I expression appeared to be reduced in this model. This finding is not consistent with the increased muscle collagen expression reported in a cardiotoxin injury model of whole life arsenic-exposed fish (Szymkowicz *et al.*, 2018), and may reflect the non-environmentally-relevant exposure paradigm. Alternatively, collagen expression in the fish model was measured using a global collagen stain, and we cannot exclude the possibility that other collagens are increasing while the collagen I is decreased. This alternative will be explored in future studies to provide mechanistic insight, as well as to help define the

arsenic-affected cell types involved in specific collagen elaboration and functional impacts of switching collagen expression.

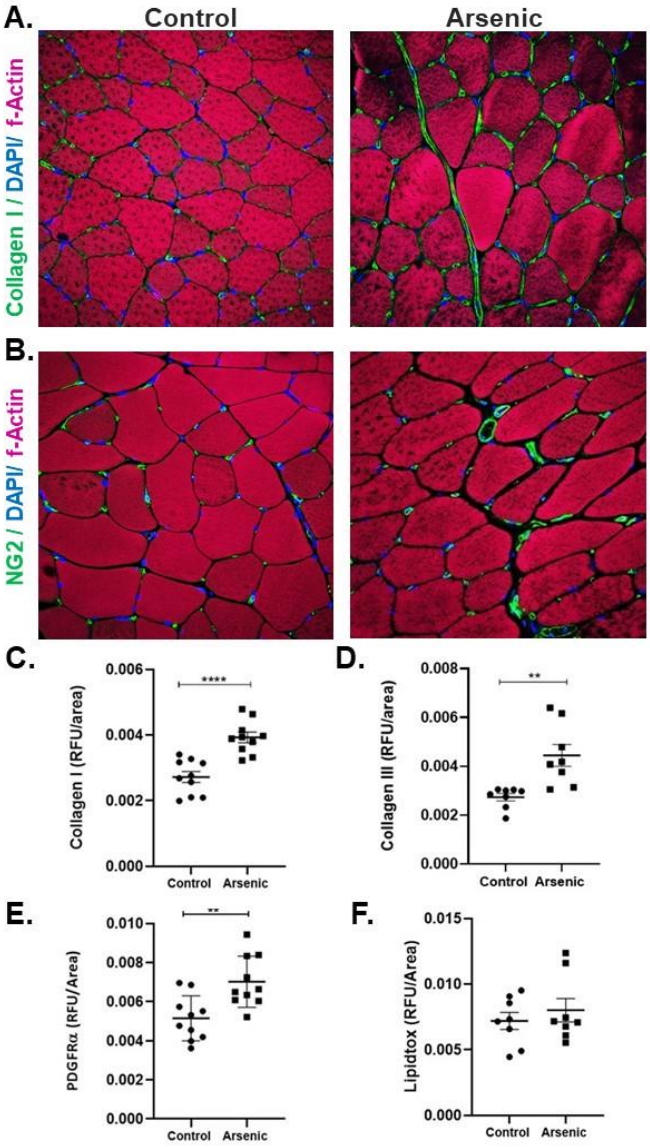

Figure 14: Arsenic-increased interstitial collagen expression in uninjured mice.
Mice were exposed to 0 or 100 μg/L arsenic for five weeks before TA muscles were excised, sectioned and processed for staining with antibodies to **(A)** collagen I, **(B)** NG2, collagen III, or PDGFRα and dyed with Lipidtox deep red, phalloidin, and DAPI. **(C-F)** Integrated densities of staining in individual 40x fields imaged from n=2 mice.

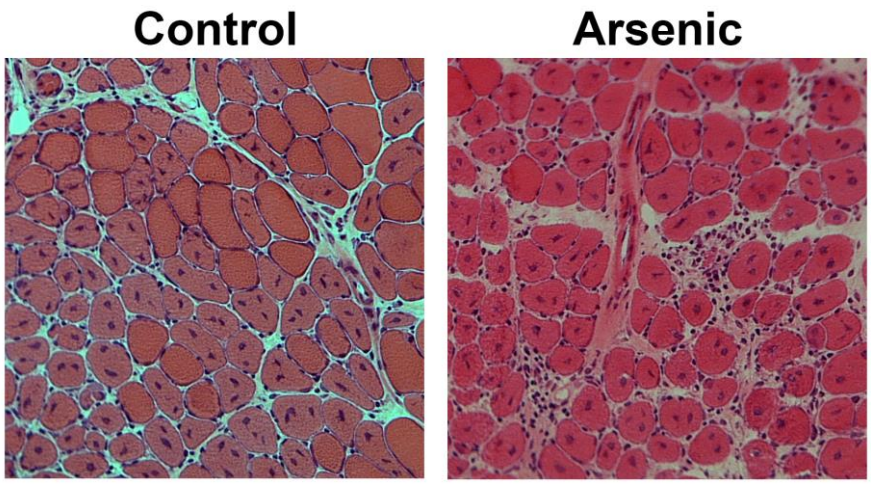

Figure 15: Hematoxylin and Eosin stain of regenerating TA muscle.

An additional preliminary finding in the studies in Figure 16 was that the PDGFRα and NG2 staining was highly colocalized. The co-localization increased with arsenic exposure (Mander's overlap coefficient: control 0.62 + 0.01, arsenic 0.72 + 0.01 (mean + s.d, $p<0.01$ for arsenic relative to control, n=6 images from 2 mice in each treatment)). This suggested that the type 1 pericytes could be expanding in the interstitium to increase the tissue adiposity. As an initial test of this hypothesis and to further characterize the muscle cells that we isolated and termed CTF ((Zhang *et al.*, 2016), Chapter 4), we stained *ex vivo* cultures for the pericyte marker NG2 and the "fibroblast" marker α-smooth muscle actin (αSMA). The images in Figure 17 suggest that the CTF isolate is a heterogeneous cell population with NG2 positive, αSMA positive, and co-stained cells. It should be noted that αSMA is not an exclusive fibroblast marker and has been shown to be expressed by a number of cells in the muscle niche including pericytes, but not FAPS or other mesenchymal progenitors (Birbrair, Zhang, Wang, *et al.*, 2014; Tedesco *et al.*, 2017). Intriguingly, the addition of arsenic to the cultures increased the NG2 staining while there was only a slight, but not significant increase in αSMA staining. The arsenic response did not appear to be dose-

dependent in the observed range, and the threshold for induction of the NG2 expression may be much lower and in the range of environmental exposures.

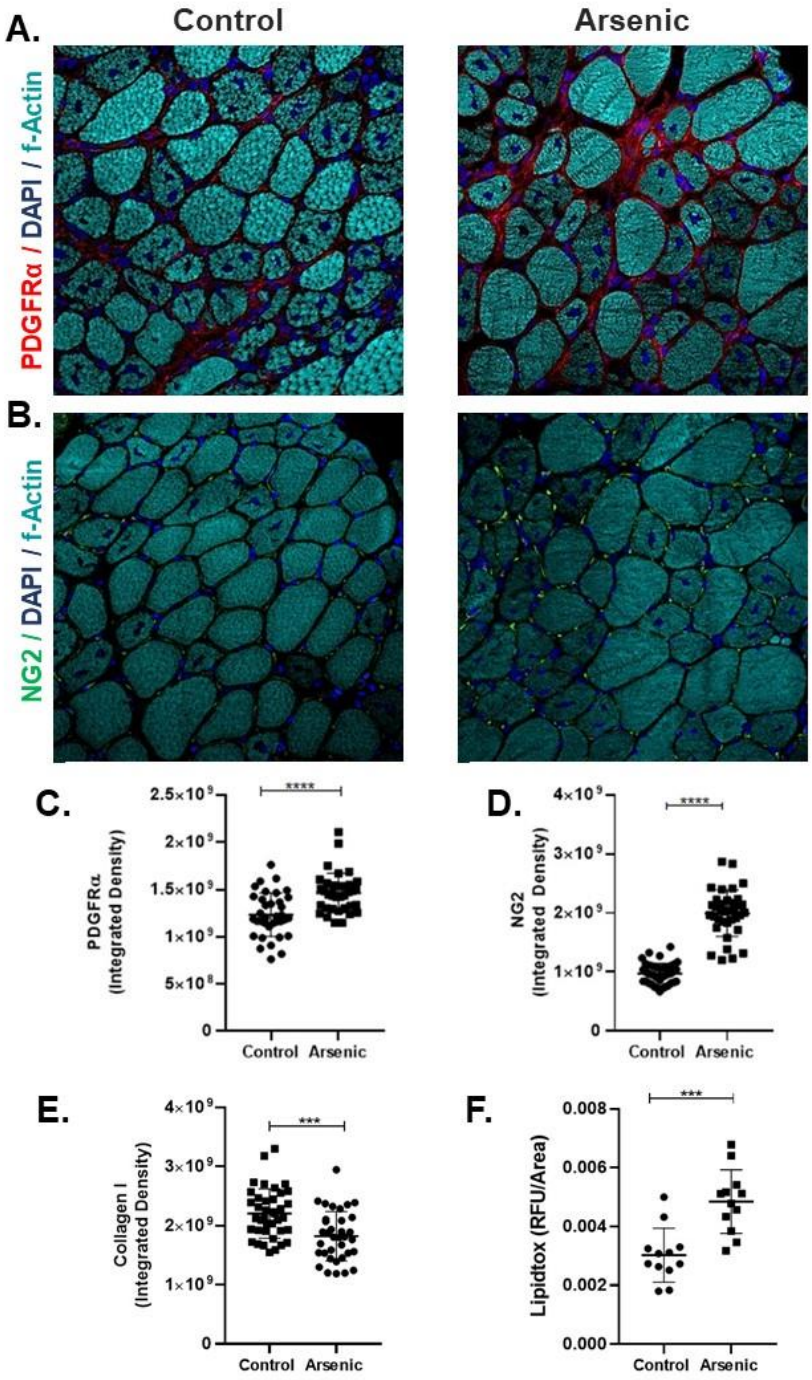

Figure 16: Arsenic exposure increases fibro-adipogenic tissue following injury.
Mice were exposed to 0 or 100 µg/L arsenic for five weeks before receiving bilateral BaCl2 injury to the TA muscles. After two week recovery, the TA were excised, sectioned, and processed for staining with antibodies to **(A)** PDGFRα, **(B)** NG2 or Collagen I and dyed with Lipidtox deep red, phalloidin, and DAPI. **(C-F)** Integrated densities of staining in individual 40x fields imaged from n=3 mice.

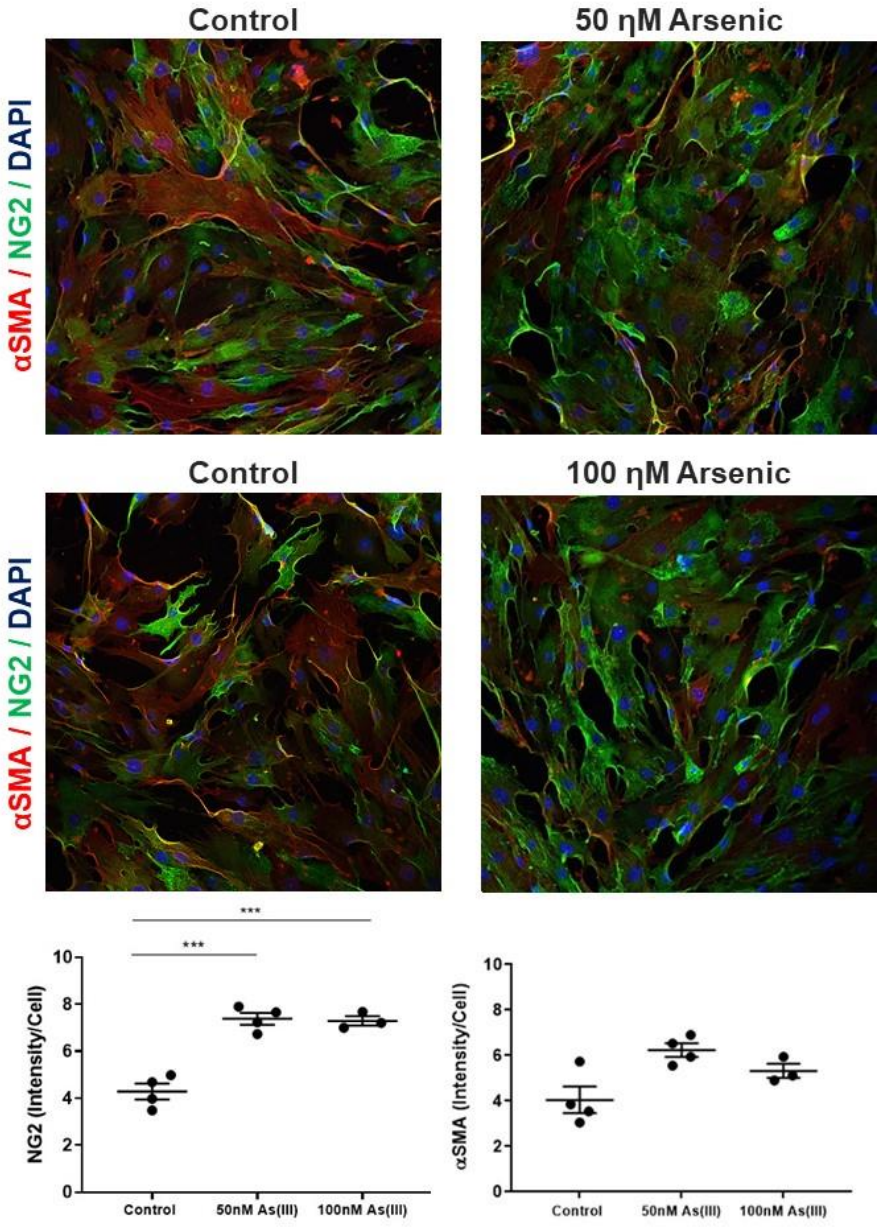

Figure 17: Effect of ex vivo arsenic exposure on CTF phenotype.
CTF isolated from control mice were plated and exposed to the indicated amounts of arsenic for 24 hours. After fixing, the cells were stained with antibodies to NG2 and αSMC, as well as DAPI.

8.0 Conclusions and Future Directions

These preliminary studies strongly suggest that arsenic may induce expansion of the NG2 positive population of muscle progenitor niche cells. However, the rigor of the observations needs to be increased with observations in more mice following both the uninjured and recovery from injury exposure paradigms. In addition, careful characterization of the CTF isolated from both control and arsenic-exposed mice will be necessary to rigorously examine the mechanisms for arsenic-induced cell phenotypes that contribute to dysfunctional tissue regeneration and advance the risk of cardiometabolic disease caused by arsenic exposure. Specifically, the questions of which niche progenitor cell is induced to promote fibro-adipogenic determination over healthy muscle maintenance and regeneration and why are essential to answer before effective intervention strategies can be identified to reduce the burden of arsenic-promoted diseases. Elucidating the mechanisms integrating cellular and ECM communications that underlie the pathogenic effects on muscle and muscle metabolism will contribute significantly to this effort.

We potentially observe the upregulation of type -1 pericyte-associated proteins among CTF^{ars}. These pericytes are capable of producing collagen in an organ-dependent manner (Birbrair, Zhang, Files, *et al.*, 2014), and type-1 pericytes delivered intramuscularly in mice generated ectopic fat (Birbrair *et al.*, 2013). We observed significant increases in PDGFRα in hMPC plated on matrix elaborated by CTF isolated from arsenic exposed mice (Figure 8), as well as in both arsenic exposed uninjured and regenerating muscle (Figures 14, 16), suggesting that arsenic is promoting phenotypic change through activation or differentiation of the interstitial cells. The high degree of co-localization in both control and arsenic-exposed muscle indicates that the pool of type 1 pericytes and their transformation into adipose-like cells may be increased to account for the

increased cellularity and apparent lipid accumulation in the regenerating muscles shown in Figures 15-16. Further, studies will be needed, perhaps with NG2 lineage-tagged transgenic mice (available commercially; (Birbrair *et al.*, 2013)) to confirm the origins of the signals for fibro-adipogenic differentiation of the muscle progenitors and the identity of the progenitors that give rise to adiposity in both uninjured and regenerating muscle.

It will be essential to identify interventions that prevent or, more importantly, reverse the effects of arsenic on the increase in NG2 positive cells and adiposity in the muscle interstitium. The studies in Chapter 4 suggest that restoring mitochondrial function with SS-31 or blocking Notch signaling can prevent or revert arsenic-promoted fibro-adipogenic tissue expansion. The interventions prevented the matrix from CTF of arsenic-exposed mice from reducing hMPC myogenesis and increasing fibro-adipogenic differentiation. Thus, it will be important to demonstrate that the interventions prevent a "priming" of the uninjured muscle that results in the expansion or activation of the pericytes to enhance fibro-adipogenic remodeling after injury. Dees *et al*. found that "primed" or activated dermal fibroblasts had high levels of Notch activation (Dees *et al.*, 2011), and it will be essential to confirm that Notch and elaboration of Notch ligands are the central axis for dysfunctional communications and misdirection of cell fate within the arsenic-exposed niche.

Finally, the studies are limited by only being conducted in male tissues, although the sex of the commercial hMPC was not determined. There are sexual dimorphisms in muscle composition, aging, and pathogenic declines (N. Miljkovic *et al.*, 2015). Unpublished studies in the Barchowsky laboratory found substantial differences in the effects of arsenic exposure on metabolism and adiposity in male and female mice. Thus, it will be important to investigate these differences in the context of muscle adiposity and the differential potential for arsenic to promote

myosteatosis in males and females. These studies would greatly impact the public health significance of the work as they would influence the development of effective interventions to reduce the burden of arsenic-promoted disease across the entire human population.

Appendix: Antibodies and Reagents

Appendix Table 1: Primary Antibodies.

Primary Antibody	Host	Company	Catalogue No.	Dilution
Anti-Actin, α-Smooth Muscle	Mouse	Sigma	A2547	1:250
CD34 Antibody [EP373Y]	Rabbit	Abcam	ab81289	1:100
Anti-Desmin	Rabbit	Abcam	ab15200	1:1000
Anti-DLL4	Rabbit	Abcam	Ab7280	1:500
DLL4 blocking antibody	American hamster	eBiosciences / ThermoFisher	16-5948-82	1:100
Anti-PDGFR alpha [16A1]	Mouse	Abcam	ab96569	1:200
Anti-PDGF Receptor α	Rabbit	Cell Signaling	3174S	1:250
MyoD (G-1)	Mouse	Santa-Cruz	sc377460	1:800
Anti-activated Notch1	Rabbit	Abcam	Ab52301	1:200
anti-EGFR	Mouse	BD Laboratories	610017	1:100
anti-pY845EGFR	Rabbit	Cell Signaling	2231	1:100
Alexa Fluor-647 anti-pY845EGFR	Mouse	BD Laboratories	Clone 12A3	1:100
Anti- Cyclin D1	Goat	R&D Systems	AF4196	1:100
Anti-MTCO2	Mouse	ThermoFisher	12C4F12	1:200
Total OXPHOS Rodent WB Antibody Cocktail	Mouse	Abcam	ab110413	6 µg/ml
Anti-VDAC	Rabbit	Abcam	ab15895	1 µg/ml

Appendix Table 2: Secondary Antibodies and Dyes.

Secondary Antibody	Host	Company	Catalogue No.	Dilution
Alexa Fluor 488 anti- Rabbit IgG (H+L)	Donkey	Invitrogen	A21206	1:2000
Cy3 Conjugated anti-Mouse Affinipure	Donkey	Jackson ImmunoResearch	715-165-151	1:800
Alexa Fluor 647-conjugated anti-goat IgG (H+L)	Donkey	Jackson ImmunoResearch	705-605-003	1:700
Cy3 anti-Rabbit IgG (H+L)	Goat	Invitrogen / ThermoFisher Scientific	A10520	1:250
IRDye® 800CW anti-Mouse IgG	Donkey	LI-COR Biosciences	926-32212	1:20,000
IRDye® 680LT anti-Rabbit IgG	Donkey	LI-COR Biosciences	926-68023	1:20,000
Alexa Fluor 647 Phalloidin		ThermoFisher	A22287	1:40
DAPI (4',6-diamidino-2-phenylindole)		Biolegend		

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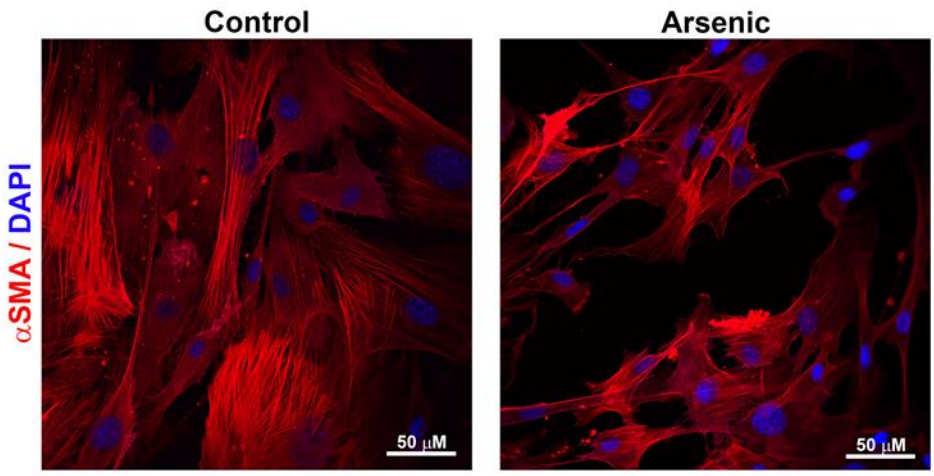
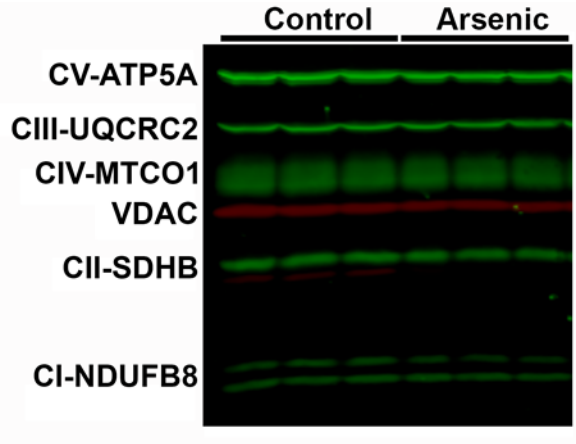
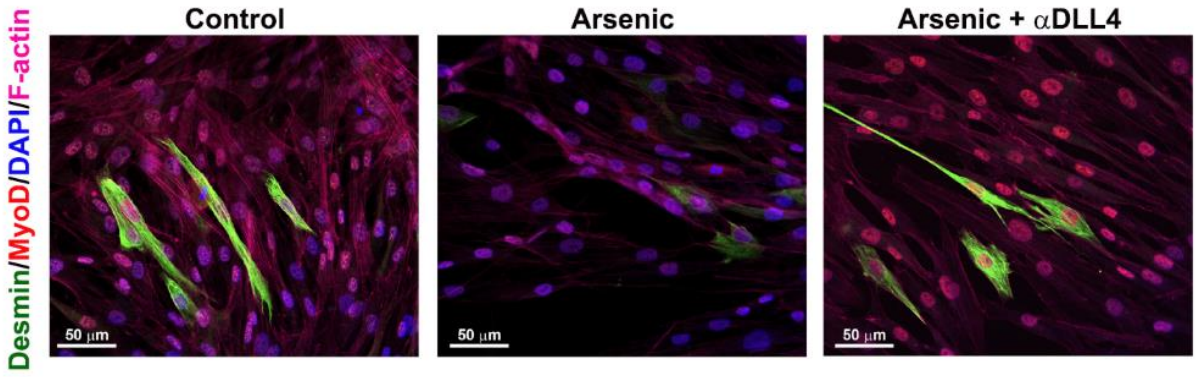


Appendix Supplemental Figure 1: CTF Characterization.
CTF were isolated from hind limb muscles of two mice in each group. The cells were fixed 24 hours after being plated in cultures and immunostained with antibody to αSMA. Confocal images were captured at 40x magnification. Greater than 95% of isolated cells were αSMA in all cultures**.**



Appendix Supplemental Figure 2: Effect of Arsenic on CTF mitochondrial respiratory protein expression.
Respiratory protein expression measured by immunoblotting in the additional three replicate cultures of CTF^{ctr} and CTF^{ars} (first three replicates shown in Figure 2B). The abundances of respiratory complex proteins (green) and VDAC (red) from all six replicate cultures are given in Table 1.



Appendix Supplemental Figure 3: Effect of ECM DLL4 inhibition on hMuSC differentiation.
CTF^{ctr} and CTF^{ars} were cultured for three days to elaborate ECM. The cells were removed and the ECM was incubated with an antibody that blocks DLL4/Notch binding. After rinsing to remove unbound antibody, hMuSC were seeded and cultured in differentiation medium for two days before fixing and immunofluorescent imaging for desmin (green) and nuclear MyoD (red) expression. Nuclei were stained with DAPI (blue) and F-actin was stained with Alexa Fluor 647-phalloidin (magenta). Images were captured at 40x magnification**.**

Quantitative immunofluorescence imaging analysis on Nikon Elements AR software.

Multiple fields were imaged per sample in the 8-well chamber culture slides using the same magnification and threshold settings. When quantifying the mean intensity per cell for several proteins (desmin, NICD, DLL4, PDGFRα, and DLL4), each image was designated as a region of interest (ROI). The mean intensity for each protein within the ROI was quantified by the Nikon Elements AR software. That value was then divided by the number of nuclei within the ROI to calculate the mean intensity per cell for each protein. The mean intensity of MyoD within cell nuclei was calculated by designating each nucleus within an image as a ROI. Each individual nuclear ROI in each field was then averaged by the Nikon Elements AR software. The average of the mean nuclear intensities from each image was graphed as a single data point.

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