ARSENIC INHIBITS P19 STEM CELL DIFFERENTIATION BY ALTERING MICRORNA EXPRESSION AND REPRESSING THE SONIC HEDGEHOG SIGNALING PATHWAY

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ARSENIC INHIBITS P19 STEM CELL DIFFERENTIATION BY ALTERING MICRORNA EXPRESSION AND REPRESSING THE SONIC HEDGEHOG SIGNALING PATHWAY

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

by
Jui-Tung Liu
December 2015

Accepted by:
Dr. Lisa Bain, Committee Chair
Dr. William Baldwin
Dr. Wen Chen
Dr. Charles Rice
ABSTRACT

Arsenic is a naturally-occurring toxicant that exists in bedrock and can be leached into ground water. Humans can be exposed to arsenic via contaminated drinking water, fruit, rice or crops. Epidemiological studies have shown that arsenic is a developmental toxicant, and in utero exposure reduces IQ scores, verbal learning ability, decreases long term memory, and increases the likelihood of dying from a neurological disorder. Arsenic can also reduce birth weight, weight gain, and muscle function after an in utero exposure. Although the mechanism behind these physiological changes is not known, in vitro studies have shown that arsenic can reduce muscle and neuronal cell differentiation. The purpose of this study was to investigate whether arsenic can disrupt signaling pathways or alter the expression of microRNAs that are important in stem cell differentiation. We used P19 murine embryonic stem cells, which were exposed to 0, 0.25, or 0.5 µM arsenite for up to 9 days, to analyze the expression of developmental-related cell signaling pathways and microRNAs by microarray and quantitative PCR. While arsenic does not appear to impact FGF, Bmp nor Notch pathways, it does reduce transcript levels within the sonic hedgehog (Shh) signaling pathway, including the ligand Shh, the key transcription factor Gli2, and its target gene Ascl1, by 2-fold, 3-fold and 5-fold respectively. GLI2 protein expression was also reduced, leading to an inhibition of transcriptional activity. Additionally, exogenous SHH protein rescued the inhibitory effects of arsenic. Based on miRNA microarray data using of 1900 feature mouse miRNAs, Several miRNAs known to be important in development and/or stem cell differentiation, including miR-9, miR-92a, miR-199a, miR-291a and miR-709, were
altered by arsenic. Interestingly, a polycomb gene Sfmbt and its hosted miR-466-669 cluster were induced from 1.5-to 3.5 fold by arsenic in a time-dependent manner. Moreover, knockdown of this cluster rescued the arsenic-mediated repression of Gli2 expression and cell differentiation. Taken together, our results imply that arsenic inhibits P19 cell differentiation through repressing the Shh signaling pathway by decreasing Gli2 expression and its activity, and the reduction of Gli2 is directly or indirectly regulated by miR-466-669 cluster. These results suggest two novel mechanisms by which arsenic disrupts cell differentiation.
DEDICATION

I dedicate this work to my family, especially to my parents.
ACKNOWLEDGMENTS

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>..................................................................................................................i</td>
<td></td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>.................................................................................................................. ii</td>
<td></td>
</tr>
<tr>
<td>DEDICATION</td>
<td>..............................................................................................................iv</td>
<td></td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>............................................................................................................v</td>
<td></td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>............................................................................................................viii</td>
<td></td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>............................................................................................................. ix</td>
<td></td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I.</td>
<td>INTRODUCTION ...............................................................................................1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arsenic .................................................................................................1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Health Effects of Arsenic ....................................................................3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arsenic-Induced Developmental Toxicology ........................................5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arsenic Effects on Muscle and Neuron Development ..............................7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P19 Cells ..............................................................................................9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonic Hedgehog (Shh) Signaling Pathway ............................................9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arsenic Effects on Epigenetic Modifications .....................................12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MicroRNAs ..........................................................................................14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dissertation Goals and Objectives ..................................................17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>References .........................................................................................19</td>
<td></td>
</tr>
<tr>
<td>II.</td>
<td>ARSENIC INHIBITS HEDGEHOG SIGNALING DURING P19 CELL DIFFERENTIAITON</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Abstract ..............................................................................................31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Introduction .........................................................................................33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Material and methods ..........................................................................37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Results .................................................................................................42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Discussions .........................................................................................47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acknowledgements ...............................................................................54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>References ..........................................................................................55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tables .................................................................................................63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Figures ...............................................................................................64</td>
<td></td>
</tr>
</tbody>
</table>

vi
Table of Contents (Continued)

III. ARSENIC INDUCES MEMBERS OF THE MIR-466-669 CLUSTER TO REPRESS P19 CELL DIFFERENTIATION ........................................... 75

Abstract ........................................................................................................ 76
Introduction .................................................................................................... 78
Material and methods .................................................................................... 82
Results ........................................................................................................... 86
Discussions ..................................................................................................... 91
Acknowledgements ....................................................................................... 96
References .................................................................................................... 97
Tables ........................................................................................................... 104
Figures ......................................................................................................... 106

IV. CONCLUSION ........................................................................................ 121

References ................................................................................................... 129
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Primer sequences for quantitative PCR</td>
<td>63</td>
</tr>
<tr>
<td>3.1</td>
<td>Primer sequences</td>
<td>104</td>
</tr>
<tr>
<td>3.2</td>
<td>Developmentally-related miRNAs significantly changed by arsenic during P19 cell differentiation</td>
<td>105</td>
</tr>
<tr>
<td>3.3</td>
<td>miR-466/467/669 cluster expression in differentiating P19 cells with or without arsenic exposure</td>
<td>105</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic diagram of the Sonic Hedgehog signaling pathway</td>
<td>11</td>
</tr>
<tr>
<td>2.1</td>
<td>Arsenic decreased hedgehog pathway gene expression during cell differentiation</td>
<td>64</td>
</tr>
<tr>
<td>2.2</td>
<td>P19 cells exposed to arsenic during differentiation repress hedgehog pathway related gene expression</td>
<td>66</td>
</tr>
<tr>
<td>2.3</td>
<td>Arsenic reduces GLI2 protein levels, but not SHH levels</td>
<td>67</td>
</tr>
<tr>
<td>2.4</td>
<td>Arsenic decreases GLI2 protein levels in day 5 embryoid bodies</td>
<td>68</td>
</tr>
<tr>
<td>2.5</td>
<td>Arsenic decreases GLI reporter activity in P19 cells</td>
<td>69</td>
</tr>
<tr>
<td>2.6</td>
<td>Arsenic exposure does not affect GSK3β phosphorylation</td>
<td>70</td>
</tr>
<tr>
<td>2.7</td>
<td>Additional Shh rescues arsenic’s inhibitory effects on cell differentiation</td>
<td>71</td>
</tr>
<tr>
<td>2.8</td>
<td>Shh rescues GLI activity after arsenic exposure</td>
<td>73</td>
</tr>
<tr>
<td>S2.1</td>
<td>Arsenic does not affect ciliary accumulation of GLI2 during cell differentiation</td>
<td>74</td>
</tr>
<tr>
<td>3.1</td>
<td>Expression profiles of miRNAs between control and arsenic exposure in differentiating P19 cells</td>
<td>106</td>
</tr>
<tr>
<td>3.2</td>
<td>Validation of miRNA transcript levels by qPCR</td>
<td>108</td>
</tr>
<tr>
<td>3.3</td>
<td>Arsenic exposure induces members of the miR-466-669 cluster along with its host gene, Sfmbt2</td>
<td>110</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4</td>
<td>Arsenic does not alter expression of the miR-466-669 cluster in C2C12 and N2A cells</td>
<td>111</td>
</tr>
<tr>
<td>3.5</td>
<td>The miR-466-669 cluster share sequence similarities</td>
<td>112</td>
</tr>
<tr>
<td>3.6</td>
<td>Inhibiting the miR-466-669 cluster during P19 cell differentiation</td>
<td>113</td>
</tr>
<tr>
<td>3.7</td>
<td>Mixed miRNA inhibitors rescue rescues arsenic’s inhibitory effects on cell differentiation</td>
<td>115</td>
</tr>
<tr>
<td>S3.1</td>
<td>Arsenic exposure decreased Neuro2A cell differentiation</td>
<td>117</td>
</tr>
<tr>
<td>S3.2</td>
<td>miR-291a was upregulated when exposed to arsenic during cell differentiation</td>
<td>118</td>
</tr>
<tr>
<td>S3.3</td>
<td>Histone modification status in the promoter region of Sfmbt2 and miR-466-669 cluster is not changed when exposed to arsenic</td>
<td>119</td>
</tr>
<tr>
<td>S3.4</td>
<td>Mixed miRNA inhibitors rescue arsenic’s inhibitory effects on Gli2 expression</td>
<td>120</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

Arsenic

Arsenic is a naturally occurring metalloid element that exists in bedrock, sediments and soils. It can be found as a by-product of industrial activity including smelting, fossil fuel combustion, and pesticide production (Vahter, 2009). By dissolving into the surrounding water, arsenic can exist in ground water and surface water (Amini et al., 2008). Since arsenic can accumulate in soil, water, and airborne particles, it can be taken up by plants and marine organisms. Drinking water derived from groundwater has been reported as the most common source of human exposure to inorganic arsenic. Studies have shown that residents in China, Argentina, Taiwan, Bangladesh and USA are exposed to high level of arsenic via drinking water (ATSDR, 2007; Blanes et al., 2011; Chen et al., 1994; Nickson et al., 1998; Ning et al., 2007).

For example, Blackfoot disease was originally found in areas of Taiwan where the concentration of arsenic in well water averages 671 ppb (Chen et al. 1994). In West Bengal, India and in Bangladesh, more than half of wells have water contaminated with arsenic, and estimates are that more than 9.5 million people could be drinking water above 10 ppb, while 53,000 people are exposed to arsenic at a level of 300 ppb (Rahman et al., 2015). Within the United States, arsenic contamination is found in southwest, upper Midwest, and in New England, with concentrations ranging from 10 to 200 ppb (Ryker, 2001). Both the World Health Organization (WHO) and U.S. Environmental Protection Agency (USEPA) have set 10 parts per billion as the maximum allowable
stand for arsenic in drinking water (WHO, 2004). Even with this limit, there are still more than one hundred million people worldwide at risk of elevated arsenic exposure.

There are many uses of arsenic in products such as pesticides, lead-acid storage batteries, anti-cancer drugs, and antimicrobial additives for animal and poultry feed (ATSDR, 2007). Recently, an increasingly important source of exposure to inorganic arsenic is food, such as cereals, crops, and vegetables, which are cultured in arsenic-rich areas where farmers use groundwater for irrigation (Vahter, 2009) or in areas with historical arsenical pesticide use. Arsenic can accumulate in rice through the silicon transport system because arsenious acid, which exists in contaminated flooded rice paddies, is indistinguishable from silicic acid to the rice plant (Ma et al., 2008). Thus, people consuming a diet rich in rice have urinary arsenic levels that are two-to-three fold higher than those consuming a wheat-based diet (He and Zheng, 2010). Moreover, another study shows that consumption of 0.56 cup per day of cooked rice was comparable to drinking 1L/day of 10 µg As/L water, the current maximum contaminant limit in US (Gilbert-Diamond et al., 2011). Another study determined that the urinary arsenic concentration in children who reported consuming rice was 1.5 fold higher among those who did not consume rice, and the total urinary arsenic concentration increased 14.2% with each 0.25 cup increase in cooked rice consumed (Davis et al., 2012). These studies suggest that there are multiple exposure routes of arsenic, which raise concern about its effects on public health.

Arsenic has two biologically important oxidation states, arsenite, the trivalent form and arsenate, the pentavalent form. In the environment, inorganic arsenic can be
transformed into organic forms, such as methyl and dimethyl arsenic, by methanogenic bacteria (Ferguson and Gavis, 1972). In mammals, inorganic arsenic can also be transformed into an organic form by metabolism. Briefly, arsenite can undergo oxidative methylation to methylarsonic acid [MMA(V)] by arsenic methyltransferases (As3MT). This pentavalent form can be reduced by glutathione to methylarsonous acid [MMA(III)], which is then transformed into dimethylated arsenic [DMA(III)] (Thompson, 1993). In general, the trivalent state is more toxic than the pentavalent state, and inorganic forms of arsenic are more toxic than methylated arsenic species. However, recent studies show that methylated trivalent arsenic metabolites have significant biological activity and may be as or more toxic than inorganic species (McCoy and Bain, 2015).

**Health Effects of Arsenic**

Chronic exposure to arsenic has been reported to cause several adverse health effects. The typical dermal effects are the hyperkeratinization of skin which is considered a precursor lesion of nonmelanocytic skin cancers (ALAIN et al., 1993). A study using human keratinocytes (HaCaT) exposed to environmentally relevant concentrations of arsenite for 14 days found that p53 levels, a tumor suppressor gene which plays a critical role in DNA repair, were reduced by arsenic, suggesting a potential mechanism by which arsenic induced keratosis (Hamadeh et al., 1999). Patients exposed to arsenic are found to have multiple hyperkeratinized corns and spots of hypopigmentation (Rakhunde et al., 2012). In West Bengal, India, residents consuming water containing at least 50 ppb arsenic had keratosis, and the incidence increases dose-dependently. Interestingly, men
have two to three times the prevalence than women with the same exposure level (Mazumder et al., 1998).

Another famous disease caused by arsenic is a peripheral vascular disease called Blackfoot disease (BFD), which was originally found in Taiwan (Tseng, 2002). In Blackfoot disease, the blood vessels in the lower limbs are severely damaged, which reduces blood circulation around the limb tissue and thus causes tissue damage. This damage can cause a progressive numbness or coldness of limbs, which may ultimately lead to gangrene. Between 1956 and 1960, the incidence of BFD was extremely high in the residents of the southwestern coast of Taiwan (Tseng, 2002). Epidemiological studies found that residents had used deep artesian wells for drinking water since 1920s, where the arsenic concentration of the well water ranged from 700 to 930 ppb (Chen and Wu, 1962). Moreover, studies showed that there is a dose-dependent relationship between the prevalence of BFD and arsenic concentrations in well water, suggesting the link between arsenic exposure and BFD (Tseng, 2002).

Other than peripheral vascular disease, arsenic exposure is also associated with cardiovascular disease. Epidemiological studies in Taiwan demonstrated that long-term exposure to arsenic causes carotid atherosclerosis, ischemic heart disease and cerebrovascular disease (Navas-Acien et al., 2005). In the United States, several studies showed a higher incidence of cardiovascular disease–related mortality, ischemic stroke admissions, hypertension, and coronary heart disease with increasing arsenic exposure (Engel and Smith, 1994; Gong and O'Bryant, 2012; Lisabeth et al., 2010). Moreover, a 7-year study measuring the blood pressure of residents living in an arsenic contaminated
area suggests that long-term arsenic exposure accelerates age-related increases in blood pressure (Jiang et al., 2015).

Inorganic arsenic is considered a human carcinogen by the International Agency for Research on Cancer, as it causes skin, lung, bladder, kidney and liver cancer (Anders et al., 2004; IARC, 2012). Convincing evidence from epidemiological studies show that arsenic can increase the risk of lung cancer in workers exposed to arsenic trioxide dust at copper smelters (Axelson et al., 1978; Järup et al., 1989; PERSHAGEN, 1985) and bladder cancers are also reported as an outcome in Argentina and Taiwan, where studies show a clear dose-response relationship between arsenic and bladder cancer (Chiou et al., 1995; Hopenhayn-Rich et al., 1996).

**Arsenic-Induced Development Toxicity**

Several studies have shown that arsenic can cause developmental defects in humans. Developing organisms are usually more vulnerable to toxicants because of their rapid cell division and differentiation. Reports indicate that arsenic readily crosses the placenta, as similar arsenic levels are in the fetus and in the mother, with 9 μg/L arsenic in cord blood and 11μg/L in maternal blood, when consuming drinking water containing 200ppb arsenic (Concha et al., 1998). A cohort study in Bangladesh showed that women drinking tube-well water with more than 50μg/L arsenic during pregnancy significantly increased their risk of fetal loss and infant death (Rahman et al., 2007). Arsenic exposure also affects the development of the central nervous system, and can cause changes in hearing and increases in learning deficits (Bencko and Symon, 1977). In Mexico,
children in an arsenic-contaminated area have lower verbal intelligence quotient (IQ) scores, decreasing from 96.7 to 91.2, along with a significant 11% reduction in long-term memory (Calderon et al., 2001). In 1955, more than 10,000 Japanese infants were exposed to arsenic through contaminated milk powder. The follow-up studies of the surviving infant victims 14 years later showed a 10-fold increase in IQ scores lower than 85, and a 150-fold increase in those with a hearing disability, which suggests that the neurotoxicity is a concern of early lifestage exposure to inorganic arsenic (Dakeishi et al., 2006).

Similar adverse effects of arsenic on development have been shown in animal models. Arsenic can easily pass through the placenta in rodents (Lindgren et al., 1984), and this reduced body weight and increased neurotoxicity in the offspring (Wang et al., 2006). For example, when pregnant rats were exposed to 0.3 and 3ppm arsenite via their drinking water, the pups showed decreased spontaneous behavior such as movement, shaking and tremors (Chattopadhyay et al., 2002). Similarly, rats exposed to arsenite from GD15 up to 17 weeks via drinking water showed increased spontaneous locomotor activity and number of errors in a delayed alteration task compared to a control group (Rodriguez et al., 2002). These data suggest that arsenic exposure during gestation affects fetal development. Arsenic can also affect embryonic development in fish. Zebrafish embryos exposed to 10mM arsenite showed abnormal development including delayed hatching, shorter body length, and morphological changes including pericardial edema, dorsal curvature, flat head and RBC accumulation. Alterations in neural development and muscle development were also observed at 2 mM arsenic exposure (Li et al., 2009).
**Arsenic Effects on Muscle and Neuron Development**

Epidemiological studies have shown that arsenic (> 50 ppb) in drinking water causes infants to be born with low birth weight (average 57g less), and the incidences of stillbirth and neonatal mortality increase 2.5 and 1.8 fold, respectively (Hopenhayn et al., 2003; Milton et al., 2005; Tofail et al., 2009; von Ehrenstein et al., 2006). The incidence of arsenic contaminated milk powder in Japan mentioned above evidences the neuronal damage caused by arsenic (Dakeishi et al., 2006). These studies all suggest that chronic exposure of arsenic may cause adverse effects on both muscle and neuron development. This has been further shown using embryonic stem cells (ECS), in which arsenic caused a significant down-regulation of gene expression in all three germ layers (Flora and Mehta, 2009). Thus, embryonic stem cells may be an appropriate model for studying how arsenic impairs developmental. It has been reported that exposure to low micromolar levels of sodium arsenite for five days reduced neurite production, outgrowth, and complexity in newly differentiating rat phaeochromocytoma-derived PC12 cells (Frankel et al., 2009). Wang et al. (2010) showed that, arsenic inhibited neurite outgrowth in Neuro-2a (N2a) neuroblastoma cells by inhibiting the LKB1 (serine/threonine kinase 11)-AMPK (adenosine monophosphate-activated kinase) pathway, which controls cell growth in response to environmental nutrient changes (Wang et al., 2010).

With respect to muscle cell differentiation Yen et al. (2010) demonstrated that 0.1–0.5 μM arsenic trioxide exposure decreased myogenic differentiation without
apparent effects on cell viability in C2C12 mouse myoblast cells, and also inhibited myotube formation and muscle regeneration after injury in mice by inhibiting Akt-regulated signaling (Yen et al., 2010). In their next study in 2012, higher concentrations (1-10 μM) of arsenic trioxide causes ROS-induced mitochondrial dysfunction by inactivating the Akt signaling pathway, which the authors hypothesized might be the reason for decreased skeletal muscle fiber formation (Yen et al., 2012). *In utero* and postnatal exposure to arsenic resulted in increases of smooth muscle actin around airways, leading to alteration of pulmonary structure and function in mice (Lantz et al., 2009). In our laboratory, C2C12 mouse myoblast cells exposed to 20nM arsenite showed delayed myotube formation due to reductions in transcription factors such as myogenin and Mef2C. The mechanism believed to be responsible for this reduction is due to changes in promoter methylation (Steffens et al., 2011). In P19 embryonic stem cells, arsenic exposure reduced both muscle and neuronal cell differentiation because of reduced neurogenic and myogenic transcription factor expression (Hong and Bain, 2012). In this study, β-catenin was down regulated, which suggested that arsenic may affect neurogenesis and myogenesis through the Wnt/β-catenin signaling pathway during embryogenesis (Hong and Bain, 2012). Although those studies indicated the adverse effect of arsenic on neurogenesis and myogenesis, the mechanism by which this occurs is not fully understood.
P19 Cells

P19 cells are pluripotent murine embryonal carcinoma cells which were originally derived from a mouse teratocarcinoma (McBurney and Rogers, 1982). They can differentiate in culture into the 3 different germ layers and are useful for investigating developmental events such as signaling pathways and cell commitment determination (McBurney and Rogers, 1982). In the presence of dimethyl sulfoxide (DMSO), aggregates of P19 cells can be induced into skeletal muscle and neuronal cells (McBurney and Rogers, 1982), while high concentration of retinoic acid can specifically induce cells to differentiate into neurons (Jones-Villeneuve et al., 1982).

Because of the established methods of cell differentiation, P19 cells have been used as an in vitro model for investigating several molecular mechanisms that play essential roles in mesoderm formation, neuronal formation, and axial elongation (Farah et al., 2000; Marikawa et al., 2009). Signaling pathways such as Wnt, Notch, BMP and Sonic Hedgehog are found involved in P19 cell differentiation (Gianakopoulos and Skerjanc, 2005; Marikawa, Tamashiro, Fujita and Alarcón, 2009; Nye et al., 1994; van der Heyden and Defize, 2003). Therefore, P19 cells can be a valuable model for studying arsenic-induced developmental effects.

Sonic Hedgehog (Shh) Signaling Pathway

During embryonic development, a limited number of signal transduction pathways are used to direct cells and control their differentiation. Sonic hedgehog (Shh) signaling is one of the evolutionarily conserved pathways and is needed for proper limb
development and neural progenitor cell survival (Anderson et al., 2012; Litingtung and Chiang, 2000; Mill et al., 2003). Studies have shown that Shh signaling can regulate the Wnt pathway to mediate neural circuit formation, indicating that Shh may play an important role in concert with the Wnt pathway (Wilson and Stocekli, 2012). As shown in Figure 1.1, the key proteins in the Shh pathway are zinc-finger transcription factors termed Gli. In the absence of Shh, the membrane receptor Patched (Ptch) inhibits Smoothened (Smo), which is held in intracellular vesicles. Gli2 protein is transferred to primary cilium by intraflagellar transport (IFT) and forms a complex with KIF7 and Suppressor of Fused (Sufu). The complex then binds to GSK3β and PKA to phosphorylate Gli2, leading to cleavage of Gli2 into a repressive form, thus inactivating the pathway. When Shh is present, it binds to Patched on the membrane, resulting in the loss of inhibitory effect between Ptch and Smo. Smo is phosphorylated by GRK2 and then binds to KIF3A, which relocates Smo to the primary cilium. The increased Smo at the cilium leads to the dissociation of Sufu and Gli2, thus releasing full length of Gli2, allowing the active Gli2 to translocate into the nucleus and further induce cell differentiation (Anderson et al., 2012; Ingham and McMahon, 2001; Jiang and Hui, 2008).
Figure 1.1. **Schematic diagram of the Sonic Hedgehog signaling pathway.**

In the absence of Shh, the membrane receptor Ptc inhibits Smo’s movement to cilium. The complex of Gli2, Kif7 and Sufu is then phosphorylated by GSK3β and PKA, which leads to the cleavage of Gli2, thus inhibiting the signaling. Shh ligand reverses the inhibitory effect of Ptch on Smo. Smo is then phosphorylated and relocated to cilium, leading the dissociation of Gli2 complex. Full length of Gli2 then translocates to the nucleus and can transcribe downstream target genes.

Shh signaling is essential for myogenesis in zebrafish. When Shh signaling is lost in fish embryos, terminal myogenic differentiation of adaxial cells is delayed (Coutelle et al., 2001). In mouse embryos, Gli2 has been shown to regulate Myf5 expression by binding specifically to the Myf5 enhancer (Gustafsson et al., 2002). Studies have shown that the lack of Shh signaling caused neural tube defects, which disrupted brain
development (Murdoch and Copp, 2010). In zebrafish, inhibition of Shh signaling using mutants reduces neural progenitor cell proliferation, thus affecting neurogenesis in the dorsal mesencephalon (Feijoo et al., 2011). Moreover, the mutation of Shh-related genes delays motor neuron differentiation in the mouse spinal cord, suggesting that Shh signaling is also important in neurogenesis (Oh et al., 2009).

**Arsenic’s Effects on Epigenetic Modifications**

Epigenetics is the study of changing gene expression without altering DNA sequences, which can result in disrupting signaling pathway and cell morphology (Moore, 2015). Epigenetic mechanisms include methylating DNA, modifying histone tails, and altering the expression of non-coding microRNAs (Moore, 2015). The developmental program of embryogenesis can be controlled by epigenetic mechanisms (Gan et al., 2007). During embryogenesis, mutation of the DNA methyltransferase gene results in embryonic lethality (Li et al., 1992). Moreover, Azuara et al. examining the histone modification patterns in mouse ESCs showed that critical transcription factors for cell lineage determination, including Sox1, Nkx2-2, Msx-1, Irx3, and Pax3, were associated with activating (H3 Lys-9 acetylation and H3 Lys-4 methylation) and repressive (H3 Lys-27 methylation) histone modifications within their promoter loci (Azuara et al., 2006). Therefore, epigenetic alterations play an important role in understanding development defects.

The epigenetic effects of arsenic have been studied in several model systems. Arsenic can modify the amount of DNA methylation. For example, Mass and Wang
found that arsenic exposure resulted in promoter hypermethylation of the tumor suppressor gene \( p53 \), resulting in arsenic-induced carcinogenesis (Mass and Wang, 1997). Zhao et al. transformed rat liver cells into malignant tumors by exposing them to 0.5 μM inorganic arsenic for 18 weeks. Interestingly, the transformed cells showed global hypomethylation compared to normal cells, indicating the potential epigenetic mechanism of carcinogenesis induced by arsenic (Zhao et al., 1997). A previous study in our lab using C2C12 mouse myoblast cells showed that arsenic can alter the methylation patterns on the transcription factor myogenin, thereby delaying their differentiation into myotubes (Steffens et al., 2011).

How arsenic alters DNA methylation is likely related to the way cells metabolize arsenic. Inside a cell, inorganic arsenic can be methylated by arsenite methyltransferase (As3MT) using S-adenosyl methionine (SAM) as the methyl donor. The depletion of SAM inhibits DNA methyltransferase (DNMT) activity by feedback inhibition, resulting in changes to DNA methylation patterns (Caudill et al., 2001).

Beyond DNA methylation, arsenic exposure can also modify histone methylation, acetylation, and phosphorylation patterns (Ren et al., 2011). Cronican et al. showed that prenatal exposure to arsenic caused a genome-wide alteration of histone H3K9 acetylation patterns (Cronican et al., 2013). Another study exposing human lung cancer cells to inorganic arsenic resulted in increased H3K9 dimethylation and decreased H3K27 trimethylation, both of which represent gene silencing marks (Zhou et al., 2008). Previous studies in our lab using C2C12 mouse myoblast cells have shown that arsenic exposure can increase H3K9 di- and tri-methylation by 4-5-fold and reduce H3K9
acetylation by 2-fold on the MyoD promoter, all of which are silencing marks. MyoD is needed to convert myoblasts into myotubes, so the histone modifications provide a potential mechanism for how arsenic can inhibit cell differentiation (Steffens et al., 2011).

**MicroRNAs**

The third epigenetic modifiers are microRNAs (miRNAs), which are small, single stranded, non-coding RNAs that regulate gene expression. Primary microRNAs are transcribed by RNA polymerase II as stem-loop structures. Following cleavage by RNAse III family protein, Drosha, in the nucleus, the primary form becomes a small hairpin that is termed as pre-miRNA (Lee et al., 2002). Pre-miRNAs are then exported to the cytoplasm and cleaved by Dicer, another RNAse III family protein, thus releasing mature miRNAs (~22 nucleotides) (Hutvagner et al., 2001; Ketting et al., 2001). MicroRNAs in animals bind imperfectly to the 3’ untranslated region (UTR) of mRNA, leading to targeted mRNA translational repression (Yang et al., 2005).

Because of its ability to regulate gene expression, microRNAs play an important role in development. In mice, deletion of the gene Dicer-1, which cleaves pre-miRNA to produce mature miRNAs, results in the embryonic lethality (Bernstein et al., 2003). Moreover, Kanellopoulou et al. showed that the lack of Dicer-1 in mouse embryonic stem cells significantly reduces cell differentiation and centromeric silencing (Kanellopoulou et al., 2005). Expressions of several miRNAs are related to cell differentiation. For instance, miR-1 and miR-133 are highly expressed in muscle lineages in several species,
indicating the evolutionary conservation of the tissue-specific expression (Kwon et al., 2005; Simon et al., 2008; Zhao et al., 2005). Both of these miRNAs are regulated by the transcription factor MyoD or MEF2 in skeletal muscle and cardiac muscle, respectively (Liu et al., 2007; Zhao et al., 2005). miR-1 and miR-133 promote mesoderm formation and are potent repressors of nonmuscle genes during cell lineage commitment (Ivey et al., 2008). miR-124 and miR-9 are both critical for neurogenesis, because they increase several transcription factors, including Foxg1, Sox9 and Pax6 (Akerblom et al., 2012; Cheng et al., 2009; Clark et al., 2010; Otaegi et al., 2011; Shibata et al., 2011).

MicroRNAs can be located in the genome separately or in the intron of a host gene (Rodriguez et al., 2004). Several miRNA families are clustered together within the genome and are transcribed as a polycistronic transcript. For instance, the miR-17-92 cluster is found within the intron region of C12orf25 and it contains six miRNAs, miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1, within 1kb on chromosome 13 (Ota et al., 2004; Tanzer and Stadler, 2004). The miR-290 cluster is identified as a 2.2-kb region on chromosome 7, and its primary transcript generates 14 mature miRNAs which can promote pluripotency maintenance by regulating cell cycle phase distribution in mouse embryonic stem cells (Judson et al., 2009; Lichner et al., 2011). The miR-466 cluster is located in the intron 10 of the mouse Sfmbt2 gene on chromosome 2 and contains more than 40 miRNAs (Zheng et al., 2011). It is reported to be involved apoptosis and osmoregulation (Druz et al., 2011; Luo et al., 2014), but there are likely to be other unknown functions and targets.
Compare to other epigenetic modifications, the effects of arsenic on miRNA regulation is relatively unexplored. A study using arsenic trioxide (ATO) as an anticancer drug found that arsenic exposure increased miR-29a expression, thereby inhibiting cell growth and inducing apoptosis (Meng et al., 2011). Moreover, Ghaffari et al. identified several miRNAs that are altered by ATO in acute promyelocytic leukemia cells, with the up-regulated miRNAs involved in tumor suppressor functions and the down-regulated miRNAs related to angiogenesis, invasion and metastasis (Ghaffari et al., 2012). Cui et al. showed that arsenite exposure induced angiogenesis by decreasing miR-9 and miR-181b expression (Cui et al., 2012). Arsenic also induced miR-190 expression in human bronchial epithelial cells, thus enhancing cell proliferation and carcinogenic transformation by target tumor suppressor gene PHLPP (Beezhold et al., 2011). Moreover, low level of arsenic exposure (2.5 µM) in human bronchial epithelial cells caused malignant transformation by reducing the level of miR-200 family members. Overexpressing miRNA-200b in this arsenite-transformed cell line reversed the transformed phenotype (Wang et al., 2011). With increasing evidence showing the importance of miRNAs in development and arsenic-induced miRNAs alteration, how arsenic alters miRNA expression and whether this change causes development defects still needs to be uncovered.
Dissertation Goals and Objectives

The goal of this dissertation is to determine the mechanism by which arsenic reduces P19 embryonic stem cell differentiation. Previous studies have shown that 0.5 μM arsenite inhibits P19 cell differentiation by reducing transcription factors, such as MyoD and myogenin, and NeuroD and neurogenin, needed for the formation of skeletal myotubes and sensory neurons, respectively. Therefore, I plan on investigating how arsenic causes transcription factor repression. I hypothesize that arsenic will alter the Shh signaling pathway, and/or alter microRNA expression in the stem cells, thereby leading to reduced neurogenesis and myogenesis in P19 cells. My goal will be accomplished by two objectives:

Objective 1: Determine if arsenic inhibits Sonic Hedgehog signaling during cell differentiation of mouse P19 embryonic stem cells. We will expose P19 stem cells to 0.25 μM or 0.5 μM sodium arsenite and examine expression and localization changes in Shh pathway genes (Shh, Gli1, Gli2, Patch1, and Sufu) during the cells’ differentiation from pluripotent cells into myotubes and sensory neurons. Cells will also be treated with Shh protein to see if they can recover from the inhibitory effects of arsenic.

Objective 2: Determine if arsenic reduces cell differentiation by altering miRNA expression. P19 stem cells are exposed to arsenic during their differentiation into skeletal myotubes and sensory neurons. Global microRNA expression will be determined by microarrays, and validated with qPCR. miRNA inhibition will be
conducted to determine if specific miRNA knock-down can recover the arsenic-mediated reduced differentiation.

By better understanding the mechanisms of action of arsenic, we can develop the potential treatments for those who are exposed to high level of arsenic around the world, especially for infant development. Moreover, we can also apply arsenic as a cancer therapy by targeting the specific signaling pathways.
References


CHAPTER TWO

ARSENIC INHIBITS HEDGEHOG SIGNALING DURING P19 CELL DIFFERENTIATION

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Abstract

Arsenic is a toxicant found in ground water around the world, and human exposure mainly comes from drinking water or from crops grown in areas containing arsenic in soils or water. Epidemiological studies have shown that arsenic exposure during development decreased intellectual function, reduced birth weight, and altered locomotor activity, while in vitro studies have shown that arsenite decreased muscle and neuronal cell differentiation. The sonic hedgehog (Shh) signaling pathway plays an important role during the differentiation of both neurons and skeletal muscle. The purpose of this study was to investigate whether arsenic can disrupt Shh signaling in P19 mouse embryonic stem cells, leading to changes muscle and neuronal cell differentiation. P19 embryonic stem cells were exposed to 0, 0.25, or 0.5 μM of sodium arsenite for up to 9 days during cell differentiation. We found that arsenite exposure significantly reduced transcript levels of genes in the Shh pathway in both a time and dose-dependent manner. This included the Shh ligand, which was decreased 2- to 3-fold, the Gli2 transcription factor, which was decreased 2- to 3-fold, and its downstream target gene Ascl1, which was decreased 5-fold. GLI2 protein levels and transcriptional activity were also reduced. However, arsenic did not alter GLI2 primary cilium accumulation or nuclear translocation. Moreover, additional extracellular SHH rescued the inhibitory effects of arsenic on cellular differentiation due to an increase in GLI binding activity. Taken together, we conclude that arsenic exposure affected Shh signaling, ultimately decreasing the expression of the Gli2 transcription factor. These results suggest a mechanism by which arsenic disrupts cell differentiation.
**Key Words:** arsenic; P19 cells; stem cells; Sonic Hedgehog; Gli2; cell differentiation

**Abbreviations:** Shh: sonic hedgehog; PTCH: patched; SMO: smoothened; SUFU: suppressor of fused; Bmp: bone morphogenetic protein; Fgf: fibroblast growth factor; Gsk3β: glycogen synthase kinase 3β.
Introduction:

Arsenic is a naturally occurring metalloid that exists in bedrock, sediments and soils. Drinking water derived from groundwater has been reported as the most common source of human exposure to inorganic arsenic. According to the U.S. Environmental Protection Agency and the World Health Organization, the drinking water standard for arsenic is 0.01mg/L (10 ppb). Even with this limit, residents in several counties, including Bangladesh, China, India, Pakistan, Taiwan, Hungary, Argentina, and Mexico, who are still at risk of elevated arsenic exposure due to high concentrations of arsenic in ground water (Naujokas et al., 2013). An increasingly important source of exposure to inorganic arsenic is food, such as cereals, crops, and vegetables, which are cultured in arsenic-rich areas where farmers use groundwater for irrigation or in areas with historic arsenic-based pesticide usage. Both epidemiological and animal studies showed that arsenic exposure causes adverse developmental effects such as fetal loss, infant death, low intelligence quotient scores, reduced birth weight, and reduced weight gain (Calderon et al., 2001; Rodriguez et al., 2002; Wang et al., 2006a; Huyck et al., 2007; Rahman et al., 2007; Li et al., 2009; Bloom et al., 2014), suggesting that arsenic exposure impacts development.

In addition to the reductions in body weight and changes in neuronal function, arsenic exposure has been shown to decrease muscle and neuron development. For example, exposure to micromolar levels of arsenite reduced neurite production, outgrowth, and complexity in differentiating rat PC12 cells (Frankel et al., 2009), while in Neuro-2a neuroblastoma cells, arsenic inhibited neurite outgrowth by inhibiting the
LKB1 AMPK pathway (Wang et al., 2010). Arsenic also induced neural tube defects in mouse embryos (Wlodarczyk et al., 1996). Exposure to arsenic trioxide exposure in 0.1-0.5 μM range decreased myogenic differentiation in C2C12 mouse myoblast cells and during muscle regeneration after injury in mice by inhibiting Akt-regulated signaling (18).

Similarly, C2C12 mouse myoblast cells to be exposed to 20 nM sodium arsenite showed delayed myotube formation due to reductions in transcription factors such as myogenin and myocyte enhancer factor 2C (Mef2C) (Steffens et al., 2011). These results suggest that arsenic inhibits skeletal muscle and neuronal differentiation without affecting proliferation.

Sonic hedgehog (Shh) signaling is an evolutionarily conserved pathway that is needed for somite formation, patterning of embryonic structures, and neural progenitor cell survival (Litingtung and Chiang, 2000; Mill et al., 2003; Anderson et al., 2012). Studies have shown that Shh signaling is essential for myogenesis in zebrafish since without Shh signaling, adaxial cells are delayed in terminal differentiation (Coutelle et al., 2001). In mouse cells, hedgehog signaling regulates both cardiac and skeletal myogenesis, as the transcription factor of hedgehog signaling, Gli2, has been shown to regulate and activate Mef2c and MyoD expression, which are transcription factors needed for myogenic differentiation of progenitor cells (Voronova et al., 2012; Voronova et al., 2013). Shh signaling is also critical for neuronal development. Studies have shown that lack of Shh signaling disrupts dorso-ventral patterning within the neural tube in mice (Chiang et al., 1996; Murdoch and Copp, 2010). In zebrafish, inhibition of Shh signaling by using mutants that lack essential components of the Shh pathway, like Smoothen (Smo)
and Gli, reduces neural progenitor cell proliferation in the dorsal mesencephalon (Feijoo et al., 2011). Moreover, mice with mutations in Shh and Gli show a delay in motor neuron differentiation in spinal cord, suggesting that Shh signaling is also important in neurogenesis (Oh et al., 2009).

The key proteins of Shh pathway are zinc-finger transcription factors family termed Gli. Gli2 is the primary transcription factor of Shh signaling pathway. It has two different activities based on post-translational modification, in which the full length protein acts as activator and the truncation of its C-terminus acts as repressor. Gli1 acts as a minor activator and is involved in cellular growth and cell cycle progression (Sun et al., 2014), and Gli3 is a transcriptional repressor, but its expression is very low (Hui and Angers, 2011). In the absence of SHH, the membrane receptor Patched (PTCH) inhibits the activity of Smoothened (SMO), a 7-pass transmembrane protein. GLI2 protein is transferred to the primary cilium and forms a complex with KIF7 and Suppressor of Fused (SUFU). The complex then binds to GSK3β and PKA to phosphorylate GLI2, leading to the cleavage of GLI2 into a repressive form and inactivating the pathway (Kim et al., 2009). When SHH is present, it binds to PTCH on the membrane, resulting in the loss of inhibitory effect between PTCH and SMO. SMO is then phosphorylated by GRK2 and translocated to primary cilium, which cause the accumulation of SMO in primary cilium. Accumulation of SMO in primary cilium leads to the dissociation of SUFU and GLI2, releasing full length of GLI2, and therefore allowing the active GLI2 to translocate into the nucleus and induce cell differentiation (Ingham and McMahon, 2001; Jiang and Hui, 2008).
Arsenic trioxide is used as a chemotherapeutic agent, and it has been recently shown to inhibit the Hedgehog pathway in cancer cells by inhibiting GLI proteins. For example, arsenic trioxide decreased Hh-induced ciliary accumulation of GLI2 and increased its rate of degradation (Kim et al., 2010). Another study found that in the Ewing sarcoma cell line, arsenic trioxide inhibited hedgehog signaling by inhibiting GLI1 protein transactivation (Beauchamp et al., 2011). Other studies have shown that arsenic trioxide exposure in several cancer cell lines decreased the transcription of GLI1, GLI2 and PTCH1, leading to the inhibition of cell growth (Nakamura et al., 2013; Yang et al., 2013). Taken together, arsenic trioxide inhibits Shh signaling in cancer cells at the level of GLI transcription factors. Thus, we were interested in whether arsenic can also alter Shh signaling in embryonic stem cells, thereby reducing cellular differentiation.

In our previous study, arsenic exposure inhibited both skeletal muscle and neuronal cell differentiation in P19 embryonic stem cells by reducing the expression of MyoD and NeuroD1, transcription factors needed for myogenesis and neurogenesis respectively (Hong and Bain, 2012). However, the mechanism by which arsenic inhibits cell fate determination is not fully understood. In the present study, we examined whether sodium arsenite affects sonic hedgehog signaling during cell differentiation. Our findings indicate that sodium arsenite specifically decreased Gli2 expression and transcriptional activity, thereby reducing the levels of several of its downstream targets. When additional recombinant SHH protein was added, SHH rescued arsenic’s inhibitory effects on cell differentiation. Taken together, our results indicate that arsenic inhibit cell differentiation into myotubes and neurons by inhibiting sonic hedgehog signaling.
Material and methods

P19 cell culture and differentiation

The mouse embryonal carcinoma P19 cell line (ATCC, Manassas, VA) was maintained in α-MEM supplemented with 7.5% bovine calf serum (Hyclone, Logan, UT), 2.5% fetal bovine serum (Mediatech, Manassas, VA), 1% L-glutamine, and 1% penicillin/streptomycin (designated as growth medium) at 37°C in a humidified incubator containing 5% CO₂. To induce differentiation, P19 cells were aggregated by the hanging drop method with some modifications (Wang and Yang, 2008). Briefly, P19 cells were trypsinized and suspended in growth medium containing 1% DMSO with 0, 0.25, or 0.5 μM sodium arsenite at a cell density of 500 cells/20μl or drop. Hanging drops were incubated for 2 days (day 2) to let cells undergo aggregation. After 2 days, each individual drop was transferred to a 96-well ultralow attachment plate containing fresh differentiation medium with or without sodium arsenite. After 3 days of culture (day 5), the embryoid bodies were transferred to a 0.1% gelatin coated 48-well plate containing fresh differentiation medium with or without sodium arsenite. Medium was then renewed every 48 hours until cells were harvested.

Developing stable Gli reporter gene transfectants

P19 cells were transfected with a Gli-luciferase lentiviral reporter (pCignal Lenti-TRE-Reporter) following supplier’s instructions (SABiosciences, CA) to develop a stably transfected cell line. Briefly, P19 cells were seeded in 24 well cell culture plate (2×10³ cells per well) for 1 day to avoid over confluency. Cells were then transfected with
Cignal lentiviral particles for 20 hours and recovered in normal growth medium afterward. To isolate stable transfectants, 2µg/mL puromycin was added into each well for a total of 14 days, while passaging the cells every two days. To assess arsenic’s effects on hedgehog signaling, the stable cells were plated at 5000 cells/well in a 12-well plate and exposed to 0, 0.25, or 0.5 µM sodium arsenite for 48 hours (n= 3). Additionally, the stably transfected cells were exposed to 0 or 0.5 µM arsenite while being differentiated into mature embryoid bodies via the hanging drop method as above (n= 3). Luciferase activity was measured using One-Glu luciferase assay kit (Promega, Madison, WI).

**qPCR**

After 2 or 5 days of exposure, ninety six aggregating embryoid bodies (1 plate) were collected as one replicate (n=3 per treatment group per day). Total RNA were extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO) and reverse transcribed using M-MLV Reverse Transcriptase (Promega) according to the manufacturer’s protocol. Real-time qPCR was conducted using RT² SYBR green master mix (Qiagen, Valencia, CA) and appropriate primers as shown in Table 2.1. A standard curve was generated via 5 concentration points (10⁻³ to 10⁻⁷ ng of cDNA) to quantify expression levels and assess reaction efficiency. Samples were run in triplicate and gene expression data was analyzed and normalized with the Gapdh housekeeper using the comparative threshold (Ct) method (Livak and Schmittgen, 2001).
**Immunoblotting**

Cells were treated with 0, 0.25, or 0.5 μM arsenite and harvested at days 2 and days 5 as described above (n=3 per group per day). Collected cells were placed in RIPA lysis buffer (Thermo Scientific, Waltham, MA) with protease and phosphatase inhibitors (Thermo Scientific). Cell culture medium was also collected from day 5 embryoid bodies to collect exosomes. Briefly, medium was passed through a 0.2μm filter and incubated with ExoQuick-TC isolation reagent overnight at 4ºC (Systems Biosciences, Mountain View, CA). Exosomes were isolated by centrifugation per manufacturer’s instructions and lysed in RIPA buffer with protease and phosphatase inhibitors. Protein concentrations were quantified using Bio-Rad’s DC protein assay kit. Immunoblots were performed according to standard methods. Dilutions for primary antibodies were as follows: anti-GLI2 (1:200, Santa Cruz, #SC-271786), anti-GSK3β (1:1000, Cell Signaling, #9315), anti-phospho-GSK3β (Ser9) (1:1000, Cell Signaling, #9323), anti-SHH (1:1000, Cell Signaling, #2207), and GAPDH (1:1000, IMGENEX, #IMG-5019A). Secondary antibodies and dilutions were as follows: goat anti-mouse-HRP (1:2000, Santa Cruz, #SC-2005) and goat anti-rabbit-HRP (1:2000, #SC-2004). The amount of protein was detected by chemiluminescence (Luminol, Santa Cruz) using BioRad’s Chemidoc imaging system. Average densitometry values were determined using BioRad’s Quantity One software and were normalized by GAPDH as loading control.
Immunohistochemistry

Differentiating P19 cells were exposed to 0, 0.25, and 0.5 μM sodium arsenite as described above. After 2 days and 5 days of cells aggregation, 96 embryoid bodies were harvested as one replicate (n=3 per day per group). Embryoid bodies were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Sections (5 μm) were placed on slides, deparaffinized and rehydrated in graded ethanol. Slides were blocked and incubated with specific primary antibodies for one hour (GLI2 (C-10) 1:400, Santa Cruz, #SC-271786) and incubated with secondary antibody conjugated to Alexa Flour 488 (Invitrogen, Carlsbad, CA). DAPI (Invitrogen) was used to stain nuclei. Immunofluorescence was measured on a Ti Eclipse Inverted Microscope (Nikon, Melville, NY). Overall fluorescence intensity was quantified and normalized with nuclear intensity using Image J to obtain the average GLI2 expression per cell (Barbachano et al., 2010). Relative quantification units were calculated after normalizing with the intensity values to the control groups. For the later stages of differentiating cells, embryoid bodies exposed to arsenic as described above were transferred to 0.1% gelatin coated glass chambers (n = 4 per treatment per group). Cells were cultured for 7 more days with the renewal of medium every two days. Cells were fixed with 4% of PFA at room temperature for 10 minutes, permeabilized and blocked with 0.2% Triton-X-100 and 1% BSA respectively. Cells were stained with the appropriate primary antibody for 1 hour (TUJ1 1:200, Millipore #MAB1637; NANOG 1:100, GeneTex #GTX100863; GLI2 1:100, Abcam #AB26056; Acetylated-Tubulin 1:5000, Sigma #T7451) and incubated
with Alexa Flour 488, 568 conjugated secondary antibody (Invitrogen, 1:2000) and DAPI. Fluorescence was measured via Nikon Ti Eclipse Inverted Microscope.

**SHH rescue**

SHH-N terminal peptide (1 or 2.5 µg/mL) (R&D System, Minneapolis, MN) was dissolved in growth medium and P19 cell differentiation was conducted via the standard method described above, with or without 0.5 µM sodium arsenite. After five days, aggregated embryoid bodies were transferred onto a 48 well 1% gelatin coated plate and cultured for additional 4 days. Eight individual differentiated embryoid bodies per treated group were imaged on day 9 (n=8 per concentration). The length and area of differentiating and proliferating cells was quantified using ImageJ. Stable GLI-luciferase reporter cells were plated at 5000 cells/well in a 12 -well plate and exposed to 0 or 0.5 µM sodium arsenite with or without 1µg/mL SHH-N terminal peptide for 24 hours (n= 3). Luciferase activity was measured using One-Glu luciferase assay kit.

**Statistical analysis**

Results are expressed as mean ± S.E.M. Data were analyzed by ANOVA followed by Tukey’s HSD Test or t-test, as appropriate. Statistical significance was achieved at \( p<0.05 \) (*) or \( p<0.01 \) (**).
**Results**

*Arsenic decreases Sonic hedgehog pathway related gene expression*

To determine what signaling pathways were involved in arsenic’s ability to reduce stem cell differentiation into skeletal myotubes and sensory neurons, we examined mRNA expression of key proteins in pathways known to be involved in cellular differentiation. P19 cells treated with 0, 0.25μM and 0.5μM of sodium arsenite were harvested at days 4 and 6 of differentiation. Key transcripts examined included *Bmp4*, *Fgf4*, *Fgf8*, *Hes1* expression for the Notch pathway, and *Shh* and *Gli2* for the Shh pathway. During the process of embryoid body formation, *Shh*, *Gli2*, and *Bmp4* expression increased by 2.5-, 6-, and 2.5-fold, respectively (Figures 2.1A-C), *Hes1* and *Fgf4* expression decreased by 3- and 8-fold respectively (Figures 2.1D and E), and *Fgf8* expression did not change (Figure 2.1F). Arsenic exposure reduced transcript levels of both *Shh* expression (2-fold) and *Gli2* expression (1.5-fold), respectively, during embryoid body formation (Figure 2.1A and B), but did not change the levels of any of the other transcription factors. To further examine Shh pathway related gene expression, P19 cells exposed to 0 or 0.5μM sodium arsenite were harvested at 2, 5, 7 and 9 days of differentiation. Transcript levels of *Shh*, the transcription factor *Gli2*, and three downstream genes: *Gli1*, *Ptch1*, and *Ascl1* were determined. Over the time of differentiation, both *Shh* and *Gli2* were significantly increased by 5 days of differentiation and expressed at high levels until day 9, supporting the role of Shh signaling in P19 cell differentiation. Arsenic exposure significantly reduced the expression of Shh and Gli2 transcripts by 2.5-fold and 3-fold, respectively, on day 5.
These reductions were maintained at days 7 and 9, indicating that the inhibitory effects of arsenic on cell differentiation are related to Shh signaling (Figure 2.2A and B). Three Gli2 downstream target genes were also examined. There was no difference in Gli1 and Ptch1 expression level when exposed to arsenic (Figure 2.2C and D). Gli1 expression was down regulated during early embryoid body formation and remained reduced throughout the differentiation period (Figure 2.2C). Ptch1 levels remained relatively constant from days 0-9 (Figure 2.2D), suggesting that it has only a minor role in cell differentiation. Ascl1 is another Gli2 downstream gene, and studies have shown that an increase in its expression can promote neuronal differentiation (Voronova et al., 2011). During P19 cell differentiation, Ascl1 expression was induced dramatically in controls cells, while arsenic exposure decreased Ascl1 expression by 2.5, 7, and 4-fold on days 5, 7, and 9, respectively (Figure 2.2E). Taken together, the data suggests that arsenic decreases Shh pathway gene expression, thus inhibiting cell differentiation.

*Arsenic exposure does not alter the levels of SHH expression nor GLI2 accumulation in the primary cilium.*

Since the genes whose expression was reduced after arsenic exposure were the Shh signal itself and the Gli2 transcription factor, we first examined SHH protein levels. Day 5 embryoid bodies exposed to 0 or 0.5 µM arsenic were collected along with the culture medium to assess secreted SHH levels contained in exosomal vesicles. In day 5 embryoid bodies, we were able to visualize both precursor and processed SHH, but arsenic exposure did not alter either one of their levels (Figure 2.3A). The secreted
precursor and processed SHH was at a much higher molecular weight, likely in the cholesterol bound form (Porter et al., 1996). However, its expression in the exosomes also remained constant between control and arsenic exposed cells (Figure 2.3B). Shh signaling activation causes ciliary accumulation of GLI2, resulting in GLI2 nuclear translocation, which further activates Shh signaling downstream genes (Chen et al., 2009; Kim et al., 2009). We investigated if arsenic inhibited GLI2 accumulation in primary cilium by differentiating P19 cells for 9 days in 0 or 0.5 µM arsenic, and co-staining them with the primary cilium marker acetylated-tubulin and GLI2. There is no difference between control and arsenic exposed cells (Figure S2.1).

**Arsenic decreases GLI2 protein levels**

GLI protein families are the key transcription factors in Shh signaling. Therefore, we determined whether GLI2 protein levels were altered after exposure to arsenic. GLI2 protein exists in a full length active (~160 kDa) form and in a repressor form (~78 kDa) after C-terminal truncation (Pan et al., 2006a). Western blot confirms that the active full-length form of GLI2 is decreased when exposed to arsenic in day 5 embryoid bodies (Figure 2.3C and D), although the antibody used cannot detect the truncated repressor form. We also examined GLI2 levels by immunohistochemistry. In day 2 embryoid bodies, GLI2 protein is expressed, but most of it remains outside the nucleus. There is also no difference in expression between control and arsenic exposed embryoid bodies (Figure 2.4). By day 5, the majority of GLI2 is translocated to the nuclei in both groups.
However, overall GLI2 expression is significantly reduced by 50% in the arsenic-exposed cells (Figure 2.4).

**Arsenic inhibits GLI activity**

Since arsenic reduced the levels of GLI2 protein, we wanted to determine if the activity of GLI2 was also inhibited after arsenic exposure. P19 cells were used to generate a stable transfectant containing the GLI protein binding element adjacent to a luciferase reporter gene. Cells were exposed to 0.25 and 0.5 µM of sodium arsenite for 48 hours prior to assessing activity. The results show that GLI activity was significantly decreased by 40% when exposed to 0.5 µM arsenite (Figure 2.5A). We further determined if GLI activity was inhibited during cell differentiation by forming embryoid bodies from the transfected cells. GLI activity was also reduced by 35% in the arsenic-exposed embryoid bodies, indicating that arsenic decreases Shh signaling activity when P19 cells are undergoing differentiation (Figure 2.5B).

**Arsenic does not disrupt GSK3β phosphorylation**

In order to generate truncated GLI2 protein, GSK3β phosphorylates GLI2, leading it to be partially processed by proteasomes (Aza-Blanc et al., 1997). GSK3β kinase activity can be inhibited by phosphorylation of its serine 9 site (Godemann et al., 1999). Therefore, we examined if arsenic disrupted GSK3β kinase activity by determining the level of total and phosphorylated GSK3β. However, there were no significant changes between control and arsenic treated day 5 embryoid bodies (Figure 2.6).
Induction of the Shh pathway rescues arsenic’s inhibitory effects on cell differentiation

To determine if stimulating the Shh pathway activity rescues the arsenic-mediated reduction in cell differentiation, 1 µg/mL of mouse recombinant SHH N-terminal domain protein was added into growth medium during cell differentiation and morphological changes were observed in the cells after 9 days of exposure. As shown in figure 2.7A, cells around the original embryoid bodies within the dashed line were defined as undifferentiated cells and were confirmed with NANOG staining (data not shown). The control group had less than 50% of undifferentiated cells and more spindle-shaped mesenchymal cells and myocytes that are migrating out from the embryoid bodies (Figure 2.7A). When cells were exposed to SHH only, neither migration distance nor percentage of differentiating cells changed, but there were more myocytes around the embryoid bodies. The cells exposed only to 0.5 µM arsenic had less differentiating cells and shorter migration distance. When cells were co-treated with 0.5 µM arsenic and SHH, there were more differentiated cells outside of the embryoid bodies and fewer undifferentiated cells compared to the arsenic-only group, indicating that SHH protein rescues the arsenic-induced inhibition on cell differentiation (Figure 2.7B).

In order to further confirm the rescue, P19 cells were co-treated with 2.5 µg/mL of recombinant SHH N-terminal domain protein and 0.5 µM of sodium arsenite during cell differentiation. After 12 days of differentiation, the number of neuronal cells was determined by stained with neuron specific marker TUJ1. The arsenic-treated cells have less neurons, but when cells are co-treated with SHH, many more neuronal cells are seen, suggesting that SHH attenuates the inhibitory effects caused by arsenic (Figure 2.7C).
To examine the mechanism by which SHH rescues the arsenic-induced inhibition of cellular differentiation, stable GLI protein binding element-luciferase cells were exposed to 0.5 μM arsenite with or without 1μg/mL SHH for 24 hours. GLI activity was significantly decreased by 20% when exposed to 0.5 μM arsenite (Figure 2.8). However, when the cells were co-incubated with arsenic and SHH, relative GLI activity was equivalent to the controls (Figure 2.8). This data suggests that the rescue of cellular differentiation by SHH is due to increases in GLI activity.

**Discussion:**

In the present study, the mechanisms by which arsenic disrupts during cell differentiation in P19 embryonic stem cells were examined, focusing on cell signaling pathways. Arsenic specifically reduces the expression of genes in the Shh signaling pathway, such as *Shh, Gli2* and *Ascl1*. GLI2 protein levels and transcriptional activity were also decreased by arsenic during cell differentiation. The inhibition of activity is not caused by changes in SHH protein levels, GLI2 nuclear translocation, GLI2 primary cilium accumulation, or GLI2 degradation. However, the arsenic-mediated reduction in cell differentiation can be recovered by treating the cells with additional recombinant SHH protein due to an increase in GLI activity. These results suggest that Shh signaling is a target of arsenic during cellular fate determination.

It has been previously shown that arsenic can inhibit cellular differentiation in P19 embryonic stem cells (Hong and Bain, 2012) as well as in human neuronal stem cells and rat cardiac myoblasts (Steffens *et al.*, 2011; Ivanov and Hei, 2013; Sumi *et al.*, 2013).
However, except for the P19 cells, these other cells are progenitor cells which have already been restricted to certain cell fates. After screening several growth signaling pathways including the Notch, Bmp, Fgf, and Shh pathways, arsenic exposure decreased the expression of Shh and its transcription factor Gli2 in P19 cells, but did not affect key proteins in the other pathways.

_Hes1_ is a bHLH transcriptional factor which is directly regulated by Notch signaling and has been shown to negatively regulate cell differentiation (Kageyama _et al._, 2005). Its expression was reduced during cell differentiation study, but was not altered by arsenic exposure. _Fgf4_ and _Fgf8_ signals are important growth factors during embryogenesis, with _Fgf4_ expression increasing early in embryogenesis and _Fgf8_ expression increasing slightly later (Wang _et al._, 2006b; Lanner and Rossant, 2010). _Bmp4_ belongs to the TGFβ superfamily, which is both critical to induce embryonic cell differentiation and is also upregulated during cardiomyocyte differentiation (Monzen _et al._, 1999; Bragdon _et al._, 2011). Our qPCR data of those genes shows patterns consistent with those studies above. Although the transcripts of these decreased and increased, respectively, during differentiation, their expression was again not impacted by arsenic.

The down-regulation of Shh signaling pathway was also examined over a longer time period. _Gli1_ and _Gli2_ are both transcription factors in Shh signaling, but their expression patterns are different based upon whether the cells are proliferating or whether they are differentiating. _Gli1_ expression is down regulated after forming embryoid bodies, indicating that is has a minor role in cell differentiation. This is similar to several studies in mice, in which _Gli2_ appears to be the primary transcriptional activator, while _Gli1_ is
not required for embryonic development (Mo et al., 1997; Ding et al., 1998; Bai et al., 2002). Indeed, in P19 cells, Gli2 is upregulated rather than Gli1 after 5 days of cell differentiation (Voronova et al., 2013), which is similar to what is seen in our study. Moreover, reports have shown that Gli2 regulates Pax3 and MyoD expression during skeletal and cardiac myogenesis (Petropoulos et al., 2004; Voronova et al., 2013). In our previous study, Pax3 and MyoD were down regulated when exposed to arsenic. Therefore, it is not surprising that arsenic only inhibits Gli2 expression. This is also similar to reductions in Gli2 transcripts in several cancer cell lines, leading to the inhibition of cellular growth (Nakamura et al., 2013; Yang et al., 2013). Ascl1 is a bHLH transcription factor that has been reported to convert embryonic fibroblasts to functional neurons and to induce neurogenesis in P19 cells (Farah et al., 2000; Vierbuchen et al., 2010), and is a target of Gli2 during Gli2-induced neurogenesis (Voronova et al., 2011). Ascl1 was also inhibited by arsenic in the same pattern as Gli2. Taken together, these results indicate that the arsenic-mediated inhibitory effect on cell differentiation is due to the inhibition of the Shh signaling pathway.

There are several potential protein targets of arsenic within the Shh pathway between the SHH ligand itself and the Gli2 transcription factor. In vertebrates, SHH protein is a morphogen that controls central nervous system and limb development in a tightly regulated dose- and time-dependent manner (Yang et al., 1997; Briscoe and Ericson, 2001; Ahn and Joyner, 2004; Harfe et al., 2004). The Shh gene has cis-regulatory elements that lie up to 1000kB upstream of its transcriptional start site, and it appears that each tissue has a means for intrinsic or self-regulation (Dessaud et al., 2007;
SHH protein is processed by cleavage, and the final product modified with cholesterol and palmitate moieties, as well as with heparin sulfate (Beachy et al., 2010). The modifications allow SHH protein to form multimeric complexes, to be anchored to the cell membrane, or to vesicle membranes, and the modifications also correlate with its signaling potency (Chen et al., 2004; Grover et al., 2011). Our results show that SHH protein expression is very robust in both the P19 cells themselves as well as in secreted, exosomal vesicles, although arsenic exposure does not alter SHH protein expression. This may be a reason why our SHH rescue required so much protein compared to other studies. In typical cell culture experiments, the concentration of additional, exogenous SHH required to induce neuronal and muscle differentiation from ES cells varies from 0.1 to 0.5 μg/mL (Dutton et al., 1999; Elia et al., 2007; Salero and Hatten, 2007). We exposed P19 cells to concentrations ranging from 0.2 to 2.5 μg/mL, but the SHH-mediated rescue only occurred at concentrations of at least 1 μg/mL.

PTCH1 is a transmembrane protein that is the receptor of SHH ligand and acts as a repressor in the absence of SHH signaling (Agren et al., 2004). However, Ptch1 expression can also be regulated in a Shh-independent manner (Agren et al., 2004). Our data indicates that Ptch1 expression is not significantly changed by arsenic and its expression does not change during the cell differentiation period. Along with PTCH1, other plasma membrane proteins can also bind to SHH. For example, Hedgehog interacting protein (HHIP) appears to compete directly with PTCH for SHH binding, resulting in an inhibition of SHH signaling activity (Chuang and McMahon, 1999). We
do not know whether HHIP levels are increased, but this may be a reason why SHH levels are not altered, but Shh signaling is reduced.

Shh signaling results in active GLI2 formation when GLI2 dissociates from the Suppressor of fused (SUFU) in the primary cilium due to the accumulation of Smoothened (SMO) (Murdoch and Copp, 2010). In the absence of SHH, SMO is prevented from entering the primary cilium, leading to GLI2 protein processing and degradation by the proteasome (Kim et al., 2009). Investigators have shown that in cancer cells, arsenic trioxide decreased the Hh-induced ciliary accumulation of GLI2 (Beauchamp et al., 2011). However, we co-stained differentiating day 9 cells exposed to arsenic with acetylated-tubulin, a primary cilium marker, and GLI2, and found no effect on the ciliary accumulation of GLI2 when exposed to arsenic (Figure S2.1).

In the primary cilium, GLI2 can be processed to a repressive form if its C-terminus is truncated (Sasaki et al., 1999). The antibody used in the GLI2 immunoblotting and immunohistochemistry assays recognizes amino acids 841-1140 of GLI2, which should identify only active GLI2. Whether it can identify the repressive form is unknown, but this is unlikely because the processing determinant domain (PDD) of GLI2 is between amino acids 648-915 (Pan and Wang, 2007) and, although processing of GLI2 into the truncated repressor form occurs in vivo, the repressor form is typically undetectable in cell culture conditions (Pan et al., 2006b). We can determine reductions in active GLI2 protein levels (Figure 2.3), but cannot visualize any cleaved repressive proteins on our blots. Phosphorylation of GLI2 by GSK3β is a critical step for GLI2 to be truncated into repressive form or cleaved by the proteasome. Our data shows no
inhibition of GSK3β kinase activity when exposed to arsenic, indicating that arsenic did not affect the GSK-mediated phosphorylation. However, more evidence is needed to determine whether arsenic targets other kinases, such as protein kinase A and casein kinase (Hui and Angers, 2011), thus increasing the phosphorylation and proteosomal degradation of GLI2 protein.

After active GLI2 is released from the primary cilium, it acts as a transcription factor. Our immunohistochemistry data indicates that GLI2 does not accumulate in the nucleus until day 5 of embryoid body formation, which indicates that the Shh signaling pathway starts between day 2 and 5. This supports our qPCR results that Gli2 and other downstream transcription factors have significantly higher expression after day 5. However, the relative nuclear translocation between control and arsenic exposed embryoid bodies were not changed. This indicates that nuclear translocation was not altered; only the total GLI2 level. But, this reduction in total GLI2 protein does correlate with the reductions in GLI2 activity seen in the reporter gene assays. In addition, exogenous SHH can rescue the loss of differentiation in P19 cells after arsenic exposure by increasing GLI activity.

Beyond the increase in GLI activity, there are several other mechanisms of regulating Shh pathway signaling. For example, we have previously published that β-catenin, a key transcription factor of Wnt signaling, is down regulated in P19 cells that are exposed to arsenic (Hong and Bain, 2012). It is reported that in P19 cells, short hairpin RNAs targeting β-catenin transfectants also decreased Gli2 expression during cell differentiation (Wong et al., 2013). Indeed, it is well-known that crosstalk exists between
the Wnt and Shh pathways (Winkler et al., 2014). Recent studies suggested that epigenetic regulation including DNA methylation, histone modification and microRNA (miRNAs) expression can be mediated by arsenic exposure (Ren et al., 2011). Indeed, arsenic is well-known to cause oxidative stress in cells, which can alter DNA and histone modifications (reviewed in Chervona and Costa, 2012). Gli2 expression can also be decreased by oxidative stress in both animal models and in cultured astrocytes (Xia et al., 2012; Ji et al., 2012). Studies have shown that microRNAs participate in development and cell differentiation through regulating growth related gene expression (Kim, 2005; Hou et al., 2012; Kawahara et al., 2012). Moreover, a recent study shows that miR-326 represses Shh signaling activity by directly targeting Gli2 expression in embryonic lung epithelium cells (Jiang et al., 2014). Taken together, arsenic-caused inhibitory effects on Shh signaling might also be due to changes in epigenetic regulation.

Results of the present study demonstrate that arsenic exposure reduces GLI2 transcript and protein expression and GLI transcriptional activity, thereby reducing P19 cell differentiation into neuronal and muscle cells. Although SHH levels are not changed, additional SHH ligand rescues the inhibitory effects caused by arsenic. We excluded other possible targets in Shh signaling such as GLI2 primary cilium accumulation, GLI2 nuclear translocation, and extracellular SHH expression. Our studies provide the insight into the mechanism by which arsenic alters during cell differentiation. However, additional targets of arsenic may still need to be elucidated.
Acknowledgements

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References


### Tables

#### Table 2.1. Primer sequences for quantitative PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td><em>Ascl1</em></td>
<td>ACTTGAACTCTATGGCGGGTT</td>
<td>CCAGTTGGTAAAGTCCAGCACG</td>
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<tr>
<td><em>Bmp4</em></td>
<td>AGAAGTCCGTCCGCCATTCACTAT</td>
<td>AGTTGAGGTGATCAGCCAGTGGAA</td>
</tr>
<tr>
<td><em>Fgf4</em></td>
<td>GGTGAGCATCTTCGGAGTG</td>
<td>GTAGGATTTCGTAGCCGGTGTGA</td>
</tr>
<tr>
<td><em>Fgf8</em></td>
<td>GCTCATTGTGGAGACCCGATAC</td>
<td>CCAGCAGATCTCTGTTGAATAC</td>
</tr>
<tr>
<td><em>Gli1</em></td>
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<td>AGCCCGCTTTTGTATTTTTGAG</td>
</tr>
<tr>
<td><em>Gli2</em></td>
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<td>TGGGCAGCCTCCATTCTGTTCAT</td>
</tr>
<tr>
<td><em>Hes1</em></td>
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<tr>
<td><em>Ptch1</em></td>
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<td>CTTCTCTATCTCGTACCGGGT</td>
</tr>
<tr>
<td><em>Shh</em></td>
<td>GCCAAGCAACCTGCTGAAAGTCTA</td>
<td>TTTACACTTTGCGGAAAAGCGAGA</td>
</tr>
</tbody>
</table>
Figure 2.1. Arsenic decreased hedgehog pathway gene expression during cell differentiation.

P19 cells treated with 0, 0.25μM and 0.5μM of sodium arsenite were harvested at days 4 and 6 of embryoid body formation (n=3 per day and concentration). The expression of key genes in differentiation signaling pathways were examined. Values were normalized with Gapdh as the housekeeping gene and average fold changes were compared to
undifferentiated P19 cells (day 0). Statistical differences were determined by Student’s t-test (*; \( p < 0.05 \)). Error bars represent ± SEM from three biological replicates.
Figure 2.2. P19 cells exposed to arsenic during differentiation repress hedgehog pathway related gene expression in a time-dependent manner.

RNA was extracted from differentiating P19 cells, with or without 0.5 μM sodium arsenite exposure, on days 0, 2, 5, 7, and 9. Transcript levels of sonic hedgehog signaling pathway genes (Gli1, Gli2, Ptch1, Shh, Ascl1) were determined by qPCR analysis (n=3 per group per day). Values were normalized to Gapdh and all expression fold changes were compared to day 0 control cells. Statistical differences were determined by Student’s t-test (*; p <0.05, **; p <0.01).
Figure 2.3. Arsenic reduces GLI2 protein levels, but not SHH levels.

P19 cells were induced to differentiate for 5 days while being exposed to 0, 0.25, or 0.5 µM arsenic. Protein was extracted from both the cells and secreted exosomes. SHH levels in the cells were assessed by immunoblotting (n=3 per treatment group) and were normalized using GAPDH (A). SHH levels in the exosomes were examined by immunoblotting (n=2 per group) (B). Full length GLI2 levels were also examined by immunoblotting, with results normalized to GAPDH (n=3 per group) (C). GLI2 protein levels were analyzed by densitometry, were averaged, and are expressed as the fold-change from control cells ± standard deviation (D). Statistical differences were determined by Student’s t-test (*, p<0.05).
Figure 2.4. Arsenic decreases GLI2 protein levels in day 5 embryoid bodies.

After exposure to 0 or 0.5 µM arsenic, day 2 and day 5 embryoid bodies were embedded in paraffin and GLI2 expression examined by immunofluorescence (A). Overall day 5 fluorescence intensity was quantified as integrated intensity per cell (n=5 slides per treatment group) (B). Results were normalized to the control group and statistical differences were determined by Student t-test (*; p<0.05).
**Figure 2.5. Arsenic decreases GLI reporter activity in P19 cells.**

P19 cells were stably transfected with a GLI binding element-luciferase plasmid. Stable transfectants were then exposed to 0, 0.25, and 0.5 µM sodium arsenite for 48 hours and luciferase activity was assessed by luminometry (A). The stable strain was also differentiated into embryoid bodies for 5 days while being exposed to 0 or 0.5 µM arsenite, and luciferase activity measured (B). Luminesce values were averaged, normalized to the controls, and are expressed as a relative reporter activity value ± SEM (n=3 per concentration). Statistical differences were determined by ANOVA analysis followed by Tukey’s HSD test or by Student’s t-test (*; p<0.05).
Figure 2.6. Arsenic exposure does not affect GSK3β phosphorylation.

Days 5 embryoid bodies were exposed to 0, 0.25 and 0.5 µM of arsenic and were harvested for protein extraction. GLI2, GSK3β and phospho-GSK3β (Ser9) expression was examined by immunoblotting. Shown is a representative blot from 3 replicates (n=3).
Figure 2.7. Additional Shh rescues arsenic’s inhibitory effects on cell differentiation.
P19 cells were aggregated via hanging drops with or without 1μg/mL recombinant SHH protein and 0.5 μM of sodium arsenite. Embryoid bodies were transferred to 0.1% gelatin coated plates after 5 days and were differentiated for an additional 4 days to observe morphological changes. The embryoid body is pictured in the top right corner, while the dashed line delineates the boundary between differentiated and undifferentiated cells (confirmed by NANOG expression). The longest distance of migration is shown as a green bar from a representative image (A). The percentage of undifferentiated cells compared to total migration area was calculated for each group, and statistical differences were determined by ANOVA followed by Tukey’s (*; p<0.05). Error bars represent ± S.D. from ten replicates (n=10) (B). P19 cells were differentiated for 12 days with or without 2.5 μg/mL recombinant SHH protein and 0.5 μM sodium arsenite. Cells were fixed on day 12 and TUJ1 expression was examined by immunofluorescence (C).
Figure 2.8. Shh rescues GLI activity after arsenic exposure.

GLI-luciferase transfected P19 cells were exposed to 0 or 0.5 µM arsenite with or without 1 µg/mL recombinant Shh protein for 24 hours. Luciferase activity was assessed by luminometry. Luminescence values were averaged, normalized to the controls, and are expressed as a relative reporter activity value ± SEM (n=3 per concentration). Statistical differences were determined by ANOVA analysis followed by Tukey’s HSD test (*; p<0.05).
Figure S2.1. Arsenic does not affect ciliary accumulation of GLI2 during cell differentiation.

P19 cells were aggregated through handing drop method in the presence or absence of 0.5 µM sodium arsenite for 9 days. Cells were fixed and stained to visualize GLI2, acetylated-tubulin, and DAPI (A). The levels of ciliary GLI2 were quantified from immunofluorescence images (n=10). Error bars indicate S.D. (B).
CHAPTER THREE

ARSENIC INDUCES MEMBERS OF THE MIR-466-669 CLUSTER TO REPRESS

P19 CELL DIFFERENTIATION

This manuscript will be submitted to *Toxicological Sciences* and is in the format required of that journal.
Abstract:

Humans can be exposed to arsenic via drinking water, rice, and fruits grown in areas with naturally-occurring arsenic or in areas with historical pesticide use. Chronic arsenic exposure can result in adverse development effects including decreased intellectual function, reduced birth weight, and altered locomotor activity. Previous in vitro studies have shown that arsenic inhibits stem cell differentiation. MicroRNAs (miRNAs or miRs) are small non-coding RNAs involved in multiple cellular processes including embryonic development and cell differentiation. The purpose of this study was to examine whether altered miRNA expression was a mechanism by which arsenic inhibited cellular differentiation. P19 embryonic stem cells were exposed to 0 or 0.5 μM sodium arsenite for 9 days during cell differentiation, and miRNA microarrays were analyzed for changes. We found that several developmental-related miRNAs, such as miR-9 and miR-199 were decreased by 1.9- and 1.6-fold respectively, while miR-92a, miR-291a, and miR-709 were increased by 3-, 3.7- and 1.6-fold, respectively. The members of the miR-466-669 cluster and its host gene, Sfmbt2, were significantly induced by arsenic from 1.5- to 4-fold in a time-dependent manner. This induction appears to be specific to stem cells, because changes in miR-466-669 members were not found in more differentiated cell lines, including neuroblast and myoblast cells. When consensus sequences targeting the whole miR-466-669 cluster were co-exposed to P19 cells, this rescued the inhibitory effects of arsenic on cell differentiation. Taken together, we conclude that arsenic induced the miR-466-669 cluster specifically in embryonic stem cells, which acts to inhibit cellular differentiation.
Keywords: arsenic, microRNA, Sfmbt2, miR-466-669 cluster, P19 cells, differentiation, myoblast, neuroblast
Introduction:

Arsenic is a ubiquitous element that is widely distributed in the bedrock, sediments and soils, and can leach into ground water and surface water (Amini et al., 2008). Humans can be easily exposed to arsenic via drinking water from contaminated wells (ATSDR, 2007). Studies have shown that residents are exposed to high level of arsenic in their drinking water in countries such as China, Argentina, Taiwan, Bangladesh and the USA (ATSDR, 2007; Blanes et al., 2011; Chen et al., 1994; Nickson et al., 1998; Ning et al., 2007). Both the World Health Organization (WHO) and U.S. Environmental Protection Agency (USEPA) have set 10 ppb arsenic as the drinking water standard (WHO, 2004). Other than drinking water, humans can also be exposed to arsenic by consuming crops, such as rice, that are grown in soil with former arsenic pesticide use (Zavala et al., 2008).

Arsenic can cross the placental barrier such that arsenic concentrations in cord blood are almost as high as in maternal blood (Concha et al., 1998). Therefore, children can be affected by arsenic during pregnancy. Epidemiological studies have shown that arsenic can increase the rates of spontaneous abortion and stillbirth with increasing maternal arsenic exposure (Quansah et al., 2015; Rahman et al., 2010). Maternal arsenic exposure in pregnancy has also been found to reduce newborn birth weight by an average of 57g (Hopenhayn et al., 2003; Huyck et al., 2007; Rahman et al., 2009). Moreover, data from more than 20 epidemiological studies of children 7 months old to 15 years old exposed to arsenic levels > 10 ppb show that arsenic reduces verbal IQ, spatial memory,
and long-term memory, indicating that arsenic can also affect intellectual function during development (Tolins et al., 2014).

Several in vitro studies have shown that arsenic impacts cell differentiation. For example, exposing C2C12 myoblasts to 0.1 μM arsenic trioxide or 20nM sodium arsenite inhibits myotube formation (Yen et al., 2010; Steffens et al., 2011). Arsenite reduced muscle cell differentiation by down-regulating the expression of transcription factors involved in myotube formation, such as MyoD and myogenin (Steffens et al., 2011). Arsenic also disrupts neuronal differentiation and neurite outgrowth in both PC12 and Neuro-2a neuroblastoma cells (Frankel et al., 2009; Wang et al., 2010). Our previous work demonstrates that arsenic inhibits the differentiation of mouse P19 embryonic stem cells into myotubes and sensory neurons by repressing the expression and nuclear translocation of β-catenin, and reducing the expression and activity of Gli2, a key transcription factor in the sonic hedgehog signaling pathway (Hong and Bain, 2012; Liu and Bain, 2014). However, the mechanism by which arsenic reduces transcription factor expression is not well understood.

One potential mechanism for altering transcript and protein expression is through the use of microRNAs (miRNAs). miRNAs are small, single stranded, non-coding RNAs that regulate gene expression by binding imperfectly to the 3’ untranslated region (UTR) of mRNA, leading to targeted mRNA translational repression (Yang et al., 2005). MicroRNAs can be located in introns or outside of genes (Rodriguez et al., 2004). If several miRNAs are clustered together, they can be transcribed as a polycistronic transcript. For instance, the miR-17-92 cluster is found within an intron of C12orf25 and
it contains six miRNAs within about 1kb, including miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1 (Ota et al., 2004; Tanzer and Stadler, 2004). The miR-290 cluster is a 2.2-kb region on chromosome 7, and the primary transcript generates 14 mature miRNAs which play an important role in embryonic stem cells (Judson et al., 2009). The miR-466 cluster is located in intron 10 of the mouse Sfmbt2 gene and contains 65 miRNA genes (Lehnert et al., 2011). There is little information reported on this cluster, other than it is involved in apoptosis and osmoregulation (Druz et al., 2011; Luo et al., 2014).

Because of their ability to regulate gene expression, microRNAs play an important role in development. In mice, deletion of the Dicer-1 gene, which cleaves pre-miRNA to produce mature miRNAs, can result in embryonic lethality (Bernstein et al., 2003). Moreover, mouse embryonic stem cells which lack Dicer-1 have significant defects in cell differentiation (Kanellopoulou et al., 2005). Several miRNAs impact stem cell differentiation into skeletal muscle and neurons. For instance, miR-1 and miR-133 are regulated by the transcription factors MyoD and MEF2 to target Hand2 and serum response factor (SRF), respectively (Liu et al., 2007; Zhao et al., 2005). miR-1 and miR-133 promote mesoderm formation and are potent repressors of nonmuscle gene during cell lineage commitment (Ivey et al., 2008). miR-124 and miR-9 are both critical for neurogenesis and are both highly expressed in the brain (Cheng et al., 2009; Shibata et al., 2011). Several neurogenic transcription factors, including Foxg1, Sox9, and Pax6, have been identified as targets of miR-9 and miR-124 (Akerblom et al., 2012; Clark et al., 2010; Otaegi et al., 2011; Shibata et al., 2011).
Compared to other epigenetic modification, the effects of arsenic on miRNA regulation are relatively unexplored. Arsenic trioxide (ATO) exposure to liver cancer cells increased miR-29a expression, which inhibited cell growth and induced apoptosis (Meng et al., 2011). ATO also alters several miRNAs in acute promyelocytic leukemia cells; with the up-regulated miRNAs related to tumor suppressor functions and the down-regulated miRNAs related to angiogenesis, invasion, and metastasis (Ghaffari et al., 2012). Beyond arsenic trioxide in cancer cells, only a few other studies indicate that inorganic arsenic can alter miRNAs levels. For example, arsenite exposure can induce angiogenesis by decreasing miR-9 and miR-181b expression in a human umbilical vein cell line (Cui et al., 2012). Arsenic also induced miR-190 expression in human bronchial epithelial cells, thus enhancing cell proliferation and carcinogenic transformation by targeting the tumor suppressor gene PHLPP (Beezhold et al., 2011). Similarly, arsenite exposure in human bronchial epithelial cells caused malignant transformation by reducing the level of miR-200 family members, (Wang et al., 2011). Since increasing evidence indicates that miRNAs are important in development, cellular differentiation, and that arsenic exposure can alter miRNAs levels, we hypothesized that arsenic could alter miRNA expression during cell differentiation, leading to the disruption of differentiation process.

In the present study, we exposed mouse P19 embryonic stem cells to arsenite during cell differentiation and determined that several miRNAs were differentially expressed. Interestingly, the miRNA-466-669 cluster was significantly increased along with its host gene Sfmbt2 following arsenic exposure. This induction appears to be an
early cell lineage response, as the levels of miR-466/467/669 did not change in myoblasts and neuroblasts. In addition, when the miR-466-669 cluster was inhibited by anti-miRs, this rescued the inhibitory effects on differentiation mediated by arsenic. These results indicate that miR-466-669 cluster is highly expressed when exposed to arsenic, which leads to the inhibition of stem cell differentiation.

**Material and Methods**

**Cell culture and differentiation**

Mouse embryonal carcinoma P19 cells (ATCC, Manassas, VA) were cultured in MEM Alpha medium (Hyclone, Logan, UT) with 7.5% bovine calf serum (Hyclone), 2.5% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 1% L-glutamine, and 1% penicillin/streptomycin (designated as growth medium). Cells were maintained in a humidified incubator containing 5% CO$_2$ at 37$^\circ$C. Embryoid bodies were formed and the cells were differentiated for up to 9 days using the hanging drop method as described previously (Liu and Bain, 2014).

Neuro2a neuroblastoma cells were cultured in DMEM medium (Hyclone, Logan, UT) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 1% L-glutamine, and 1% penicillin/streptomycin (designated as growth medium). To induce differentiation, growth medium was replaced with DMEM medium containing 2% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin and 20 μM all-trans retinoid acid (designated as differentiation medium) (Frankel et al., 2009). Cells were exposed to 0, 1, or 2.5 μM arsenite during this process. The mouse myoblast C2C12 cell line (ATCC,
Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin (growth media). To differentiate the cells, the medium was replaced with DMEM supplemented with 2% horse serum, 1% L-glutamine, and 1% penicillin/streptomycin (differentiation media) (Steffens et al., 2011). Cells were exposed to 0 or 20nM arsenite during this process.

miRNA microarrays

P19 cells were exposed to 0 or 0.5 μM arsenic as sodium arsenite and induced to differentiate for 9 days (n=3). Cells were harvested, and total RNA was extracted with TRIzol (Life Technologies, Grand Island, NY) with extra ethanol cleaning step to improve the quality of RNA used. Three replicates samples were pooled together equally and hybridized onto two LC Sciences murine miRNA microarray chips (Houston, TX) per exposure group. The array included probes for all available mature mouse miRNAs (miRBase database, Release 21, July 2014). Fluorescence intensity was corrected by background subtraction and normalized with the statistical mean of all detectable transcripts. Intensity values for the control and 0.5 μM arsenic-exposed cells were averaged, and statistical differences assessed using a Student’s t-test.

miRNA reverse transcription and miRNA specific qPCR

Total RNA (1 μg) was used to reverse transcribe miRNA (QuantiMir RT kit, System Biosciences, Mountain View, CA). Quantitative real-time PCR was performed with RT² SYBR green master mix (Qiagen, Valencia, CA) using adapter-specific reverse
primers. Gene specific miRNA forward primers are listed in Table 3.1. A standard curve was generated by serial dilution to determine PCR efficiency. Samples were run in triplicate and the expression data was analyzed and normalized with U6 as a housekeeper using the comparative threshold (Ct) method (Livak and Schmittgen, 2001).

**Real-time quantitative PCR**

P19 cells were exposed to 0 or 0.5μM arsenic, induced to differentiate, and collected at day 0, 2, 5, and 9 (n=3 per treatment group per day). Total RNA were extracted using TRizol, and 2μg RNA were reverse transcribed using M-MLV reverse transcriptase (Promega). Real-time qPCR was conducted using RT² SYBR green (Qiagen) and gene-specific primers (Table 3.1). A standard curve was generated with 5 concentration points (10⁻³ to 10⁻⁷ ng cDNA) to quantify expression levels and assess reaction efficiency. Samples were run in triplicate and gene expression data was analyzed and normalized with the Gapdh housekeeper using the comparative threshold (Ct) method.

**miRNA inhibition assay**

Four consensus sequences which target the miR-466-669 cluster were selected for anti-miRNA generation (Figure 3.4). The oligonucleotides (Integrated DNA Technologies, Coralville, Iowa) were modified by employing 2'-O-methyl RNA nucleotides linkages positioned at both ends to block exonuclease attack (Lennox et al., 2013). P19 cells were transfected with 100 nM miRNA inhibitor using Lipofectamine
2000 (Invitrogen), and allowed to differentiate as above for 5 days with or without 0.5μM sodium arsenite. At day 5, the embryoid bodies were transferred into gelatin coated plate and cultured for 4 more days without additional anti-miR.

**Chromatin immunoprecipitation (ChIP) assays**

P19 cells were induced by aggregation with or without 0.5 µM sodium arsenite as described above and were harvested at differentiation day 5 (n=3). Chromatin was extracted and sonicated using a Vibra Cell probe (Sonic and Materials Inc, Danbury, CT) using 25% power output for 450 seconds and run on a gel to ensure appropriate fragmentation. Both chromatin extractions and immunoprecipitations were performed according to standard protocols (Abcam Inc., Cambridge, MA). The antibodies used for ChIP assays were anti-H3K9 Ac (ab4441, Abcam) and H3K9 Me2 (ab1220, Abcam). Normal mouse IgG (sc-2025, Santa Cruz) was used as a negative control. Quantification of precipitated DNA was performed by qPCR using SYBR green with miR-466-669 cluster and Sfmbt2 promoter-specific primers (Table 3.1). Percentage of input method was used to normalize threshold (Ct) values with input samples [100*2^(Ct (Adjusted input) - Ct (IP))].

**Statistical analysis**

Results are expressed as mean ± S.E.M. Data were analyzed by ANOVA followed by Tukey’s or Student’s t-test, as appropriate. Statistical significance was achieved at p<0.05 (*) or p<0.01 (**).
Results

Arsenic alters the microRNA expression profile during P19 cell differentiation

A murine microRNA microarray containing 1900 mature transcripts (miRBase Release 21.0) was used to determine which miRNAs were altered by arsenic after 9 days of P19 cell differentiation. A total of 59 miRNAs were significantly changed, with 27 miRNAs decreased and 32 miRNAs increased, in the arsenic-exposed cells (Figure 3.1). Several of these miRNAs are known to impact development and stem cell differentiation (Table 3.2). miR-92a is highly expressed in stem cells and its expression decreases when cells undergo differentiation. miR-291a belongs to the miR-290~295 cluster, which promotes pluripotency and prevents cells from differentiating. miR-709 inhibits adipocyte differentiation by targeting the GSK3β/Wnt signaling pathway. Arsenic exposure to P19 cells increased these transcripts, which is consistent with the repression of differentiation (Figure 3.1). These changes were validated by miRNA qPCR, in which miR-92a, miR-291a, and miR-709 were all significantly induced in arsenic treated cells by 3-, 3.5-, and 1.6-fold, respectively (Figure 3.2 A,B,C). In contrast, miR-9 induces neuronal cell differentiation and miR-199a increases chondrogenesis in the skeletal system (Table 3.2). qPCR demonstrated that miR-9 and miR-199a were significantly reduced by 2-and 2.5-fold, respectively (Figure 3.2 D,E). These data suggest that during cell differentiation, arsenic exposure increases miRNAs associated with pluripotency and decreases those associated with cell lineage commitment.
**Arsenic induced miR-466-669 cluster expression**

Clustered microRNAs are usually co-expressed, and several studies have shown that they have a small but significant tendency to co-target the same genes and therefore jointly regulate specific molecular pathways (Hausser and Zavolan, 2014). As shown in Table 3.3, seven miRs in the miR-466/467/669 cluster were significantly induced in the arsenic-exposed cells, and five of them are increased by more than two-fold. To verify the expression of these clustered miRNAs, qPCR was used. All the miRNA 466/467/669 family members are significantly induced in the arsenic exposed cells from 1.7- to 3.7-fold, except miR-466i (Figure 3.3A). Among those transcripts, miR-466g, -467d, -467a and -669p have more than 3-fold increase in day 9 cells exposed to arsenic.

**Arsenic induced the miR-466-669 cluster and its host gene, sfmbt2, in a time-dependent manner.**

The miR-466/467/669 cluster is located on chromosome 2 within the 10th intron of the Sfmbt2 gene. Thus, the expression of the host gene was also assessed by qPCR, to see if co-regulation of Sfmbt2 and the miR-466-669 cluster existed. Indeed, on day 9, Sfmbt2 expression is induced 2-fold in the arsenic-exposed cells (Figure 3.3B). We further wanted to determine if the miRNA cluster and host gene expression patterns were similar during embryoid body formation and differentiation, so transcript levels from day 0, 2, 5 and 9 were analyzed by real-time PCR. The levels of miR-467d, miR-669p, and Sfmbt2 were dramatically induced by 11-, 40-, and 55-fold, respectively on day 2 of embryoid body formation as compared to the day 0 cells (Figure 3.3 C,D,E). As time
goes on, expression of all three transcripts decreases in the control cells, while the levels remain higher and decline more slowly in the arsenic-exposed cells. At all time points, the cells exposed to arsenic have significantly increased transcript expression of miR-467d, miR-669p, and Sfmbt2. For example, at day 5, the highest point of transcript expression, miR-467d, miR-669p and sfmbt2 were induced by arsenic by 3-, 3- and 2-fold, respectively (Figure 3.3C,D,E). Sfmbt2 and miR-467d and miR-669p showed the similar pattern of expression, indicating that Smbt2 induction likely drives the expression of this miRNA cluster.

**Induction of miR-466-669 cluster is specific to stem cells**

In our model of differentiation, P19 stem cells can differentiate into neurons and skeletal myotubes. Thus, we sought to determine if the miR-466-669 cluster played a role in specific cell lineages. We evaluated the expression of this miRNA cluster in the C2C12 myoblast cell line, which can be induced to differentiate into skeletal myotubes and in Neuro2A neuroblast cells, which can differentiate into neurons. Our lab has previously shown that 20nM arsenite inhibits the differentiation of C2C12 cells (Steffens et al., 2011). Neuro2A cells exposed to 1µM and 2.5µM of arsenite during differentiation also show decreased neuron formation (Figure S3.1), which consistent with other studies (Wang et al., 2010). Both cell lines were exposed to arsenic in either growth medium or differentiation medium, and RNA was extracted. miR-466g, -467d, and -669p expression did not change when the cells were undergoing differentiation (Figure 3.4A,C) nor when they were simply proliferating (Figure 3.4B,D). Likewise, the
levels of these miRs did not change when the cells were exposed to arsenic (Figure 3.4). These results suggest that the induction of miR-466/467/669 cluster expression controls lineage commitment earlier in the cell differentiation process.

**miR466-669 cluster members share multiple consensus sequences**

In order to determine sequence similarity, we used all mature miRNA sequences within this cluster from the updated miRBase Sequence Database (version 21) to perform multiple sequence alignment by Clustal Omega. The cluster sequences included 26 mature miRNA sequences in the miR-466 family, 13 sequences in the miR-467 family, and 26 sequences in the miR-669 family. A total of 24 miRNA-3p members have high degree of similarity, as shown in its consensus sequence (Figure 3.5A). This first consensus sequence is modified from another study which aligned all miRNA-3p members together (Luo *et al.*, 2014). Interestingly, 15 of the -3p members share the seed sequence, UAUACAU, even those are in different subfamilies (Figure 3.5A). The miRNAs-5p members clustered together, but did not have the same degree of similarity to each other. So these were subdivided into 3 additional clusters with miR-466 (h-5p/j/m-5p) grouping together (Figure 3.5B), miR-467(c/e/g/d/a/b-5p) grouping together (Figure 3.5C), and miR-669 (c/n/e/b/l/d/a/l/o-5p) grouping together (Figure 3.5D). These sequence similarities suggest that these miRNA families may work together and target similar transcripts.
Inhibition of the miR-466-669 cluster rescues the inhibitory effects of arsenic on stem cell differentiation

To evaluate the effects of the miR-466-669 families on stem cell differentiation and its inhibition by arsenic, consensus anti-miRs targeting consensus sequence 1, 2, 3, and 4 (Figure 3.5) were added during cell differentiation from day 0 to day 5, when cells were cultured with or without 0.5 μM arsenic. A portion of day 5 embryoid bodies were harvested for marker gene determination and the rest were grown out to day 9 to observe morphological changes. The 4 individual consensus sequence anti-miRs did not rescue the arsenic-mediated reduction in cellular differentiation (Figure 3.6C-J), as few neurons and myoblasts were observed. The lack of morphological rescue was confirmed by the lack of Pax3 expression changes (Figure 3.7 A-B). However, when cells were transfected with all four consensus inhibitors, there was a rescue effect, in that cells exposed to arsenic differentiated into neuronal and muscle cells (Figure 3.6L). Pax3 expression was also recovered by the anti-miR mix (Figure 3.7C).

In order to verify if miRNAs were also knocked down by inhibitors, we designed primers for these consensus sequences. As shown in figure 3.7, only consensus sequence-1 (Figure 3.7D) and 3 (Figure 3.7F) are significantly induced by arsenic, indicating that only those two subfamilies are involved in arsenic-induced effects. Notably, the induced expression by arsenic was knocked down to the unexposed level when treated with inhibitors (Figure 3.7D, F). To determine if the inhibitory effects are due to the miRNAs themselves or their host gene, we collected day 5 transfected cells and determined Sfmbt2 expression by qPCR. Arsenic induced Smbt2 expression whether or not the anti-miRs
were added, suggesting that the host gene itself is not involved in the differentiation process nor is its expression inhibited by the anti-miRs (Figure 3.7H).

Discussion

The present study demonstrates that arsenic can alter miRNA expression, including miR-9, miR-92a, miR-199a, miR-291a, miR-709, and the miR-466-669 cluster, which leads to the repression of P19 embryonic stem cell differentiation. This study highlights the role microRNAs play in arsenic-mediated repression of stem cell differentiation and ascribes a novel function for the miR-466-669 cluster in embryonic stem cells.

Several studies report that arsenic alters miRNA expression. For example, exposure of bladder or liver cell lines to 5 μM arsenic trioxide (ATO) can reduce miR-19a and miR-29a expression, which inhibits cell proliferation and induces apoptosis (Cao et al., 2011; Meng et al., 2011). Neither one of these miRs is expressed in our embryonic stem cells. Chronic exposed to 1μM sodium arsenite for 26 weeks induced transformation in lung epithelial cells and miR-199a was significantly reduced in transformed cells, suggesting the roles of miR-199a in carcinogenesis (He et al., 2014). Interestingly, the reduction of miR-199a is consistent with our expression data, which may indicate that miR-199a expression is simply be indicative of arsenic exposure or of a de-differentiation process of the lung cells. Exposure of chick embryos to 100 nM arsenite reduced miR-9 and miR-181b, which target neuropilin-1(Nrp1) to induce angiogenesis (Cui et al., 2012). According to our microarray data, miR-181b levels are
fairly high and are reduced slightly by arsenic, but this is not statistically different. As measured by the arrays and by qPCR, miR-9 is significantly reduced by 2- to 3-fold in arsenic exposed embryoid bodies. miR-9 is known to be induced during neural lineage differentiation, and its inhibition blocks neurogenesis in embryonic stem cells (Krichevsky et al., 2006). This indicates that miR-9 plays an important role during embryonic development and reduction by arsenic might be involved in the inhibitory effects seen in our study.

miRNAs can regulate specific gene expression during different development stages and in different cell types. In mouse embryonic stem cells, miR-134, miR-296 and miR-470 are induced during differentiation because they target three critical transcription factors, Nanog, Oct-4 and Sox2, which are downregulated when cells undergo differentiation (Tay et al., 2008). In our day 9 cells, miR-470 is not expressed, and while the levels of miR-134 and miR-296 are not changed when exposed to arsenic, they are only expressed at low levels. Because we plate the embryoid body on day 5 and allow cells to proliferate and differentiate away from it during the next 4 days of differentiation, part of the initial embryoid body, consisting of undifferentiated cells, remains. Indeed, we can still see the stem cell marker Nanog being expressed in the center of the embryoid body (Hong and Bain, 2012). While we definitely see changes in miR expression, the levels of transcript may be diluted out because of the mixed population of cells.

During skeletal muscle formation, many miRNAs control the core myogenic transcriptional network, including miR-1, -27, -133, -206, -208, and -486 (Braun and Gautel, 2011). Increased expression of miR-1, -27b, and -206 can bind to and inhibit
Pax3 expression in myoblast cells (Crist et al., 2009). In our day 9 cells, miR-1 is not expressed and miR-206 is expressed, but at very low levels. miR-27b is increased by 1.4-fold (p=0.1), which may be suggestive of Pax3 inhibition. Because these miRs were discovered in myoblast cells, the lack of concordance may suggest that these miRs regulate further stages of differentiation, from myoblasts to myotubes. The miR-290 cluster is important in maintaining stem cell pluripotency and inhibiting embryonic stem cell differentiation (Sinkkonen et al., 2008). Interestingly, several members of miRNA-290 cluster were induced by arsenic in our microarray, including a 4-fold induction of miR-291a and miR-291b, a 2-fold induction of miR-290a, a 2.8-fold induction of miR-292a, and a 4.7-fold fold induction of miR-294. We further determined the time-dependent expression of the most significantly induced family member, miR-291a by qPCR. As the cells were undergoing differentiation, miR-291a levels decreased by 3.2-fold on day 5 and 4-fold on day 9. However, arsenic exposure kept miR-291a at nearly the same levels as in day 0 stem cells (Figure S3.2). This indicates that cellular pluripotency is maintained when cells are exposed to arsenic. However, the exact mechanisms by which arsenic can induce the miR-290 cluster expression still remains to be uncovered.

The miRNA 466-669 cluster is one of the largest miRNA clusters in mice and rats, and is located in the 10th intron of the Sfmbt2 gene, a polycomb group protein (Lehnert et al., 2011). There are few investigations on the function of this cluster. miR-466h is induced in Chinese hamster ovary cells treated with nutrient-depleted media, which induces apoptosis (Druz et al., 2011). Another study demonstrated that miR-466h
is increased due to the downregulation of HDAC activity in its promoter region, caused by low glucose-derived oxidative stress (Druz et al., 2012). miR-467a can promote the growth and survival of mouse ES cells by targeting Lats2 and Dedd2, which are well conserved inducers of apoptosis (Zheng et al., 2011). These investigators predicted a number of genes that were targets for the entire cluster (Zheng et al., 2011). We examined several of their top predicted genes, ones that are related to cell differentiation pathways and histone modification, including Hdac4, Myst4, Hes6, Fzd1, and Fzd7, in day 5 and day 9 arsenic-treated P19 cells by qPCR. However, none of them were significantly changed by arsenic (data not shown), nor were the expression of Hdac4 and Myst4 altered when the cells were exposed to miR-466-669 cluster inhibitor (data not shown). These results suggest that arsenic does not target these genes, nor do specific clusters members target Hdac4 or Myst4.

Additionally, when mouse medullary epithelial cells were exposed to high sodium chloride levels, the increased levels of miR-466(a/e)-3p bound to and down-regulated renal Nfat5, an osmoreponsive transcription factor (Luo et al., 2014). Although arsenic exposure has not been associated with hypertonic stress, arsenic has been reported to inhibit histone deacetylase activity (Ramirez et al., 2008). Therefore, arsenic’s effects on the miR-466-669 cluster might be due to epigenetic regulation. We did examine H3K9 acetyl and dimethyl marks on several regulatory regions of the cluster. However, there is no detectable acetylation and arsenic exposure did not alter H3K9 methylation (Figure S3.3). These results suggest that the induction of the miR-466-669 cluster by arsenic is not due to histone modification on its promoter region.
In a previous study, we determined that arsenic inhibited the Shh signaling pathway by decreasing Gli2 expression and activity in P19 cells (Liu and Bain, 2014). Thus, Gli2 transcript levels were examined cells exposed to anti-miRs targeting the miR-466-669 cluster exposed with or without arsenic. Interestingly, Gli2 expression was increased almost back to control levels when the miR-466-669 cluster was inhibited (Figure S3.4). There is no evidence showing that this cluster directly target Gli2 gene or decrease Gli2 activity. By comparing the consensus sequence and Gli2 mRNA 3’UTR region and based on the algorithm from Zheng’s study, Gli2 is not the predicted target of miR-466-669 cluster (Zheng et al., 2011). However, Gli2 expression is regulated by other signaling pathways, including TGF-β and Wnt (Borycki et al., 2000; Dennler et al., 2007). Based on the prediction algorithm from Zheng’s study, Wnt4 has been predicted as the target of miR-466-669 cluster (Zheng et al., 2011). This suggests that miR-466-669 might act upstream of Shh signaling pathway, although more studies are needed to see whether this signaling is regulated directly or indirectly by the cluster.

Collectively, our results indicate that low-level arsenic exposure to P19 embryonic stem cells during their differentiation alters the expression profile of miRNAs, including several development related miRNAs. Specifically, members of miRNA-466-669 cluster were significantly induced by arsenic, but this induction did not occur in myoblast or neuroblast cells. The host gene Sfmbt2 was also induced by arsenic a time-dependent manner, which is consistent with the cluster expression. However the acetylation and methylation status on its promoter region is not changed, indicating that induction is not due to changes in histone modification. Interestingly, the inhibition of
the whole cluster rescued the inhibitory effects of arsenic, although the further studies are needed to uncover potential target genes for this cluster, such as Gli2. Our results indicate that the miR-466-669 cluster plays a role in stem cell differentiation and is also a target of arsenic effects.

Acknowledgements

The authors would like to thank Dr. Feliciano’s Lab from Clemson University for kindly providing N2A cell line. Funding for this study was provided by NIH (ES023065).
Reference


### Table 3.1. Primer sequences

<table>
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<th>Gene Name</th>
<th>Forward</th>
<th>Reverse</th>
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</thead>
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<tr>
<td>miR-9</td>
<td>ATA AAG CTA GAT AAC CGA AA</td>
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</tr>
<tr>
<td>miR-92a</td>
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<tr>
<td>miR-199a</td>
<td>ACA GTA GTC TGC ACA TTG GTT A</td>
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<td>miR-291a</td>
<td>CAT CAA AGT GGA GGC CCT CT</td>
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<tr>
<td>miR-466b</td>
<td>CCA AGC CAA CTT TAT GTC AGG G</td>
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<td>miR-466f</td>
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<td>miR-466g</td>
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<td>miR-466h</td>
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</tr>
<tr>
<td>miR-466i</td>
<td>GCC AAG CAA CCT GCT GAA AGT CTA</td>
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<td>miR-467d</td>
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<tr>
<td>miR-669f</td>
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<td>miR-669p</td>
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<td>Consensus-4</td>
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<td>CAC CTG TCC CTG ACA CCA CTC T</td>
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Universal Reverse Primer
Table 3.2. Developmentally-related miRNAs significantly changed by arsenic during P19 cell differentiation

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>As(III) exposure</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-92a</td>
<td>Up regulated</td>
<td>Highly expressed in induced pluripotent and embryonic stem cells</td>
<td>(Wilson et al., 2009)</td>
</tr>
<tr>
<td>miR-291a</td>
<td>Up regulated</td>
<td>Promotes pluripotency maintenance in mouse embryonic stem cells</td>
<td>(Lichner et al., 2011)</td>
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<tr>
<td>miR-709</td>
<td>Up regulated</td>
<td>Inhibits adipocyte differentiation through targeting GSK3β</td>
<td>(Chen et al., 2014)</td>
</tr>
<tr>
<td>miR-9</td>
<td>Down regulated</td>
<td>Induced during neuronal cell differentiation</td>
<td>(Zhao et al., 2009)</td>
</tr>
<tr>
<td>miR-199a</td>
<td>Down regulated</td>
<td>Induced during embryonic development and regulates chondrogenesis</td>
<td>(Lee et al., 2009; Lin et al., 2009)</td>
</tr>
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</table>

Table 3.3. miR-466/467/669 cluster expression in differentiating P19 cells with or without arsenic exposure

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold Change (control/As)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>miR-669f-3p</td>
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<td>0.013</td>
</tr>
<tr>
<td>miR-466g</td>
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<td>0.015</td>
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<td>miR-669p-3p</td>
<td>1.87</td>
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<tr>
<td>miR-669e-3p</td>
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<td>miR-466h-3p</td>
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<td>0.078</td>
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Figures

Control As(III)

1: mmu-miR-466m-3p  33: mmu-miR-8104
2: mmu-miR-6348  34: mmu-miR-8093
3: mmu-miR-291a-5p  35: mmu-miR-101a-3p
4: mmu-miR-6981-5p  36: mmu-miR-8100
5: mmu-miR-467a-3p  37: mmu-miR-199a-5p
6: mmu-miR-294-5p  38: mmu-miR-133a-3p
7: mmu-miR-291b-5p  39: mmu-miR-6926-5p
8: mmu-miR-290a-5p  40: mmu-miR-1892
9: mmu-miR-292a-5p  41: mmu-miR-153-3p
10: mmu-miR-6946-3p  42: mmu-miR-10a-3p
11: mmu-miR-92b-3p  43: mmu-miR-7221-3p
12: mmu-miR-466q  44: mmu-miR-8110
13: mmu-miR-466f-5p  45: mmu-miR-6937-5p
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17: mmu-miR-133b-5p  49: mmu-miR-7020-5p
18: mmu-miR-92a-3p  50: mmu-miR-125a-3p
19: mmu-miR-7028-5p  51: mmu-miR-3473g
20: mmu-miR-574-3p  52: mmu-miR-93-5p
21: mmu-miR-669e-3p  53: mmu-miR-7085-5p
22: mmu-miR-494-3p  54: mmu-miR-705
23: mmu-miR-31-5p  55: mmu-miR-9-3p
24: mmu-miR-669p-3p  56: mmu-miR-7047-5p
25: mmu-miR-363-5p  57: mmu-miR-9-5p
26: mmu-miR-294-3p  58: mmu-miR-6239
27: mmu-miR-3097-5p  59: mmu-miR-106b-5p
28: mmu-miR-669b-3p
29: mmu-miR-669f-3p
30: mmu-miR-709
31: mmu-miR-467d-3p
32: mmu-miR-378c
Figure 3.1. Expression profiles of miRNAs between control and arsenic exposure in differentiating P19 cells.

P19 cells were induced to differentiate with or without 0.5 µM of arsenic for 9 days. miRNA expression was detected via a miRNA microarray and plotted as a heat map. Green indicates reduced expression due to arsenic and red indicates increased expression. Only statistically different expression (Student’s t-test; p-value < 0.05) are listed in the map. The miRNAs with similar expression signals and fold-changes are clustered together as shown in the left panel.
Figure 3.2. Validation of miRNA transcript levels by qPCR.

Day 9 differentiated P19 cells were used to confirm miRNA expression by qPCR (n=3 per treatment). Significantly changed miRNAs with known roles in development were examined (Table 2), including miR-92a (A), miR-291a (B), miR-709 (C), miR-199a (D), and miR-9 (E). Expression values were normalized with U6 snRNA and fold differences
calculated from control cells using the delta Ct method. Values are expressed as mean ± S.D. and statistical differences were determined by Student’s t-test (*; \( p < 0.05 \)).
Figure 3.3. Arsenic exposure induces members of the miR-466-669 cluster along with its host gene, Sfmbt2.

P19 cells were differentiated and RNA was extracted from cells exposed to 0 or 0.5 μM arsenite on days 0, 2, 5, 7, and 9 (n=3 per treatment per day). miRNA or mRNA expression was determined by qPCR. Day 9 samples were used to determine the expression of miRNA-466-669 cluster genes (A) and the host gene Sfmbt2 (B). Samples from days 0, 2, 5, 7, and 9 were used to determine the expression of miR-467d (C), miR-669p (D), and Sfmbt2 (E). Expression values were normalized with U6 snRNA for the miRNAs, and Gapdh for Sfmbt2. Fold changes were compared to unexposed cells, and time-dependent qPCR expression fold changes were compared to day 0 unexposed cells. Data is shown as mean ± S.D. Statistical differences were determined by ANOVA followed by Tukey’s test or by Student’s t-test (*; p <0.05).
Figure 3.4. Arsenic does not alter expression of the miR-466-669 cluster in C2C12 and N2A cells.

C2C12 myoblast cells were exposed to 20 nM arsenite in growth medium (GM) or differentiation medium (DM) for 4 days (n=3 replicates per treatment) (Figure A-B). Neuro2A neuroblast cells were exposed to 1 μM or 2.5μM arsenite in growth medium (GM) or differentiation medium (DM) for 2 days (n=3 replicates per treatment) (Figure C-D). qPCR was used to determine the expression of miR-466g, miR-467d, and miR-669p. All expression values were normalized with shRNA U6 and fold changes were compared to unexposed cells. Data is shown as mean ± S.D., and statistical differences were determined by ANOVA followed by Tukey’s test (*; p<0.05).
Figure 3.5. The miR-466-669 cluster share sequence similarities.

Sequences of mature miRNA from the miR-466-669 cluster were aligned using Clustal Omega. All mature sequences were initially aligned together and then were subdivided into four major groups which have high similarity. The highlighted nucleotides were used to derive the consensus sequences (as light blue). An asterisk indicates the sequence identity among all miRNAs within the group.
**Figure 3.6. Inhibiting the miR-466-669 cluster during P19 cell differentiation.**

P19 cells were transfected with 100 nM anti-miRNA oligonucleotides which target 4 individual consensus sequences of the miRNA-466/467/669 cluster. Cells were co-exposed to 0 or 0.5 μM arsenic for the 5 days of embryoid body formation. Only the arsenic exposure was maintained for the entire 9 days of differentiation, after which cell morphology was observed. Transfections include oligonucleotides sequences that do not target any miRNAs, designated as negative control, (A-B), consensus-1 (C-D),
consensus-2 (E-F), consensus-3 (G-H), consensus-4 (I-J), and mixed transfection that combined all consensus anti-miRs (K-L). Arrows indicate neuronal cells and arrow heads indicate myotubes.
Figure 3.7. Mixed miRNA inhibitors rescue arsenic’s inhibitory effects on cell differentiation.

P19 cells were transfected with miRNA inhibitors, with or without 0.5 μM arsenic (n=3 replicates per anti-miR and per exposure group), and allowed to form embryoid bodies for 5 days. A portion of the day 5 embryoid bodies was collected for RT-qPCR. Pax3 expression, an early differentiation marker gene, was determined in the cells transfected individually with the consensus anti-miRs (A-B) or transfected with a combined mixture of the 4 anti-miRs (C). Rescue of the four individual consensus sequences and the mixture were assessed by qPCR (D-G), as was the effect on the host gene, Sfmbt2 (H). mRNA expression levels were normalized with Gapdh and miRNA expression levels were normalized with shRNA U6 using the comparative delta Ct method. Fold changes were compared to negative control (NC). Data is shown as mean ± S.D., and statistical differences were determined by ANOVA followed by Tukey’s test (*; p <0.05).
Figure S3.1. Arsenic exposure decreased Neuro2A cell differentiation.

Neuro2a neuroblastoma cells were cultured in DMEM medium with 10% fetal bovine serum (growth medium) with or without 1 and 2.5 μM sodium arsenite for 48 hours (A-C). To induce differentiation, growth medium was replaced with DMEM medium containing 2% fetal bovine serum and 20 μM all-trans retinoid acid (differentiation medium), with or with or without 1 and 2.5 μM sodium arsenite for 48 hours (D-F). Representative images are shown (n=3).
Figure S3.2. miR-291a was upregulated when exposed to arsenic during cell differentiation.

P19 cells were differentiated and RNA was extracted from cells exposed to 0 or 0.5 μM arsenite, on days 0, 5, and 9 (n=3 per treatment per day). miR-291a expression was determined by qPCR. Expression values were normalized to U6 snRNA, and fold changes were compared to day 0 unexposed cells. Data is shown as mean ± S.D. Statistical differences were determined by one-way ANOVA followed by Tukey’s test (*; p <0.05).
Figure S3.3. Histone modification status in the promoter region of Sfmbt2 and miR-466-669 cluster is not changed when exposed to arsenic.

Day 5 embryoid bodies with or without arsenic exposure were harvested for ChIP assay. A schematic diagram of Sfmbt2 and the miR-466-669 cluster promoter region shows the predicted promoter site (Marson et al., 2008), CpG island, transcription start site and the location of ChIP assays (A). ChIP assays were performed with antibodies against dimethylated H3K9 (H3K9 Me2), and acetylated H3K9 (H3K9 Ac) histones. Enriched DNA fragments from the ChIP assays were analyzed by qPCR, and are expressed as a percentage of input. The values from replicate experiments were plotted as the mean ± S.D. (n=3) and statistical differences were determined by ANOVA followed by Tukey’s test (B; *, p<0.05).
Figure S3.4. Mixed miRNA inhibitors rescue arsenic’s inhibitory effects on Gli2 expression.

P19 cells were transfected with all four miRNA inhibitors, with or without 0.5 μM arsenic (n=3 replicates per exposure group), and allowed to form embryoid bodies for 5 days. A portion of the day 5 embryoid bodies was collected for RT-qPCR. Gli2 expression was determined by qPCR, and transcript levels normalized with Gapdh using the comparative delta Ct method. Fold changes were compared to negative control (NC). Data is shown as mean ± S.D., and statistical differences were determined by ANOVA followed by Tukey’s test (*; p <0.05).
CHAPTER FOUR

CONCLUSION

Previous studies in our laboratory have shown that arsenic represses neurogenesis and myogenesis in P19 embryonic stem cells (Hong and Bain, 2012). Therefore, the goal of the present study was to discover the mechanisms by which arsenic reduces embryonic stem cell differentiation. Our results from Chapter 2 indicate that arsenic inhibits the sonic hedgehog (Shh) signaling pathway during cell differentiation. The expression of the critical transcription factor Gli2 is reduced at both the mRNA and protein level, leading to its reduced transcriptional activity when exposed to arsenic. Treatment with exogenous Shh can rescue the inhibitory effects by arsenic on cell differentiation. In Chapter 3, we further discovered that specific microRNAs are altered by arsenic during P19 cell differentiation. Several members of miRNA-466-669 cluster, along with the host gene Sfmbt2, were significantly induced by arsenic in a time-dependent manner. Moreover, this induction is specifically in embryonic stem cells, and inhibition of this cluster can rescue the inhibitory effects caused by arsenic. Taken together, the inhibition effects on cell differentiation by arsenic is due to the repression of transcription factor Gli2. Even though the inhibition mechanism of Gli2 is unknown, we found that arsenic induces the expression of miR-466-669 cluster, which regulates Gli2 expression directly or indirectly, leading to the inhibition of cell differentiation.

Epidemiological studies have shown that arsenic causes developmental toxicity, in that children exposed in utero to arsenic have lower intelligence quotient scores, alterations in verbal learning, and decreases in long term memory (Jiménez-Capdeville
and Giordano, 2003). Arsenic exposure also reduced birth weight and weight gain (Hopenhayn et al., 2003). When conducting our experiments examining stem cell differentiation, we wanted to use arsenic levels similar to that which an embryo might be exposed to. While there is limited available exposure data, a study in Argentina shows that mothers exposed to ≤ 200 ppb arsenic in their drinking water had 34 μg/kg arsenic in their placentas and 9 μg/L arsenic in cord blood (Concha et al., 1998). Because embryonic exposure levels should reflect the amount of arsenic in cord blood and placentas, rather than in drinking water, our study exposed P19 embryonic stem cells to levels of arsenic ranging from 7.5 to 37.5 μg/L. In vivo, inorganic arsenic can be transformed into methylated metabolites, including monomethylarsonic acid (MMAIII) and dimethylarsinic acid (DMAIII). Although we have previously shown that As3MT is robustly expressed in P19 cells, we have not determined the amount of methylated arsenical species formed in P19 cells when exposed to inorganic arsenite. However, another study from our laboratory indicates that MMAIII and DMAIII repress cell differentiation and reduce myogenic and neurogenic transcription factor expression in a manner similar to inorganic arsenite, albeit at lower concentrations, which suggests that exposure of P19 cells to inorganic arsenic should still be reflective of an in vivo exposure scenario (McCoy et al., 2015). Studies have shown that maternal exposure to 100 ppb of arsenic via drinking water results in urinary arsenic levels that are 43% DMA, 31% MMA, and 26% iAs (Hall et al., 2007). Since the methylated species impair differentiation at lower concentrations than inorganic arsenic, in vivo exposures might be
more toxic and detrimental to embryonic development than our study reveals, since there would be more methylated metabolites of arsenic present.

Several in vivo studies have looked into the gene and signaling pathways altered by arsenic embryonic development and fetal growth. For example, a study determining expression of several key developmental genes in human placenta from an in utero arsenic exposure found out that Gli3 expression was negatively associated with arsenic exposure and positively associated with infant birth weight (Winterbottom et al., 2015). Another study exposing mice to 50 ppb arsenic during pregnancy found out that glucocorticoid signaling in the brain was disrupted by reducing the enzyme 11β-HSD expression during embryonic day 14, which plays a critical role of learning and memory (Caldwell et al., 2015). These studies suggest that arsenic alters signaling pathway in vivo, leading to the dysfunction of organism. In order to elucidate the mechanism by which arsenic affects signaling, several in vitro studies have determined the effects on apoptosis pathways, oxidative stress pathways (Chattopadhyay et al., 2002; Cheung et al., 2007; Martinez et al., 2011). Moreover, signaling pathways related to cell growth such as Akt, Ras, ERK, JNK and p38 pathways are also altered when exposed to arsenic (Liu et al., 1996; Souza et al., 2001). To our knowledge, our study is one of the few which is examining arsenic’s effects on developmentally-important signaling pathways.

In order to elucidate the main pathway targeted by arsenic, we first examined key proteins in the Notch, Bmp4, Fgf, and Shh signaling pathways. Previous studies in our laboratory have indicated that arsenic can modulate β-catenin transcript and nuclear localization during stem cell differentiation, which works via the Wnt signaling pathway.
(Hong and Bain, 2012). Our preliminary experiments indicated that in addition to Wnt, arsenic can down-regulate the expression of Gli2, thus inhibiting the Shh signaling pathway. The Shh signaling pathway has been shown to induce the expression of several genes of the myogenic regulatory factor family including, Pax-3, MyoD, and myogenin (Duprez et al., 1998). It also induces neurogenic bHLH factors such as NeuroD, Neurog1, and Ascl1 (Voronova et al., 2011). In addition to the changes in Gli2 expression, we also examined other Shh signaling proteins such as the expression of Smo, Sufu and Ptc1, the phosphorylation and cleavage of GLI2, and its localization within the primary cilium, but none of these were altered by arsenic. Therefore, our study indicates that decreased expression of those neuronal or myogenic transcription factors by arsenic might be due to the inhibition of Shh signaling via Gli2. However, why arsenic specifically reduces this signaling pathway, as opposed to other pathways, is still unknown. There are other signaling pathways that help to regulate Gli2 expression. During somite formation, Wnt signaling was shown to induce Gli2 expression (Borycki et al., 2000), and in several human cell types, TGF-β induces Gli2 independent from Shh receptor signaling (Dennler et al., 2007). Therefore, studying further upstream pathways can help us elucidate how arsenic inhibits Gli2 expression.

Since several studies show that microRNAs inhibit Shh signaling, we hypothesized that alterations in developmentally-important signaling pathways are due to changes in miRNA expression (Ferretti et al., 2008; Katoh and Katoh, 2008). Based on our miRNA microarray data, none of the miRNAs reported in the literature to regulate Shh signaling, including miR-125b, miR-324 and miR-214, were altered by arsenic.
However, several other miRNAs were changed, including miR-9, miR-92a, miR-199a, miR-291a and miR-709, all known to be involved in stem cell pluriopotency maintenance or muscle and/or neuronal cell differentiation (Chen et al., 2014; Lee et al., 2009; Lichner et al., 2011; Wilson et al., 2009; Zhao et al., 2009). Changes in these specific miRNAs validate that arsenic is indeed working to impair stem cell differentiation, rather than proliferation. We chose to focus our further studies on a novel set of microRNAs, the miR-466-669 cluster. This cluster has 65 members within intron 10 of the host gene Sfmbt2 (Lehnert et al., 2011), and of the 65 members on the microRNA array used, 20 of these genes were increased by 1.5-to 4-fold. This study further determined that the use of 4 consensus anti-miRs together, that should inhibit 60 % of the cluster, can effectively rescue the inhibitory effects by arsenic. While the exact function of the miR-466-669 cluster is not known, other studies have determined that it can regulate osmoregulation and urine concentration in mice (Luo et al., 2014).

Interestingly, Gli2 transcript levels are also recovered when knocking down the miR-466-669 cluster. Arsenic reduces Gli2 expression by 2.5-fold and the consensus anti-miRs bring the expression back up to 0.9-fold of control. These data suggest that the arsenic’s induction of the miR-466-669 cluster can directly or indirectly regulates Gli2 expression. This is also supported by the time-dependent expression data in P19 cells which shows that Gli2 expression starts at day 5, and miRNA-466-669 cluster expression starts to decrease at day 5. However, by comparing the consensus sequences with the Gli2 3’UTR, and using the algorithm from Zheng’s study, Gli2 is not the predicted target of miR-466-669 cluster (Zheng et al., 2011). Therefore, we hypothesize that the miR-
466-669 cluster might act upon a protein upstream of Gli2. As mentioned above, two other signaling pathways, Wnt and Tgf-β, can regulate Gli2 expression. Wnt4 is predicted as a possible target of the miR-466-669 cluster (Zheng et al., 2011). Therefore, studying the effects of the cluster upstream of Gli2 is something that should be done in future studies. In conclusion, it appears that arsenic repressed the sonic hedgehog signaling pathway, and induced expression of the miR-466-669 cluster, which may both work together to inhibit stem cell differentiation.

Ultimately, our data should be useful to determine whether the current drinking water standard for arsenic, 10ppb, is protective of embryonic health, and whether a standard for arsenic in foods, such as rice, needs to be set. As part of setting these standards, cells are often exposed to the toxicant of interest and then examined for morphological changes, such as proliferation (Vega et al., 2001). Because low levels of toxicants may not show significant morphological effects, and because many times only proliferation, rather than differentiation, is assessed, the use of these data for risk assessment is often difficult (Gibb et al., 2011). Recently, researchers are trying to develop better toxicity assessment methods using cell lines to quickly screen chemicals. National programs such as Tox21 from NIEHS and ToxCast from EPA screen chemicals using a variety of differentiated human cell lines to examine reporter gene activity, gene expression, kinase/protease activity, and G-protein coupled receptor signaling. Work incorporating mouse stem cell differentiation screening assays is being finally conducted under this program (Sipes et al., 2013). Other investigators outside of EPA and NIEHS are also using stem cell differentiation assays as screening tools to identify developmental
toxicants (Palmer et al., 2013). Therefore, studies using mouse embryonic stem cells appears to be gaining favor as a model to perform chemical assessments for development toxicity. In addition, our miRNA microarray data provides a list of miRNAs whose expression level are affected by arsenic treatment, which can serve as potential biomarkers of arsenic effect with useful value for risk assessment on developmental toxicity.

Several studies indicate that low levels of arsenic exposure increase the risk of carcinogenesis by inducing the sonic hedgehog signaling pathway (Fei et al., 2010; Tokar et al., 2010). However, our study shows that arsenic impairs Shh signaling during development and cell differentiation. The results of our study may be useful when thinking about potential therapies for arsenic-induced developmental defects. For example, Shh gene therapy has been shown to increase skeletal and cardiac muscle regeneration after injury (Kusano et al., 2005; Piccioni et al., 2014). The sonic hedgehog pathway agonist SAG has also been used to rescue the phenotypes associated with hippocampal deficits in mice, leading to the improvement of learning and memory behavior (Das et al., 2013). The results of our study may provide a potential therapeutic method for those who were exposed to arsenic during development.

Taken together, this study provides a mechanistic approach on how arsenic affects stem cells differentiation. However, there are still questions needed to be elucidated. For example, we need to understand how the altered miRNAs regulate the downstream signaling (ie, what are the target genes) and how does this link back to arsenic and reduced stem cell differentiation? Future studies should also investigate whether the
altered mechanisms seen in our stem cell culture system mimics that which is seen *in vivo* during embryogenesis.
References


