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Effects of Environmentally Relevant Transplacental Arsenic Exposure on Mouse (Mus musculus) Hepatic Protein Expression

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EFFECTS OF ENVIRONMENTALLY RELEVANT TRANSPLACENTAL ARSENIC EXPOSURE ON MOUSE (*MUS MUSCULUS*) HEPATIC PROTEIN EXPRESSION

by

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Abstract

Inorganic arsenic is a well-known toxic element found around the world, but the molecular mechanisms involved in arsenic toxicity are currently poorly understood. Arsenic has been linked to several types of cancer, diabetes, cardiovascular disease, and other metabolic diseases. This project explores the toxic effects of arsenic using mouse (*Mus musculus*) as a mammalian model organism. Preliminary data from the Van Beneden lab has shown that mice respond to low-dose, transplacental arsenic exposure in a dose-, sex-, and generation-dependent manner. The current study addresses a potential mechanism of toxicity by determining relative expression levels of pAKT/AKT[1], a serine/threonine kinase that is activated via phosphorylation. In previous Van Beneden lab studies, arsenic exposure was linked to altered expression of several gene products that are involved in cell cycle regulation as well as glucose uptake and lipid transport; each of which is known to be regulated in part by AKT. Many of these pathways are highly conserved, making AKT the subject of a significant amount of cancer and diabetes research. We hypothesized a dose-dependent increase in $\frac{\text{pAKT}}{\text{AKT}}$ expression, suggesting more AKT pathway activity in response to arsenic exposure. Initial data show a trend of reduced AKT activity at 50ppb and 500ppb transplacental arsenic exposure, but no sex-dependent response or statistically significant effects of the treatment levels. Analysis of $\frac{\text{pAKT}}{\text{AKT}}$ expression provides insight to the molecular pathways involved in arsenic toxicology when partnered with existing literature and results of the ongoing study in the Van Beneden laboratory.

[1]When referring to gene products, uppercase represents protein, whereas lowercase italics represent mRNA.

Acknowledgements

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Lastly, I would like to thank my friends and family for the support as I found myself struggling to accept the realities of laboratory research: most procedures are time consuming and few produce ideal results at first.

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Introduction

This study was done to further assess the effects of arsenic on the hepatic system. The study was based on previous work performed in the Van Beneden lab by Patrick Carlson. Pat identified several gene products (on both the mRNA and protein scale) with altered levels of expression in response to low-dose transplacental arsenic exposure in mice, all of which are involved either in cell cycle regulatory pathways or in metabolic pathways related to glucose or lipids (Carlson, 2013c). AKT (formerly Protein Kinase B or PKB) plays a role upstream of these signaling pathways, making it interesting to study for its implications associated with cancer formation and diabetes development. Of further fascination is the well-documented connection between arsenic, cancer, and diabetes (Zimmerman, 1999). Given this information, it was decided that this project would focus on determining AKT activity in the same animals used in the preceding studies. This is relevant because arsenic is a common toxicant found in drinking water around the New England region and around the world, and the rates of both cancer and diabetes have been on the rise in recent years (Ayotte *et al.,* 2003; CDC Diabetes Public Health Resource; NCI Stat Fact Sheet). Because of the link between arsenic exposure and these chronic diseases, and because of the role of AKT, it was hypothesized that relative $\frac{\text{pAKT}}{\text{AKT}}$ expression would increase with arsenic exposure, suggesting more AKT pathway activity. This hypothesis was derived from proposed mechanisms laid out by Patrick Carlson in his dissertation (Carlson, 2013c). With this study we hope to gain further insight to the molecular mechanisms involved in arsenic toxicity, which have been complicated by a non-linear dose-response curve.

Arsenic:

Arsenic, a naturally occurring metalloid found in bedrock around the world, is found throughout the environment in its inorganic form. The World Health Organization (WHO) and United States Environmental Protection Agency (US EPA) have set a current allowable exposure level of 10 parts per billion (ppb). This regulation is a result of significant research performed around the turn of the millennium, which led the acceptable limit in U.S. drinking water to be dropped from 50ppb to 10ppb in 2001 (EPA, 2007).

Inorganic arsenic is odorless and colorless and is commonly found in private drinking water wells as a result of leaching from bedrock, and, to a lesser extent, through industrial and agricultural waste and runoff (EPA, 2007). Naturally occurring arsenic in groundwater varies with respect to both climate and geology. Globally, the highest levels of exposures can be found in Mexico, Chile, Argentina, Taiwan, Thailand, Bangladesh, and India. However, there is also widespread occurrence at less extreme levels found in Japan, Poland, Hungary, Canada, and the United States (Basu *et al*., 2001). In the U.S., it has long been recognized that groundwater arsenic concentrations are high in the Interior Plains region, throughout much of the Rocky Mountain System, and in many southwestern states, though in recent years it has been shown that several other regions—including New England—have higher concentrations than previously believed (Welch *et al.,* 2000). Throughout eastern New England, it is estimated that over 100,000 people with private wells are exposed to arsenic in their drinking water at levels above the

current EPA standard (Ayotte *et al.,* 2003). This recorded distribution is shown in Figure 1.

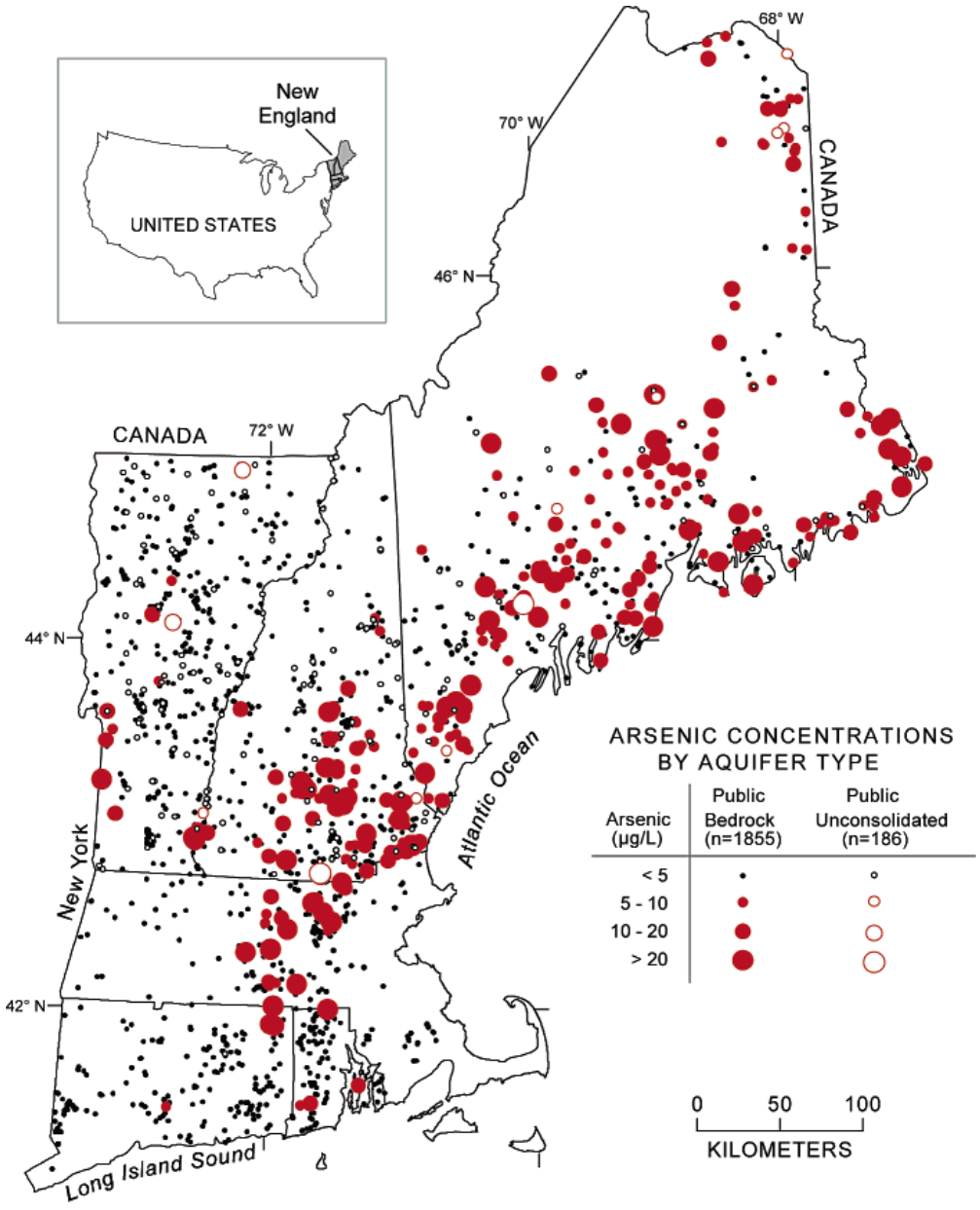


Figure 1: Arsenic concentrations found throughout New England (Ayotte *et al.,* 2003).

In the United States, the first regulation of arsenic exposure occurred in 1974 with the Safe Drinking Water Act, which established maximum contaminant level goals

(MCLGs) for individual contaminants. This allows for a baseline assessment for utilities to have as a resource when ensuring safe potable water. Interestingly, the MCLG for arsenic has been set at zero, yet the EPA has based the 10ppb standard on the feasibility of utility systems to attain a concentration of 0ppb effectively. In 2002, a year after the lowered acceptable limit, the Arsenic and Clarifications to Compliance and New Source Contaminants Monitoring Final Rule became effective, which allows the EPA to continually review and revise standards based on emerging scientific data as frequently as every six years. For this reason, the study of arsenic toxicology has legitimate potential to have a broad effect on public health standards (EPA, 2007).

Inorganic arsenic has been shown to have a wide range of effects on human health, including carcinogenesis, cardiovascular disease, diabetes, peripheral neuropathy, skin lesions, hypertension, and other metabolic diseases (Ratnaike, 2003; Duker, 2005). With specific respect to cancer, it has been shown that arsenic promotes tumor angiogenesis (affecting the ability of cancerous growths to obtain nutrients), alters matrix-associated proteins (enabling metastasis), and alters expression of cell-cycle regulatory genes (Soucy *et al*. 2003, 2005; Straub *et al*. 2008; Carlson *et al.,* 2013b,c). Less is known about arsenic's impacts on lipid metabolism. However, epidemiological studies have shown that the presence of diabetes is positively correlated to high levels of arsenic in drinking water and with arsenic metabolites in urine (Navas-Acien *et al.,* 2008; Del Razo *et al*., 2011). Many studies have explored the pathways of arsenic metabolites—notably, methylated arsenites—and their effects on glucose metabolism (Del Razo *et al.,* 2011; Paul *et al.*, 2011; Swaran, 2011). It has been shown that exposure to inorganic arsenic inhibits insulin signaling and glucose uptake in an AKT-mediated

pathway (Paul *et al.,* 2011). Studies such as this laid the foundation for prior Van Beneden lab studies linking arsenic exposure to cancer and diabetes, and provide further rationale for the purpose of this study.

Hepatic Relevance:

The liver is responsible for several important functions, with the most important to this study being metabolism. The organ produces enzymes critical to digestion, toxicant metabolism, and glucose metabolism (Burke, 1975). Consequently, it is of interest to study hepatic gene product expression in toxicological studies, which have come to rely on integrated transcriptomics, proteomics, and metabolomics. This has led to exploration of functional genomics involved in arsenic toxicology, with particular respect to diabetes in hepatic studies. In recent years, the field of hepatic toxicogenomics has developed significantly, supporting traditional toxicology measures to advance the field from hazard identification to hazard characterization (Kienhuis *et al.,* 2010). The mouse (*Mus musculus*) is a useful model organism to study due to extensive homology associated with mammalian phylogeny. For this study the animal care and treatment were performed in previous years, with harvested tissue samples stored at -80ºC.

Prior Studies:

The Van Beneden lab designed the transplacental study based on existing literature citing effects of *in utero* exposure on development of chronic adult diseases (Waalkes *et al.,* 2003; Ferrario *et al*., 2008; States *et al.,* 2011). The study also sought to explore the documented effects of arsenic in a sex-dependent manner as observed in literature of the field (Ferrario *et al*., 2008). Furthermore, the study was also designed to allow for observation of the effects of transplacental arsenic exposure in a generation-

dependent manner, which is cited frequently not only with inorganic arsenic, but with many other toxicants as well (Paul *et al.,* 2011; States *et al*., 2011; Stueckle *et al.,* 2012). For this project, generation-dependent effects were not explored, though much of the preparation to do so was performed. The Van Beneden lab is positioned to continue studying the effects of inorganic arsenic exposure on relative AKT activity in the mouse hepatic system of the F_2 offspring.

Of the prior work done in the Van Beneden lab, the most notable and relevant to this project was performed by Patrick Carlson, whose studies explored the effects of arsenic on cell cycle regulation as well as glucose and lipid metabolism. It was observed that arsenic exposure in zebrafish resulted in several dozen proteomic expression differences (Carlson *et al*., 2013a). Upon further investigation, it was shown that low-dose inorganic arsenic exposure in zebrafish altered mRNA expression of a selected subset of genes, notably *Pparγ* and *Wee1*, which are known to be regulated in part by AKT (Carlson *et al.,* 2013b). Furthermore, it was shown in separate studies that similar exposures in mice (administered transplacentally) alter expression of *Pparγ* and *Wee1*, as well as other genes involved in similar pathways. PPARγ, a peroxisome proliferator activated receptor, is integrated along the growth hormone—Insulin-like growth factor (GH-IGF) axis and is therefore involved in growth factor responses related to cell cycle progression and metabolism (Scarth JP, 2005; Stueckle *et al.,* 2012; Carlson *et al.,* 2013b). WEE1 is critical to regulation at the G2/M checkpoint for the cell cycle, making it of interest in early-stage cancer development (Katayama *et al.,* 2005; Carlson *et al.,* 2013b). Due to the relative importance of AKT signaling upstream in both PPARγ and WEE1 activity, the observed changes in their expression in response to arsenic exposure

drove the rationale to study the effects of arsenic exposure on AKT activation.

AKT:

In the 1980's, several research groups exploring insulin signaling pathways identified what appeared to be a novel kinase similar to protein kinases A and C (PKA and PKC; Jones *et al.,* 1991; Brazil *et al.*, 2001). Upon cloning and further identification, AKT was termed Protein Kinase B (PKB) due to its structural similarities to PKA and PKC. In the 1990's, three isoforms of AKT were identified, now known as AKT1, AKT2, and AKT3 (Franke, 2008). For the purposes of this study, the three isoforms are discussed as a unit simply as AKT. This is because they share similar function and their structure is so homologous that the antibodies used for western blotting were not specific to any individual isoform.

AKT is a serine/threonine kinase with a plethora of downstream targets. AKT is activated via phosphorylation by the membrane-bound phosphatidylinositol 3-kinase (PI3K) as shown in Figure 2.

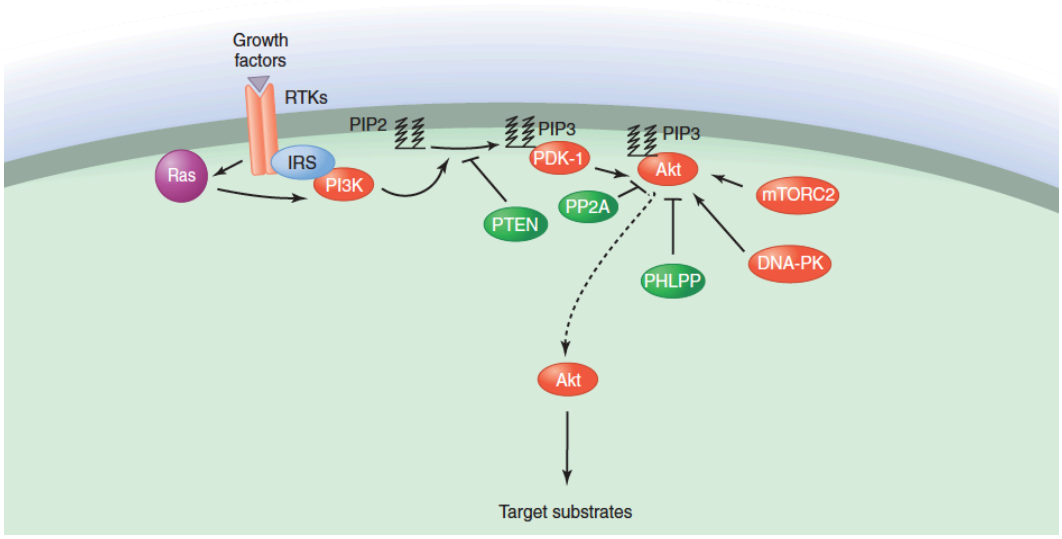


Figure 2: Upstream AKT signaling (Hemmings *et al.,* 2012). Arrows indicate activation, while bars indicate inhibition.

In its inactive state, AKT is free in the cytoplasm, where if it is "damaged" (*i.e.* mis-folded, improperly translated, etc.) it is ubiquitinated, which acts as a molecular tag for degradation (Liao *et al.,* 2010). The signal transduction cascade can begin in several ways in response to growth and stress factors. Most commonly, receptor tyrosine kinase (RTK) subunits bind the signal ligand, stimulating the recruitment of phosphoinositides and PI3K (Engelman *et al.*, 2006; Hawkins *et al.* 2006). PI3K is then phosphorylated, activating its kinase activity to initiate the downstream cascade. PI3K phosphorylation is also regulated (in what is proposed to be a lesser extent) by detached subunits in G protein-coupled receptor (GPCR) pathways and by small signaling proteins such as Ras. It should be noted that GPCR-mediated PI3K regulation has significant importance to the mitogen activated protein kinase (MAPK) cascade, which can be activated downstream from PI3K kinase activity (Lopez-Ilasaca *et al.*, 1997). The MAPK cascade is a well-documented set of pathways associated with cellular proliferation, differentiation, survival, and death (Kim *et al.,* 2010).

Activated PI3K induces phosphorylation of phosphoinositides—most notably PIP_3—which recruits both phosphoinositide-dependent kinase (PDK1) and AKT in proximity to each other. Phosphorylation of AKT takes place once PDK1 is bound to the membrane in proximity to AKT, which allows for its dissociation from PIP_3, and thus stimulates its kinase activity.

AKT has several phosphorylation sites, with the most important being at Ser473 (which is the phospho-site targeted by the active-state antibody used for this study). Once phosphorylated, AKT can act on a number of substrates involved in diverse biological responses, ranging from primarily metabolic functions such as glucose

transport, glycolysis, glycogen synthesis and the suppression of gluconeogenesis to protein synthesis, increased cell size, cell-cycle progression and apoptosis suppression (Franke, 2008). The identified motif that AKT acts on is R-X-R-X-X-S/T-B, where X represents any amino acid and B represents bulky hydrophobic residues [and S/T represents the serine/threonine phosphorecognition site] (Alessi *et al.*, 1996). This identified motif is similar to other protein kinases, such as PKA and PKC, unique only by the denoted R-residues [Arginine] (Manning *et al.,* 2007). However, at high levels of substrate and longer time interval, AKT can also act on the less specific R-X-X-S/T site, which gives an added level of complexity to the study of downstream AKT signaling. For this study, it is imperative to note that AKT can also act on the less specific site because *in vivo* cellular environments can have significant variation in the concentration of proteins with AKT recognition sites relative to *in vitro* studies (Manning *et al.,* 2007). Literature review reveals over 100 reported AKT substrates, though not all contain the specific kinase motif, and many have not been studied beyond *in vitro* kinase assays. Figure 3 shows the diverse downstream signaling of pAKT.

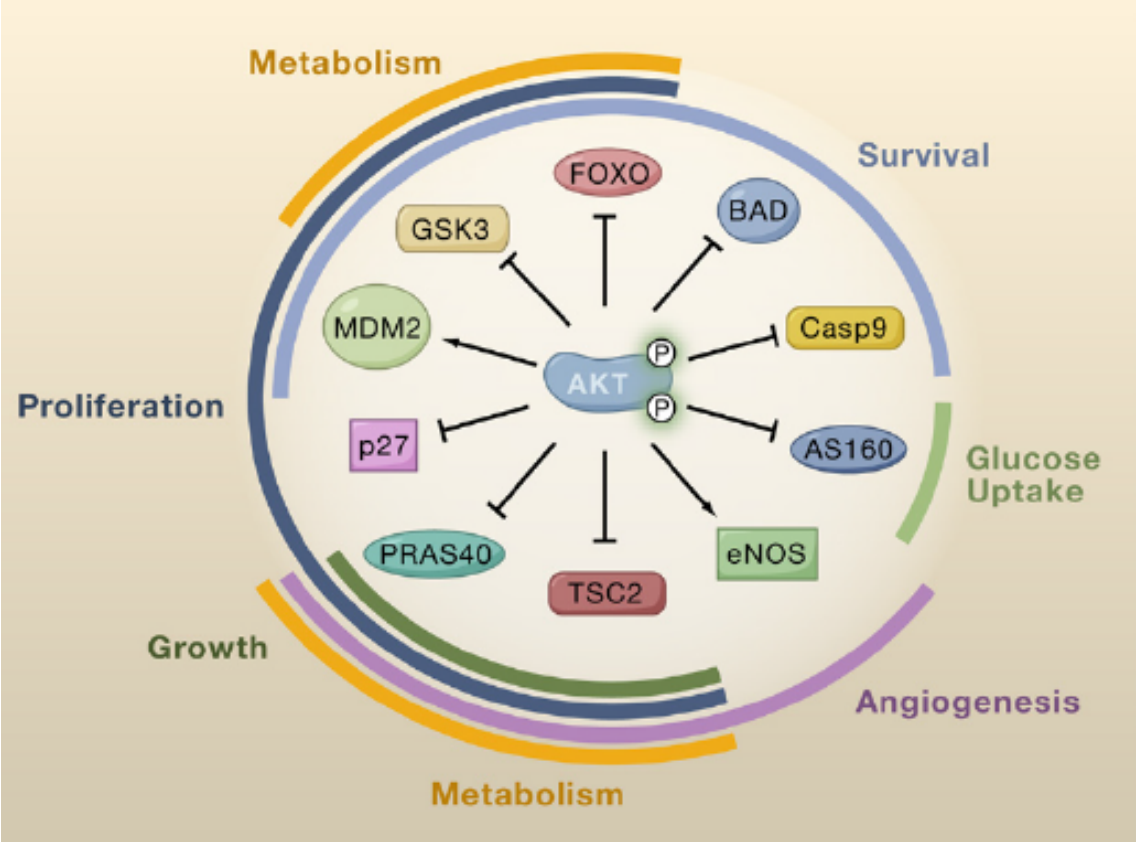


Figure 3: Downstream AKT signaling (Manning *et al.,* 2007). Arrows indicate activation, while bars indicate inhibition.

Prior Van Beneden lab studies identified altered gene expression of WEE1, BRCA2, and PPARγ in response to arsenic, each of which is proposed to be regulated in part by AKT, making them of particular importance to this study. Other associated gene [products] that are relevant to this study include GSK3, FOXO1, and PFK (Phosphofructokinase; Manning *et al.,* 2007; Hemmings *et al.* 2012) that are associated with glucose metabolism. As mentioned previously, the role of the liver includes producing enzymes critical to toxicant metabolism and glucose metabolism (Burke, 1975). For this reason, and given the cited connection between arsenic exposure and diabetes (Navas-Acien *et al.,* 2008; Del Razo *et al*., 2011; Paul *et al.*, 2011; Swaran, 2011), we hypothesize expression of GSK3, FOXO1, and PFK to be affected in response

to arsenic exposure, potentially leading to altered glucose metabolism. AKT-mediated down-regulation of GSK3 prevents activation of the enzyme glycogen synthase, which lowers the rate of gluconeogenesis and raises glucose concentrations (Cohen *et al.*, 2001). Inhibited FOXO1 lowers hepatic glucose production and interferes with the differentiation of cells involved in metabolic control (Acilli *et al.,* 2004). Conversely, up-regulated PFK stimulates glycolysis, further raising glucose levels in hepatic tissue (Ono *et al.* 2003). The combined effects of these downstream targets of AKT in hepatic tissue suggest strong connection to the role of AKT in regulating glucose metabolism. Over-activated AKT (higher $\frac{\text{pAKT}}{\text{AKT}}$) can therefore potentially result in pathway disruptions associated with the development of type II diabetes mellitus (via a desensitization of insulin as a result of elevated glucose levels; Ono *et al.* 2003; Manning *et al.* 2007).

The diversity of downstream targets of AKT shown in Figure 3 also further shows how AKT acts as a regulatory connection between both metabolic activity and cell cycle progression. As noted earlier, AKT interconnected with upstream regulation of the MAPK cascade, which is involved in cellular proliferation, differentiation, survival, and death (Lopez-Ilasaca *et al.*, 1997; Kim *et al.,* 2010). Regulation of cell growth appears to occur primarily via activation of the mTOR complex 1 (mTORC1), which is involved in a negative feedback loop (Manning *et al.,* 2007). mTORC1 is a critical regulator of translation initiation that is extensively characterized, detailing its highly conserved role in cell growth control (Wullschleger *et al.,* 2006). AKT has also been proposed as a regulator of cellular proliferation via phosphorylation of MDM2. This phosphorylation is known to downregulate p53, which minimizes p21 expression (Mayo *et al.,* 2001; Zhou *et al.,* 2001). Tumor suppressor p53 is commonly recognized as one of the most

significant proteins involved in regulation of cellular growth and division, and has been labeled as "the guardian of the genome" (Lane, 1992). The p21 protein regulates the cell cycle at the G_1/S phase transition by inhibiting cyclin-dependent kinase complexes necessary for cell cycle progressions. In humans, p21 is encoded by the gene *Cdkn1a*. Patrick Carlson has shown that mice transplacentally exposed to 500ppb sodium arsenite exhibit a 3-fold decrease in *Cdkn1a* expression (Carlson, 2013c), which is consistent with results found in the literature (Mayo *et al.,* 2001; Zhou *et al.,* 2001). These findings support the rationale for studying AKT activity in response to arsenic exposure; this study is an attempt to further characterize the connection between arsenic, diabetes, and cancer.

Western Blotting:

All modern blotting protocols stemmed from the originally described Southern blots used to detect DNA fragments that had been separated by electrophoresis, named after the inventor Edwin Southern in the mid 1970's (Southern, 1975). By the end of the decade, Henry Towbin's lab at the Friedrich Miescher Institute in Switzerland had described the technique now known as western blotting (also immunoblotting), which has not changed much to this day (Towbin *et al.*, 1979). The technique was developed to meet the need for a visual assay for antigen specificity of monoclonal antibodies, though today its implications are far broader for immunodiagnostics, making it a ubiquitous research tool in molecular biology (Burnette, 2009). It should be noted that technological advancements have allowed for more effective detection techniques utilizing digital imaging systems, which has in turn allowed western blotting to be incorporated into molecular biology research to a much greater extent.

Major Findings:

Western blot analysis showed that low-dose transplacental arsenic exposure has minimal effects on mouse F_1 hepatic AKT and pAKT relative protein expression. No statistically significant differences were observed in a sex- or dose-dependent manner. However, an apparent decrease in the relative concentration of AKT in the active form was observed in animals exposed to sodium arsenite at 50ppb. There are several ways in which arsenic could induce AKT signaling via a dynamic web of inter-related pathways (Fig. 3), but it is important to note that unregulated activation of AKT generally leads to signal transduction promoting processes associated with cancer progression as well as disrupting processes associated with glucose metabolism and homeostasis.

Materials and Methods

Animals and arsenic exposures: Previous studies provided a foundational framework and tissues used for this study (Van Beneden *per. comm*.; Carlson *et al.,* 2013a,b,c). In short, 5-week-old C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and acclimated to a 14/10h light/dark cycle at 22ºC for 3 weeks in the University of Maine Small Animal Facility. Female mice (F_0) were fed standard mouse chow (5POO ProLab RMH 3000, Purina Mills LLC, St Louis, MO, USA) and exposed to 0, 10, 50 or 500 ppb sodium arsenite (Sigma, St. Louis MO, USA) in drinking water *ad libitum* beginning four days prior to mating. Eight males were used to mate with sixteen females; all females had successful litters. Direct arsenic exposure to the F_0 dams continued throughout mating, gestation and during nursing for 21 days post birth of the F_1 generation. Three male and three female F_1 pups from separate litters at each treatment level were moved to an arsenic-free water supply; the remaining animals were sacrificed.

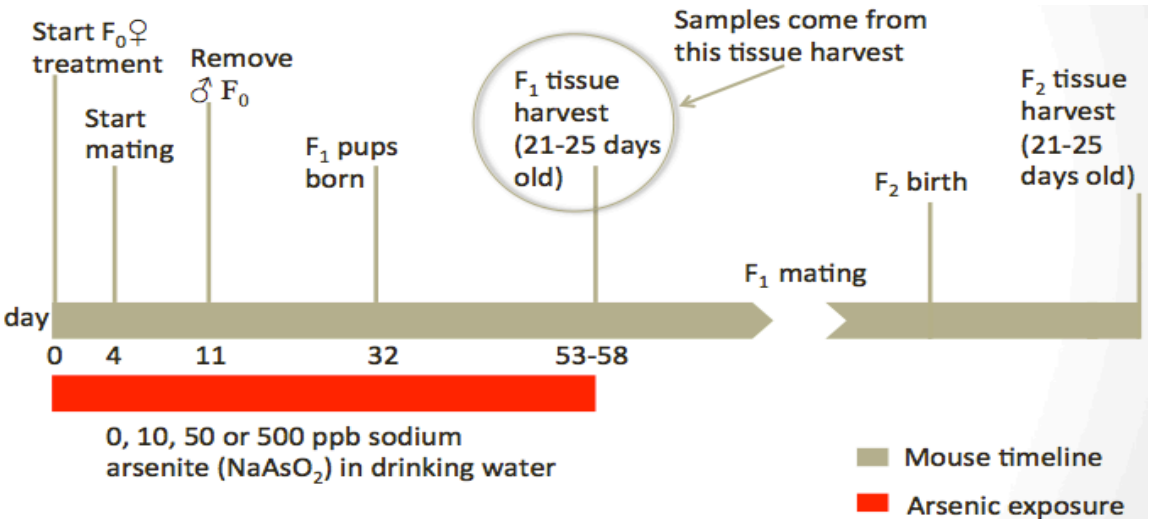


Figure 4: Timeline of transplacental mouse study experimental setup (modified from Carlson, 2013c).

A portion of the liver tissue was flash frozen in liquid nitrogen and stored at -80°C for gene expression analysis; a second portion was placed in 10% buffered formalin for histological analysis. F_1 mice were mated to produce an F_2 generation with no further arsenic exposure. At 21 days post birth, F_2 mice were sacrificed and liver tissue was collected as described above. All animals were handled in accordance with protocols approved by the University of Maine Institutional Animal Care and Use Committee (IACUC).

Sample Preparation: Hepatic tissue samples (3 males, 3 females at each exposure level in F_1) identified for study (Fig. 5) were taken from the same individuals previously tested for altered expression of other genes (Carlson *et al.,* 2013a), which allows us to increase the combined data set. The samples were labeled individually by harvest date and exposure level (*e.g.* the first animal sacrificed in December of 2010 that was exposed to arsenic at a concentration of 50ppb was labeled as 122010-50-1).

	0ppb	**10ppb**	**50ppb**	**500ppb**
Blot 1 (male)	102010-0-11	122010-10-07	122010-50-10	122010-500-08
Blot 3 (male)	122010-0-10	122010-10-05	122010-50-09	122010-500-07
Blot 5 (male)	122010-0-09	122010-10-06	122010-50-08	122010-500-06
Blot 2 (female)	122010-0-19	032011-10-02	032011-50-03	122010-500-17
Blot 4 (female)	122010-0-05	032011-10-04	032011-50-02	122010-500-15
Blot 6 (female)	122010-0-17	032011-10-05	042011-50-18	122010-500-19

Figure 5: Male and female animals from which hepatic samples were obtained, organized by exposure level and blot number. Note: each blot consists of a sample from each exposure level.

Homogenization: Tissue samples (prepared from F_1 hepatic tissues stored at -80ºC in the Van Beneden lab) with mass of approximately 5mg were homogenized on ice in 200µL PhosphoSafe buffer (EMD Millipore, Dermstadt, Germany) with added protease inhibitors (Complete Protease Inhibitors, Roche Diagnostics, Mannheim, Germany) using a micro-pestle. Homogenates were then centrifuged at 14,000xg for 10 minutes at 4ºC; the supernatant was extracted and the pellet was discarded. Protein supernatants were stored at -80ºC.

Protein Quantification: Total protein concentration was determined via BCA assay (Pierce Biotech, Rockford, IL) following supplier's protocol.

Western Blot Analysis: Loading Samples (20µL total volume) were prepared to 40µg total protein in distilled water with 5µL NuPage loading dye (Life Technologies, Carlsbad, CA) and 2µL reducing agent (Life Technologies). Controls were prepared using 10µL Jurkat cell extracts treated with Calyculin A (Cell Signaling, Danvers, MA), 3µL distilled water, 5µL dye, and 2µL reducer. Samples were heated for 10 minutes at 70ºC to denature proteins and spun at 14,000xg for 30 seconds before loading onto Novex NuPAGE pre-cast SDS-polyacrylamide 10% bis-tris gels (Life Technologies). Protein size-standard markers added to marker lanes were a SeeBlue Plus2 Prestained Standard (Life Technologies) and a SuperSignal Molecular Weight Protein Ladder chemiluminescent marker (ThermoScientific, Philadelphia, PA). Proteins were separated by SDS-PAGE using MOPS running buffer (stock 20X: 50mM MOPS, 50mM Tris Base, 0.1% SDS, 1mM EDTA, pH 7.7; Life Technologies; diluted to 1X concentration in distilled water) at 160v for approximately 1 hour or until the loading dye reached the foot of the gel. Proteins were then transferred to an Immobilon PVDF membrane (Millipore)

using transfer buffer (50 mL 20x stock, 100mL methanol, 850mL dH_2O; Life Technologies) at 35v for 1 hour. Electrophoresis and transfer were performed using an XCell *SureLock* Novex Mini-Cell apparatus (Life Technologies) following manufacturer's protocol.

Post-transfer gels were stained in Coomassie staining solution (1g Brilliant Blue R, 500mL methanol, 100mL acetic acid, 400mL dH_2O) and subsequently placed in de-staining solution (450mL methanol, 100mL acetic acid, 450mL dH_2O) to observe band separation. Gels were preserved using a gel drying solution (368mL ethanol, 25mL glycerol, 607mL dH_2O) and clamped overnight between sheets of gel drying cellulose film (Promega, Madison, WI). A Ponceau stain was performed to determine transfer efficiency, prepared using Ponceau S stock solution (2.5mL 20x stock, 47.5mL dH_2O; Sigma). Briefly, membranes were placed in stain for 5 minutes before being rinsed with dH_2O to observe bands. With bands present, membranes were marked to be cut after blocking, which allowed for separate primary incubations. Membranes were then rinsed with 0.1M NaOH to remove stain and subsequently washed in dH_2O with frequent water changes to remove residual NaOH.

Non-specific protein binding sites were blocked with 2.5% bovine serum albumin (BSA) in TTBS (90g NaCl, 50mL 1M Tris, pH 7.6, 10mL Tween 20, dH_2O to 1L) for 1hr at room temperature before membranes were cut and segments were separately incubated in primary antibody (AKT and pAKT 1:500, β-Actin 1:1000 in 2.5% BSA) overnight at 4ºC. Membranes were then washed 4 times with TTBS before incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Jackson Immuno Inc., West Grove, PA) for 1hr at room temperature. Final washes with TTBS

(4x 5min) followed with TBS (NaCl, Tris, pH 7.6; 5x 5min) were performed before imaging. Proteins were visualized using LumiGLO chemiluminescent substrate (KPL, Gaithersburg, MD) and a digital imager (FUJIFilm LAS4000). Finally, membranes were stained in Coomassie staining solution and compared to gels for qualitative assessment of transfer efficiency.

Primary antibodies used for analysis were anti-β-Actin (Cell Signaling, Danvers, MA), anti-AKT (Cell Signaling), and anti-Phospho-AKT (Ser473; Cell Signaling). All membranes were incubated with the same volume of luminescent substrate and imaged with a five second exposure time. Gel Analyzer software was used to generate densitometry values for relative expression of protein bands on the western blots. β-Actin values were normalized to an average expression derived from a pAKT-stimulated Jurkat cell extract control used for each blot to adjust for blot-to-blot variability. These values were then used to normalize AKT and pAKT values on each blot. Relative AKT activity was determined using the ratio of relative $\frac{\text{pAKT}}{\text{AKT}}$ expression generated by densitometry analysis; significance was determined using a two-tailed t-test, one-way ANOVA, and Tukey's test.

Results

Western blot analysis produced signal bands, representing relative targeted protein expression, for each sample tested. However, the antibody targeting AKT produced a much stronger "signal" than the antibody for pAKT. The exception of this is found in the pAKT-stimulated control Jurkat cell extracts, as shown in Figure 6. Jurkat extracts were used to normalize for blot-to-blot variability and to determine antibody specificity. As shown in Figure 6, the pAKT-stimulated controls also displayed signal for inactive AKT. This could be due to the actual presence of AKT despite pAKT stimulation, or it could be due to a lack of pAKT antibody specificity. Because of this, samples were analyzed for relative $\frac{\text{pAKT}}{\text{AKT}}$ rather than determine relative pAKT expression alone.

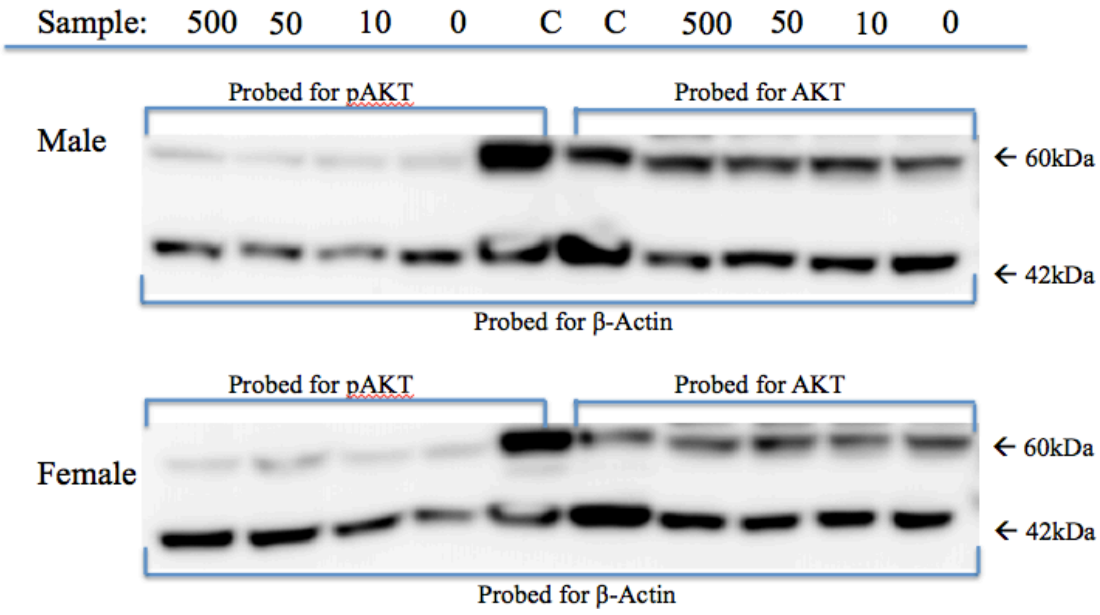
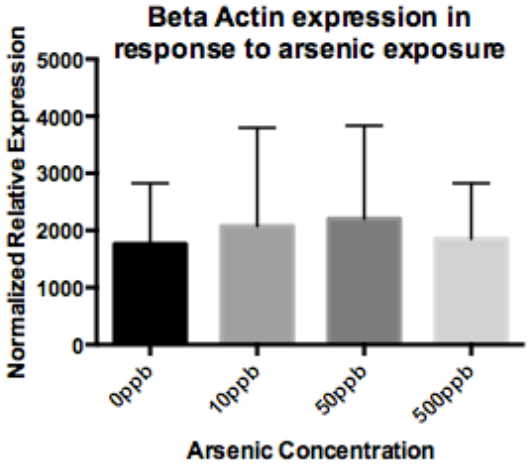


Figure 6: Representative pAKT-, AKT-, and β-Actin targeted western blot bands at different exposures. Note: the same sample is run in separate lanes to target AKT and pAKT individually (Molecular weights are 60kDa and 62kDa respectively, making them difficult to distinguish when probed together).

Before normalizing AKT and pAKT expression values to β-Actin, it was important to explore the effects of arsenic treatment on β-Actin expression. Given cited findings of actin cytoskeletal rearrangement in response to arsenic exposure (Bernstam *et al.,* 2010), it is critical that AKT expression is not normalized to β-Actin if β-Actin itself exhibits a response to arsenic. One-way ANOVA followed by Tukey's test showed no significant difference in β-Actin expression at any arsenic exposure level, as shown in Figure 7.



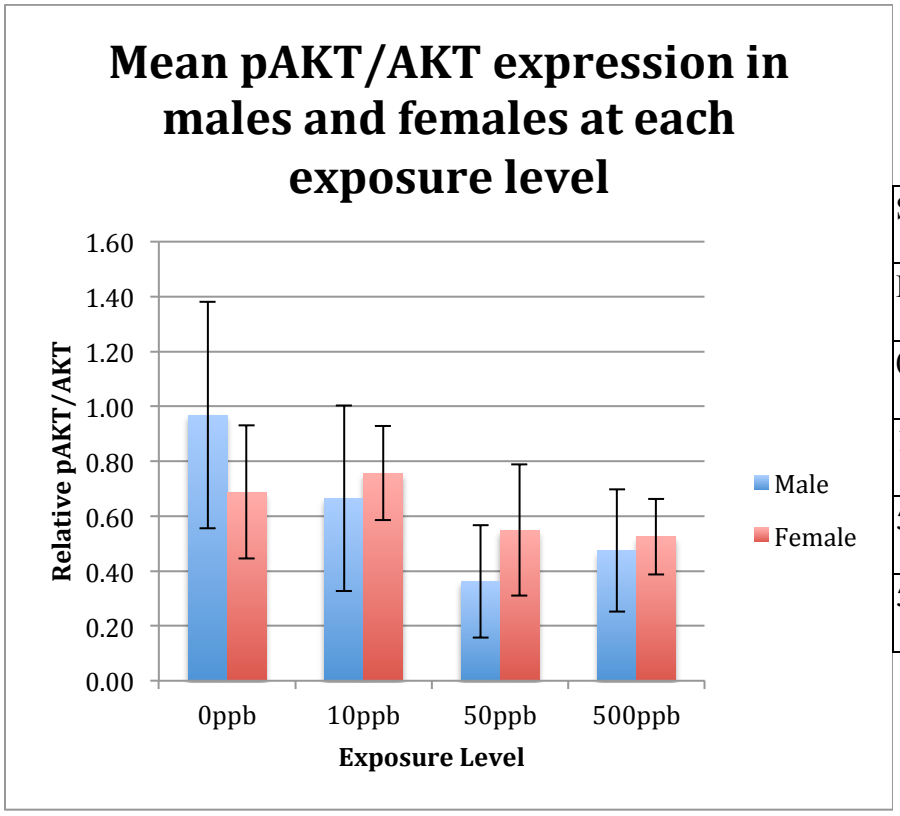
ANOVA summary	
F	0.2128
P value	0.8869
P value summary	ns
Are differences among means statistically significant? (P < 0.05)	No
R square	0.01742

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0ppb vs. 10ppb	-312.9	-1983 to 1357	No	ns
0ppb vs. 50ppb	-442.9	-2113 to 1227	No	ns
0ppb vs. 500ppb	-92.42	-1763 to 1578	No	ns
10ppb vs. 50ppb	-130.0	-1800 to 1540	No	ns
10ppb vs. 500ppb	220.5	-1450 to 1891	No	ns
50ppb vs. 500ppb	350.5	-1320 to 2021	No	ns

Figure 7: Analysis of β-Actin expression in response to arsenic exposure shows no significant dose-dependent changes when normalized to Jurkat cell extract controls. Bars represent ±SEM, n=6 for each exposure, sexes combined.

With determination of β-Actin as a reliable control for lane-to-lane variability within each blot, AKT and pAKT values (also normalized to the average of the Jurkat control to account for blot-to-blot variability) could then be normalized to β-Actin expression for each sample. Normalized AKT and pAKT expression values were then compared relative to each other to determine a value of $\frac{\text{pAKT}}{\text{AKT}}$ that was used as a representation of AKT activity in each sample. It should be noted that AKT and pAKT expression were also both analyzed independently, and no significant effects were observed in a sex-dependent or dose-dependent manner in response to arsenic exposure.

Sex-dependent change in related gene product expression was observed in previous studies (Carlson, 2013b,c) so a paired t-test was performed between males and females at each exposure level. The results showed no significant differences (Fig. 8).

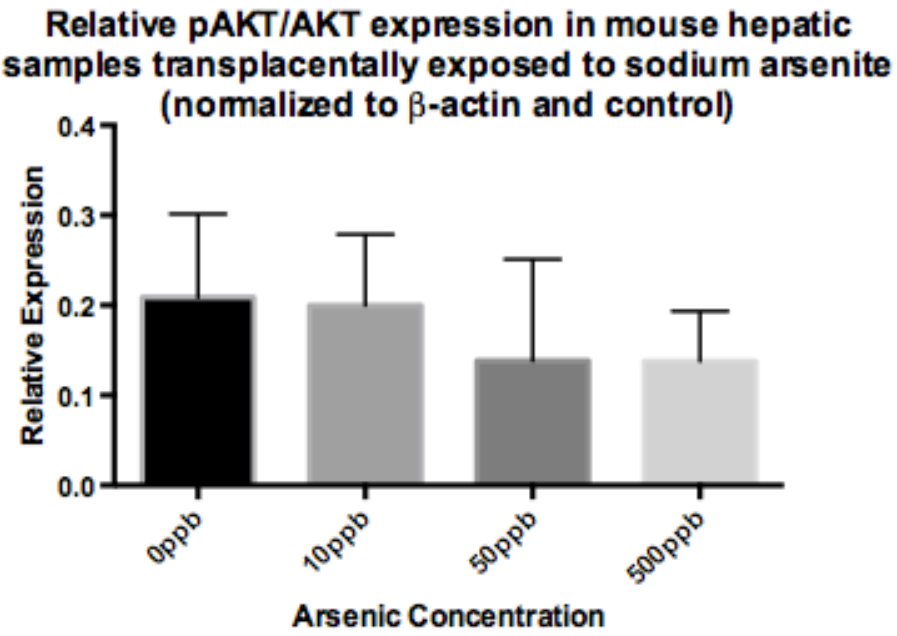


Student's t-Test	
Exposure	P-Value
0ppb	0.2942
10ppb	0.7266
50ppb	0.5025
500ppb	0.7702

Figure 8: Sex-dependent analysis of pAKT/AKT expression after arsenic exposure. Bars represent ±SEM, n=3 for each sex in each exposure group.

By determining that there is no statistical difference in relative $\frac{\text{pAKT}}{\text{AKT}}$ expression in a sex-dependent manner, the data for both sexes could be combined to increase the sample size for further analysis of dose-dependent response to arsenic exposure.

It was hypothesized that arsenic exposure would alter relative $\frac{\text{pAKT}}{\text{AKT}}$ expression in a dose-dependent manner, though not necessarily in a linear fashion. Previous studies have shown significant dose-dependent expression changes in a non-linear fashion (Carlson, 2013b,c). Because arsenic exposure has been linked to several types of cancers as well as increased rates of diabetes (Ratnaike, 2003; Duker, 2005), it was further hypothesized that more active-state AKT would be found in arsenic-exposed animals than in the control group. This is because of the association of AKT with pathways involved in cellular growth and division as well as glucose metabolism (Manning *et al*., 2007). A one-way ANOVA with Tukey's test showed no significant difference in $\frac{\text{pAKT}}{\text{AKT}}$ expression at any level of arsenic exposure (Fig. 9).

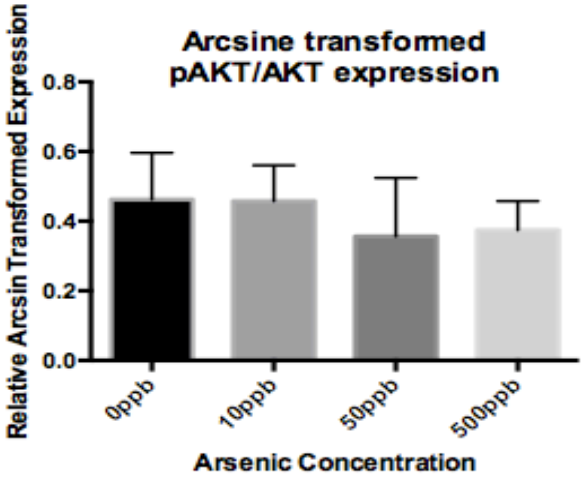


ANOVA summary	
F	0.9546
P value	0.4379
P value summary	ns
Are differences among means statistically significant? (P < 0.05)	No
R square	0.1518

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?
0ppb vs. 10ppb	0.008600	-0.1498 to 0.1670	No
0ppb vs. 50ppb	0.0701	-0.08835 to 0.2285	No
0ppb vs. 500ppb	0.07048	-0.08797 to 0.2289	No
10ppb vs. 50ppb	0.06150	-0.09695 to 0.2199	No
10ppb vs. 500ppb	0.06188	-0.09657 to 0.2203	No
50ppb vs. 500ppb	0.0003800	-0.1581 to 0.1588	No

Figure 9: Dose-dependent analysis of pAKT/AKT expression after arsenic exposure. Bars represent ±SEM, n=5 for each exposure, sexes combined.

The data show that transplacental arsenic exposure at 50ppb and 500ppb appears to result in lower relative $\frac{\text{pAKT}}{\text{AKT}}$ expression, though the results are not statistically significant. Notably, the same results are generated when using the accepted arcsine data transformation, suggesting that data distribution does not impact apparent findings. This is shown in Figure 10.



ANOVA summary	
F	0.9424
P value	0.4434
P value summary	ns
Are differences among means statistically significant? (P < 0.05)	No
R square	0.1502

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?
0ppb vs. 10ppb	0.00544	-0.2228 to 0.2337	No
0ppb vs. 50ppb	0.1057	-0.1226 to 0.3339	No
0ppb vs. 500ppb	0.08758	-0.1407 to 0.3158	No
10ppb vs. 50ppb	0.1002	-0.1280 to 0.3285	No
10ppb vs. 500ppb	0.08214	-0.1461 to 0.3104	No
50ppb vs. 500ppb	-0.01810	-0.2464 to 0.2102	No

Figure 10: Dose-dependent analysis of arcsine transformed data showing pAKT/AKT expression after arsenic exposure ±SEM, n=5 for each exposure, sexes combined.

Data transformations are useful for biological data because natural variability will oftentimes produce data sets without homogenous standard deviations. Making comparisons between one set of data with a small deviation to another set with a large deviation cannot be accurately done with an ANOVA because it depends on those deviations to produce results. Arcsine transformations are frequently used for analysis of proportion statistics with values ranging from zero-to-one (McDonald, 2014). These data look at the proportion of pAKT found in a sample relative to the total amount of AKT found in the same sample, with values ranging from zero-to-one. For that reason, it was useful to observe any differences in statistical analysis using arcsine-transformed data.

The results of this study show via western blot analysis that AKT activity in mouse livers is not significantly altered in a sex- or dose-dependent manner after transplacental arsenic exposure. These results are upheld with and without normalization techniques as well as with and without data transformation techniques.

Discussion

Chronic arsenic exposure has historically been linked to skin diseases, such as black foot disease (Basu *et al.,* 2001). In more recent years, studies have described correlations between arsenic exposure and cancers and diabetes mellitus (Ratnaike, 2003; Duker, 2005). Understanding of the link between arsenic and these diseases has been limited by the complicated molecular mechanisms of action. Arsenic exposure has been shown to have sex-, generational-, and dose-dependent effects, but the apparent complex and inter-connected pathways have proved difficult to marry into a holistic description of molecular toxicological effects (Ferrario *et al*., 2008; Paul *et al.,* 2011; States *et al*., 2011; Stueckle *et al.,* 2012; Carlson *et al.,* 2013b,c). Because the Van Beneden lab had observed several arsenic-induced expression changes of gene products involved in cancer-associated pathways as well as glucose metabolism (diabetes-associated) pathways, exploring AKT activity showed potential to help explain the link between these two major pathways (Carlson *et al.,* 2013b,c).

Although no significant expression changes were observed, it should be noted that more studies are necessary to support these findings before AKT response to arsenic can be fully understood. This study is a useful pilot that shows no major response at the protein level, but that does not necessarily mean there is no significant change in relative expression on the protein level at all, and it does not address a potential arsenic-induced expression response on the mRNA transcript level. It is well known that there is often a difference in relative transcript expression when compared to relative protein expression. As the field of functional genomics continues to expand, the dynamic regulation of expression at all levels becomes further complicated— only adding to the importance of

exploring responses beyond the protein product level.

Prior Studies:

Patrick Carlson observed increased *Pparγ* expression and decreased expression of *Wee1* and *Brca2* in response to environmentally relevant arsenic exposure. He suggested that these expression changes were AKT-mediated, and proposed the following pathway:

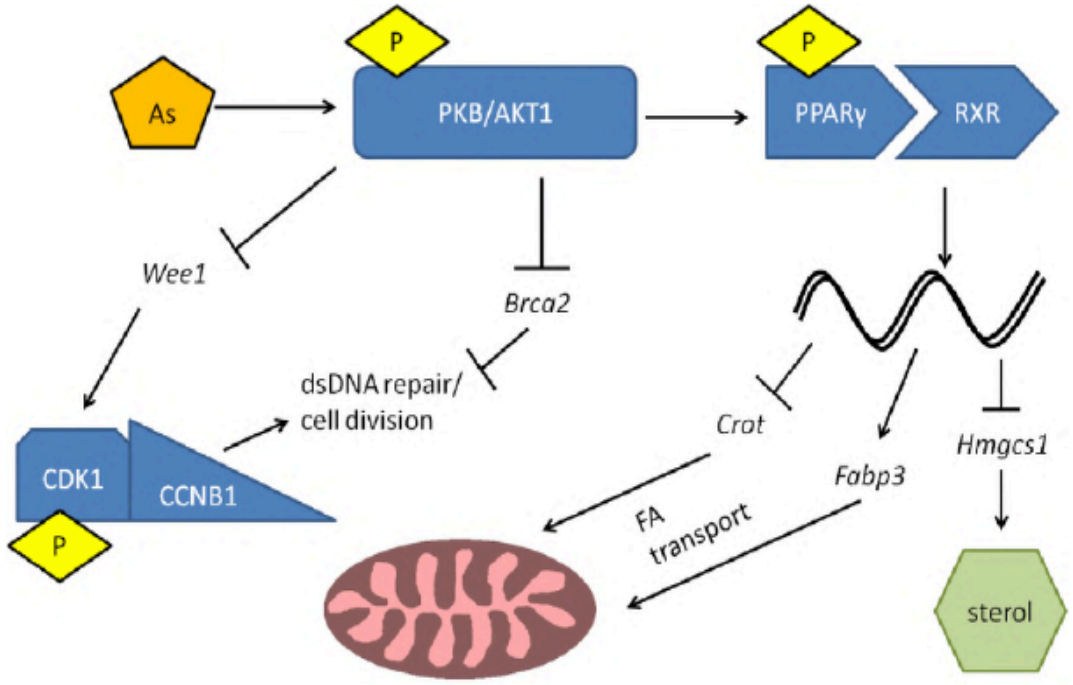


Figure 11: Proposed mechanism of arsenic toxicity. Arrows indicate activation, while bars indicate inhibition. Abbreviations: As, arsenic; P, phosphate; RXR, retinoid X receptor; CDK1, cyclin dependent kinase 1; CCNB1, cyclin B1; FA, fatty acid (Carlson, 2013c).

Aberrant AKT phosphorylation in response to arsenic is an instrumental point to the pathway proposed above. Carlson hypothesized that AKT phosphorylation could be altered in response to mutation of its upstream regulators, such as RAS, PTEN, and PI3K, which are cited as commonly mutated (Manning *et al.,* 2007; Yuan *et al.*, 2008; Carlson, 2013c). Studies exploring the effects of arsenic exposure on the abovementioned protein

expression could be instrumental in further explaining the outlined response pathway, as they would provide insight to AKT regulation. In the hypothesized pathway (Fig. 11) the downstream effects of AKT activity range from PPARγ activation (leading to suppression of *Crot* and *Hmgcs1*, potentially deregulating fatty acid transport mechanisms as well as sterol synthesis) to repression of *Wee1* and *Brca2* (potentially leading to impaired DNA repair mechanisms and the onset of cellular division). This proposed pathway—based on results of prior studies—further demonstrates the significance of AKT as a link between cell cycle regulation and metabolism.

Western Blotting:

Western blotting is a well-known diagnostic technique to analyze relative expression of specific target antigens and are generally "semi-quantitative" in nature. Reasons for this include variations in loading and transfer rates from lane-to-lane and blot-to-blot that are accounted for only with imprecise normalization techniques, as well as non-linear detection of the signal generated (Mahmood, *et al.* 2012). Furthermore, western blotting is notorious for its many problems that can arise during a relatively simple procedure. Many of these problems were observed throughout the development of this project and led to a slight change in experimental plan. For example, high background noise and non-specific binding was observed, which led to an extensive trial-and-error process of determining proper blocking buffer, antibodies and dilutions, and wash procedures. Also, it was expected that blots would be visualized using a LiCor C-DiGit scanner, but after a month of troubleshooting no workable images were produced. For these reasons and more, the project was limited to exploring relative AKT and pAKT

expression only in F_1 samples, though F_2 samples were also identified for study.

Experimental Design:

It should be noted that AKT phosphorylation state has been described as temporary and is often reversed within minutes (Fig. 12), though the mechanisms of this negative regulation are not fully understood (Yuan *et al.*, 2011).

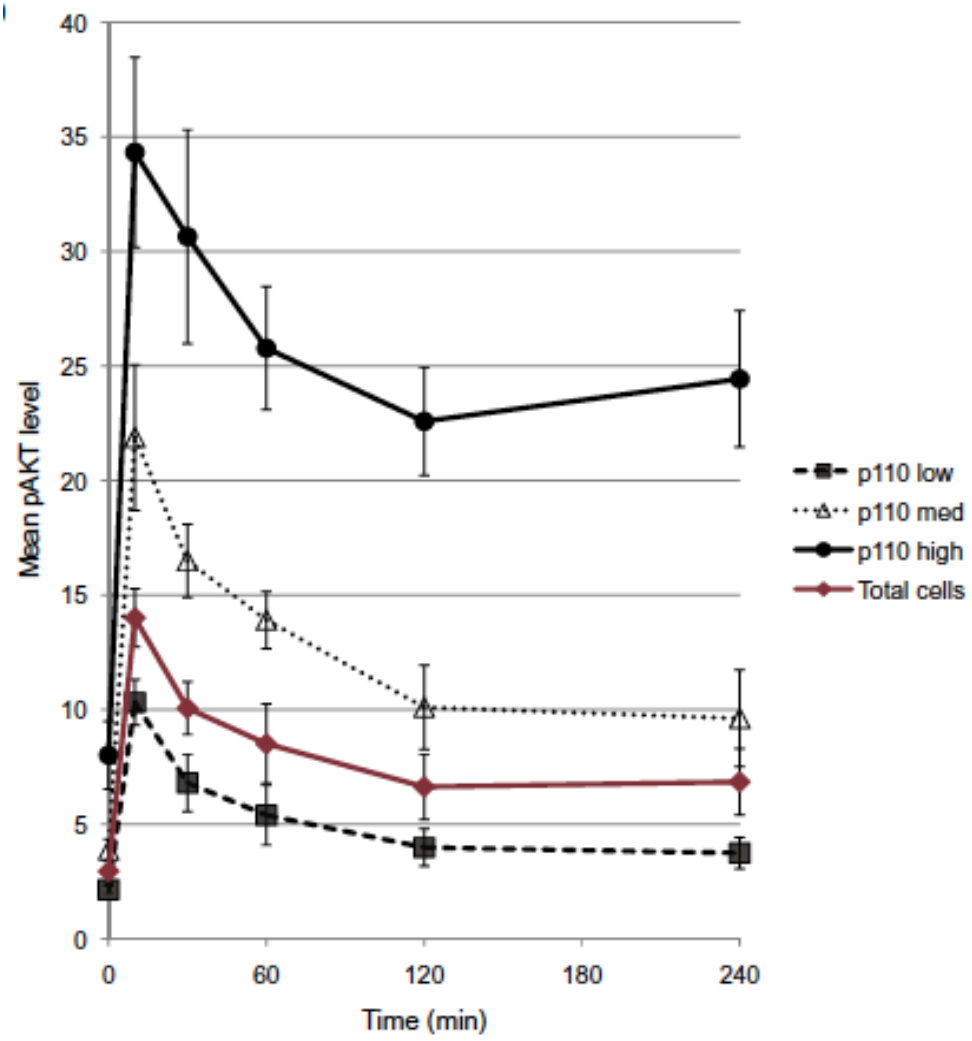


Figure 12: pAKT level observed over time after EGF stimulation of growth factor-starved cell populations (Yuan *et al*., 2011). Cells were characterized by level of p110 (catalytic subunit of PI3K; see figure 2) expression. Note "Total cells" pAKT level over 2 hour period as well as the apparent importance of p110 in pAKT regulation.

Yuan, Cantley, and colleagues show not only that relative pAKT expression is variable over a short time frame, but also that it is highly variable among subpopulations of cells within the same tissue. This has implications for this study based on the experimental design. For example, if one mouse had recently eaten a lot in the time before sacrifice, it could have vastly different relative AKT activity than a mouse in the same exposure group that had not recently eaten. Also, western blot analysis shows relative expression of the entire population of cells in the sample tested. Cell-to-cell variability shows potential to introduce further complexity to the understanding of AKT regulation (Yuan *et al*., 2011). Furthermore, this study was performed using hepatic tissue samples derived from different lobes of the liver, which can have significantly different relative protein expression. Mammalian studies addressing expression profile differences between liver lobes caution to recognize a lack of homogeneity throughout the organ (Cox *et al.,* 2006). Given the small sample sizes used, this study would benefit from a more thorough analysis using more specific samples (*i.e.* all samples taken from the same lobe of the liver) from a much larger sample size.

Future Directions:

As mentioned, the Van Beneden lab is now positioned to continue studies of mouse hepatic tissue transplacentally exposed to environmentally relevant levels of inorganic arsenic. Next steps should include further analysis of AKT activity. This project only addressed the F_1 generation, but it would be useful to explore the F_2 generation as well. It would be useful to explore generation-dependent expression in response to arsenic to further understand any direct insult response or epigenetic response

spanning across generations (Carlson, 2013c). It is also important to expand the study to a larger sample size to increase significance, and the Van Beneden lab has the tissues prepared to do just that. Furthermore, it would also be useful to partner western blot analyses with other laboratory techniques (*ie*. ELISA, IHC, and qPCR) in order to come to a more definitive conclusion.

Beyond further analysis of AKT activity, this study would also be supported greatly by exploring upstream and downstream expression. Upstream, it would be of significant benefit to this project to have information about PI3K response because it is the most significant and well-cited upstream regulator (Engelman *et al.,* 2006; Franke, 2008; Hemmings *et al.,* 2012). Downstream, it would be useful to expand on the understudied subject of glucose metabolism in response to arsenic. As mentioned previously, because this project examines hepatic tissue, AKT pathway analysis is poised to give information on arsenic-induced diabetes development. Suggestions of genes to look at include those noted earlier— GSK3, FOXO1, and PFK, as shown in Figure 13. These gene products are involved (directly or indirectly) in the regulation of glucose metabolism, and they are known to be targets of pAKT, making them particularly relevant to this project.

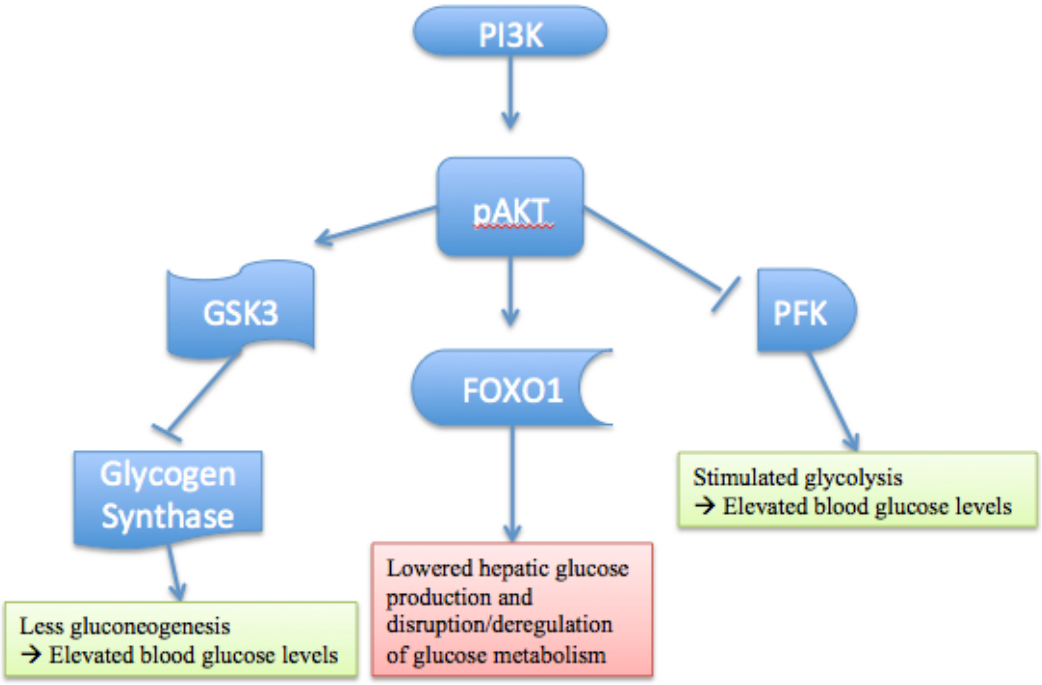


Figure 13: Example of pAKT downstream signaling in glucose metabolism pathways.

All of these studies would be very time demanding and would require significant skills in acquired laboratory techniques to generate reproducible results, but would "tell the story" of arsenic response more fully. I hope that this project will be followed up in the future because investigations into how environmental toxicants impact our health are of critical importance to public health measures.

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Author's Biography

Jay Knowlton was born in Portland, Maine on November 11th, 1993. He grew up in the mid-coast community in Camden, Maine, attending Camden Hills Regional High School for three years before being admitted to the University of Maine to study biology. Jay has participated as active member of the Senior Skull Society, the Phi Kappa Sigma fraternity, the Student Portfolio Investment Fund, the Honors College Student Advisory Board, and several other organizations. He sees opportunities for extra-curricular involvement as one of the strongest attributes to the University and has enjoyed developing his passions in Orono. He also had the incredible opportunity to spend a Semester at Sea, traveling to fifteen countries in the fall of 2013. Jay will graduate with Honors on May 9th, 2015 with a Bachelor of Science in Biology. He was accepted to the University of Southern Maine for a Masters of Public Health program with a specialization in health management beginning in the fall of 2015.

Jay enjoys traveling, spending time with family and friends, and being outdoors. He is an avid skier, golfer, and hiker, as well as a boating and auto enthusiast. Jay is passionate about health and medicine, and looks forward to a career working to reform the United States health care system in an effort to improve care at a sustainable cost. He hopes to work around the country and around the world, but looks forward to eventually returning to Maine to raise a family.