The Epigenetic Effects of Prenatal Folate Supplementation on Male CD1 Mouse Fetuses Exposed *in utero* to Arsenic

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Abstract

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(Under the direction of Miroslav Styblo and Zuzana Drobna)

Inorganic arsenic (iAs) is a common drinking water contaminant and transplacental carcinogen in mice, and possibly in humans. Recent animal studies suggest the mechanism of iAs carcinogenesis may include competition for S-adenosylmethionine required for both iAs and fetal DNA methylation, causing aberrant gene expression and cancer in adulthood. We exposed mouse dams to 0 or 85ppm iAs in drinking-water while feeding them a diet containing either 2.2 or 11mg/kg of folate. At gestational day (GD) 18, we examined DNA methylation patterns in fetal livers using CpG-island microarrays. Our results show that compared to folate supplementation alone, combined exposure to iAs and folate dramatically increased the number of CpG islands with altered methylation patterns. The most affected genes were associated with the cancer, neurological development, and cell signaling networks. This data raises concern about the efficiency and potential risks associated with folate supplementation in human populations chronically exposed to iAs.

Sarah, Mom, and Dad,

thank you for your unwavering support;

thank you for helping me to be so proud of whom I am today.

Acknowledgements

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List of Abbreviations

5-MTHF	5-Methyltetrahydrofolate
Ahcy	Adenosylhomocysteinase
ALL	Acute Lymphoblastic leukemia
APC	adenomatous polyposis coli
As3mt	Arsenic (+3 oxidation state) methyltransferase
Cbs	Cystathionine-β-synthase
Cdkn	Cyclin-dependent kinase
CKI	Cyclin dependent kinase inhibitor-1
Cnnd1	Cyclin-D1
CT	Control group
Ctnnb1	β-catenin
Daxx	Death domain associated protein
DI	Deionized
DKK3	Dickkopf-3
Dlk1	Delta-like homologue-1
DMAs	Dimethyl-arsenic
Dnmt	DNA methyltransferase
FA	Folic acid
FS	Folate-supplemented group
Fzd	Frizzled
GD	Gestational day
GSK3β	Glycogen synthase kinase 3β
HCC	Hepatocellular carcinoma
Hcy	Homocysteine
HPLC	High performance liquid chromatography
iAs	Inorganic arsenic
Igf2bp1	Insulin-like growth factor binding protein 1
Igf2r	Insulin-like growth factor 2 receptor
IP	Immunoprecipitation

IPA	Ingenuity Systems Pathway Analysis
LINE-1	Long interspersed nuclear element 1
MAs	Monomethyl-arsenic
Mat	Methionine adenosyltransferase
Met	Methionine
MS	Methionine synthase
Mtrr	Methyltetrahydrofolate-homocysteine methyltransferase
PCR	Polymerase chain reaction
ppb	Parts per billion
ppm	Parts per million
qRT-PCR	Quantitative reverse-transcription PCR
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SAM-software	Significance Analysis of Microarrays
SFRP	Secreted frizzled-related proteins
TBS	Tris-buffered saline
TBST	Tris-buffered saline 0.1% Tween
THF	Tetrahydrofolate
TMAs	Trimethyl-arsenic
TMeV	TIGR Multi-experiment viewer

Chapter I – Arsenic Metabolism and its Epigenetic Effects

Introduction

Inorganic arsenic (iAs) is a common drinking water contaminant and potent carcinogen (NRC 1999, IARC 2004). As such, iAs contamination poses a significant global health problem, particularly in developing countries. In Bangladesh, about half of the ten million tube wells installed during the last 30 years deliver water above the World Health Organization (WHO) recommended limit of 0.01 micrograms of iAs per liter of water (μg/L), or 10 parts per billion (ppb) (WHO 2004, Jakariya 2005). Even in developed countries like the United States, iAs is delivered via drinking water at levels higher than the WHO limits; data gathered by the United States Geological Survey estimates that over 7.5% of all public water supply systems exceed the target concentration of 10 ppb (Focazio 2000), the maximum contamination level also established by the United States Environmental Protection Agency (US EPA 2006).

Prolonged iAs exposure results in the complex disease arsenicosis; advanced stages are associated with cancers of the skin, lungs, liver, urinary bladder, prostate, and kidney (IARC 2004). An estimated 700,000 people are afflicted with arsenicosis in South and East Asian nations (The World Bank, 2005). 21 million people in Bangladesh are exposed to arsenic concentrations at or above 50 ppb; that figure would double if the WHO standard of 10 ppb were adopted (Smith 2000). The contamination of groundwater by iAs in Bangladesh is considered to be the largest poisoning of a population in human history (Smith 2000), where an estimated 6,500 people will die from iAs-related cancers every year, and 2.5 million people will develop some form of arsenicosis over the next fifty (Maddison 2004). Even in the United States, the WHO and EPA limits of 10ppb arsenic in water is difficult to achieve, particularly in western states like Utah (Focazio 2000, Steinmaus 2005).

iAs affects DNA methylation, an epigenetic change that can lead to cancer

Especially in the pre- and post-natal environments, exposure to various chemicals capable of modifying DNA methylation can adversely affect the adult phenotype (Baccarelli 2009). The mechanism(s) by which exposure to iAs causes cancer are unclear, though a growing body of evidence suggests that its carcinogenicity can be attributed, in part, to an epigenetic mode of action. Arsenic has been observed to induce changes in DNA methylation. For example, iAs exposure in HaCaT cells is associated with genome-wide hypomethylation, presumably due to down-regulation of DNA methyltransferases (DNMTs) (Reichard and Puga 2007). Similarly, in mouse models, sodium arsenite in drinking water increased genome-wide hypomethylation, and gene-specific hypomethylation of *Ha-ras*, an oncogene (Okoji 2002).

DNA methylation is an epigenetic process that involves the enzymatic addition by DNA methyltransferases of methyl groups to the 5-position carbon in the pyrimidine ring of cytosine in cytosine-guanine (CpG) dinucleotides located along the genome. Methylation of clusters of CpG dinucleotides (known as CpG islands) located in the promoter region of genes reduces access of transcription factors to the promoter, preventing transcription of downstream genes. By affecting gene transcription and subsequently inducing differential gene expression, cells of different tissues with identical genetic material can use DNA methylation to create varying phenotypes without altering the underlying DNA sequence. Proper DNA methylation is essential for tissue differentiation during critical growth periods like embryogenesis.

Improper gene expression resulting from aberrant DNA methylation during these critical periods is implicated in a variety of diseases, most notably cancer. For example, global genomic hypomethylation is exhibited in many human cancers, including prostate tumors, leukemia, liver carcinomas, and cervical cancer (Bedford 1987, Erlich 2002). On the other hand, hypermethylation has also been linked to a variety of cancers. Increased CpG island methylation causes inactivation of the tumor suppressor p16; subsequent loss of transcription is associated with lung cancer, gliomas, and oropharyngeal squamous cell carcinomas, and is characteristic of many cancer cell lines (Herman

1995). Methylation of the von Hippel-Lindau gene, a tumor suppressor, is associated with transcriptional inactivation and development of clear-cell renal carcinomas (Herman 1994). Exposure to chemicals that inhibit DNA methylation like 5-azacytidine, 5-azadeoxycytidine and adenosine dialdehyde has been shown to potentiate carcinogenesis in the rat liver (Rao 1989).

Arsenic has been shown to cause changes in DNA methylation, disrupting transcriptional activity and downstream gene expression (Reichard 2007, Okoji 2002, Liu 2006, Xie 2007, Chanda 2006). DNA extracted from blood samples of Bengali people exposed to arsenic in drinking water showed significant promoter-region hypermethylation of the tumor suppressors p53 and p16 in a dose-dependent manner (Chanda 2006). A number of studies have demonstrated that prenatal exposure of mice to iAs is associated with development of hepatic, adrenal, pulmonary and urogenital cancers in adult offspring. *In utero* iAs exposure is further associated with activation of oncogenes such as Ha-ras, C-myc and Cyclin-d1, resulting in hepatocarcinogenesis in male fetuses (Okoji 2002, Liu 2006, Davis 2004, Waalkes 2003). These and other literature has provided evidence for a potential mechanism by which iAs induces cancer.

SAM, SAH, and one-carbon metabolism

Accurate maintenance of DNA methylation requires several factors working in concert, including DNMT activity, methyl group availability, DNA integrity, and cell proliferation (Laird and Jaenisch 1996). DNMTs use the universal methyl donor S-adenosylmethionine (SAM) for enzymatic methylation reactions (Zeisel 2009, Chiang 1996). SAM is synthesized by the methionine adenosyltransferase (MAT) enzyme family from methyl groups derived from methionine (Chiang 1996). Nearly half the methionine from the diet is converted to SAM in the liver (Lu 2008).

As SAM is consumed for transmethylation reactions, S-adenosylhomocysteine (SAH), an inhibitor of MAT and DNMT enzymes, is produced (Duerre 1981, Cox 1977). SAH elimination is accomplished via hydrolysis to adenosine and homocysteine (Hcy) by adenosylhomocysteinase (*Ahcy*). Hcy can then be methylated to re-form methionine by methionine synthase, which derives methyl groups from folate, or betaine Hcy methyltransferase (*Bhmt*), which uses methyl groups

donated by betaine, a metabolite of choline (Zeisel 2009, Chiang 1996, Lu 2008). These two enzymes, along with the substrates and nutrients choline and folate, play an integral role in the maintenance of SAM levels in the liver (folate contributions, Figure 1). Therefore, dietary availability of choline and folate can directly affect DNA methylation patterns of the genome and subsequently, the epigenetic profile of an organism.

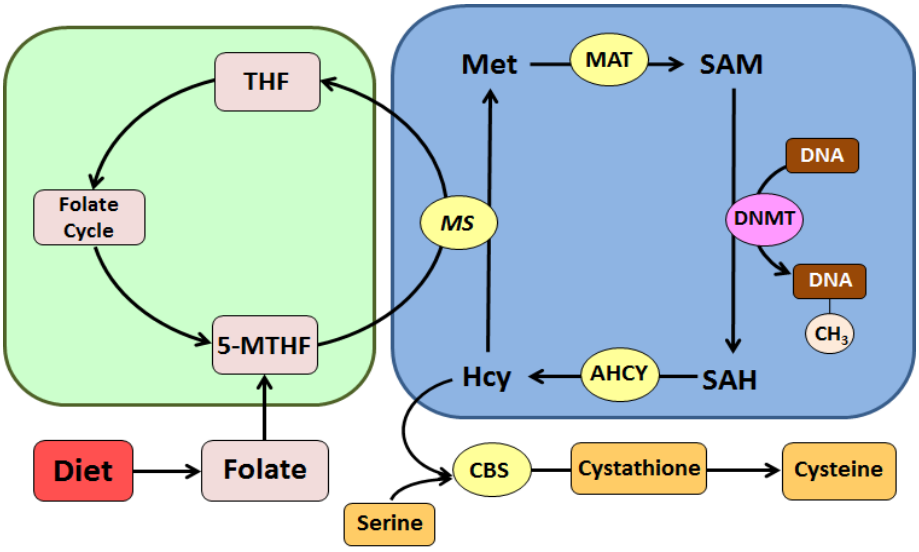


FIGURE 1. A schematic outlining the role of dietary folate in the folate cycle with some of the enzymes (embedded in ovals) responsible for one-carbon metabolism regulation. (THF, tetrahydrofolate; 5-MTHF, 5-methyl-tetrahydrofolate; MS, methionine synthase; Met, methionine; MAT, methionine adenosyltransferase; DNMT, DNA methyltransferase; AHCY, adenosylhomocysteinase; CBS, Cystathionine-β-synthase)

Dietary influences on DNA methylation

Inadequate intake of methyl group donors like choline and folate can alter epigenetic processes involving DNA methylation. For example, differences in folate status in humans were shown to correlate with genomic DNA methylation and inversely with plasma homocysteine levels (Friso 2002). C57BL/J6 mice fed folate-deficient diets exhibited reduced levels of SAM in the flat small intestine, resulting in significant DNA hypomethylation (Sibani 2002). Of groups of Fischer 344 rats fed either supplemented or deficient choline diets, those given deficient diets demonstrated DNA hypomethylation in hepatocytes (Locker 1986). Oppositely, choline-supplemented diets given to Sprague Dawley rats on gestational days 11 to 17 altered gene expression in mammary tumors in

offspring and resulted in their prolonged survival (Kovacheva 2009). Taken together, these studies demonstrate that DNA methylation is affected by the availability of methyl groups derived from the diet, and can influence a variety of clinical outcomes.

Arsenic methyltransferase requires SAM to methylate iAs

Inorganic arsenic and its trivalent methylated metabolites are potent inhibitors of enzymes as well as modulators of key signal transductions pathways in mammalian cells including the NF-κB, MAPK and apoptotic pathways (Hu 2002, Kumagai 2007, Zhong 2010). For this reason, excretion of iAs is vital. iAs is metabolized to mono-, di-, and tri-methylated arsenicals (MAs, DMAs, and TMAs, respectively) in the trivalent and pentavalent (i.e., MAs^{III} and MAs^{V}) oxidation states (Figure 2). Methylation is carried out by arsenic (+3 oxidation state) methyltransferase (AS3MT), and requires SAM as the methyl group donor (Thomas 2007). Inhibition of AS3MT activity results in the accumulation of iAs in tissues (Marafante 1985, Vahter 1987). Chronic low-dose exposure to arsenic has been shown to reduce cellular SAM concentration in human keratinocytes (Reichard 2007). Reduced intake of choline, folate or methionine results in impaired iAs methylation as suggested by decreased urinary excretion of DMAs in rabbits (Vahter 1987); in hamsters, decreased urinary excretion of methylated arsenic metabolites was associated with increased organ toxicity (Hirata 1990). Alternatively, dietary supplementation with folate has been shown to increase the proportion of DMAs excreted in urine of human subjects, suggesting that more iAs is being methylated and detoxified more efficiently (Gamble 2007).

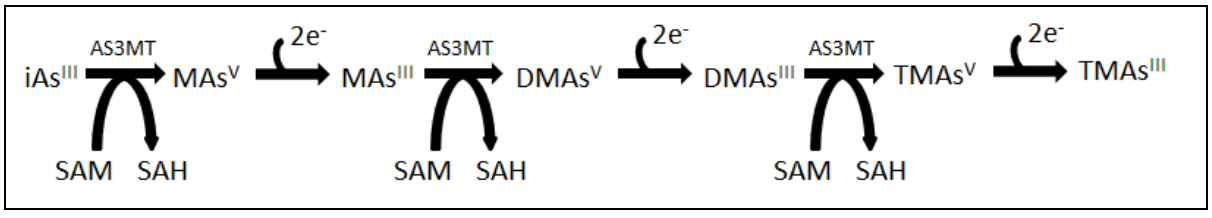


FIGURE 2. A schematic modeling the alternating enzymatic methylation of trivalent inorganic arsenic by arsenic (+3 oxidation state) methyltransferase (AS3MT) with the reduction of pentavalent arsenicals to their respective trivalent species. Arsenite (iAs^{III}) is methylated to form pentavalent monomethyl-arsenic (MAs^{V}), and reduced to trivalent MAs (MAs^{III}). MAs^{III} is methylated to $DMAs^{V}$ and reduced to $DMAs^{III}$. Finally, in some species, $DMAs^{III}$ is methylated to pentavalent trimethylated-arsenic ($TMAs^{V}$) and reduced to $TMAs^{III}$.

iAs Exposure alters the global DNA methylation profiles in animal models

Until recently, no appropriate animal models were available to help elucidate the mechanism of arsenic-induced carcinogenesis. Humans appear to be much more sensitive to arsenic toxicity than other mammals (Vahter 2000). Furthermore, primates like marmosets and chimpanzees do not appear to methylate arsenic (Vahter 1985). This variability in toxicity and methylation capacity of iAs reduces the translational significance of animal studies. However, gestation has been identified as a period of high sensitivity to chemical carcinogenesis in rodents; evidence suggests that the same applies for humans (Waalkes 2004b, Anderson 2000). Studies have demonstrated that arsenic is able to cross the placenta and enter the fetus during gestation in mice (Chattopadhyay 2002, Devesa 2006) and in humans (Concha 1998).

Recent work by Dr. Waalkes and colleagues (NCI/NIEHS) with pregnant female C3H and CD1 mice exposed to iAs in drinking water (42.5 or 85 parts per million, ppm) from gestational days 8 through 18 demonstrated a dose-dependent occurrence of hepatic and adrenal cancers in adult male offspring; in adult female offspring, pulmonary and urogenital cancers were identified (Waalkes 2006). Tissues affected by transplacental carcinogenesis in this mouse model were analogous to those affected in human arsenic carcinogenesis (Waalkes 2004b), thus providing a viable mouse model for human arsenic carcinogenesis. It should be noted that although concentrations of iAs given to pregnant mice were much higher than what is considered typical for human exposure, mice metabolize iAs more efficiently, and are less susceptible to iAs-induced cancers than are humans (Basu 2001).

Epigenetic changes have been implicated in the genesis of arsenic-related cancers. Hypomethylation of the promoter regions of estrogen receptor-α (*Er-α*) and cyclin D1 (*Ccnd1*) due to *in utero* arsenic exposure are responsible for increased expression of both genes leading to an aberrant estrogen signaling and cyclin D1 expression, with subsequent development of hepatocellular carcinomas (Waalkes 2004a, Deane 2001). As a cell cycle regulator, cyclin D1 is a known hepatic oncogene (Deane 2001). Elevated levels of both cyclin D1 and ER-α transcripts were found in liver

samples from adult men with a history of arsenicosis (Waalkes 2004a), further solidifying the relevance of the transplacental mouse model in studying human iAs exposure.

Impact of iAs metabolism on DNA methylation

Thus far, the mechanism by which arsenic modifies DNA methylation in the mouse transplacental carcinogenesis model has not yet been characterized. The hypothesis proposed over a decade ago (Goering 1999) describes two potential mechanisms diagrammed in Figure 3. First, methylation catalyzed by AS3MT competes with DNMTs for SAM and second, iAs and/or its metabolites modify the expression or activity of DNMTs and other one-carbon metabolism genes. These mechanisms can result in hypomethylation (by limiting SAM availability for DNA methylation) or hypermethylation (by causing a compensatory increase in SAM synthesis or in DNMT expression); both scenarios are known to cause cancer (Erlich 2002). It is also possible that the mechanisms work in tandem, resulting in a mosaic of hyper- and hypomethylated genes or clusters of CpG islands in a tissue and gene specific manner consistent with published data.

The efficacy of either mechanism is at least somewhat dependent on the availability of SAM and the efficiency of its synthesis. Abundant SAM availability (e.g., through supplementation with choline or folate) could limit the deficiency of SAM arising from competition for methyl groups between DNMT and AS3MT. The efficiency of iAs methylation would increase, limiting the accumulation of iAs and its metabolites in tissues as well as subsequent effects of these arsenicals on DNMT expression or activity. Conversely, SAM deficiency (e.g., due to dietary deficiencies of choline or folate) could exacerbate the competition for methyl groups and impair the conversion of iAs to DMAs, allowing iAs and its metabolites to accumulate in tissues. In either case, the availability of SAM precursors folate, choline, and methionine should modify the effects of iAs exposure on DNA methylation and alter carcinogenesis associated with iAs exposure.

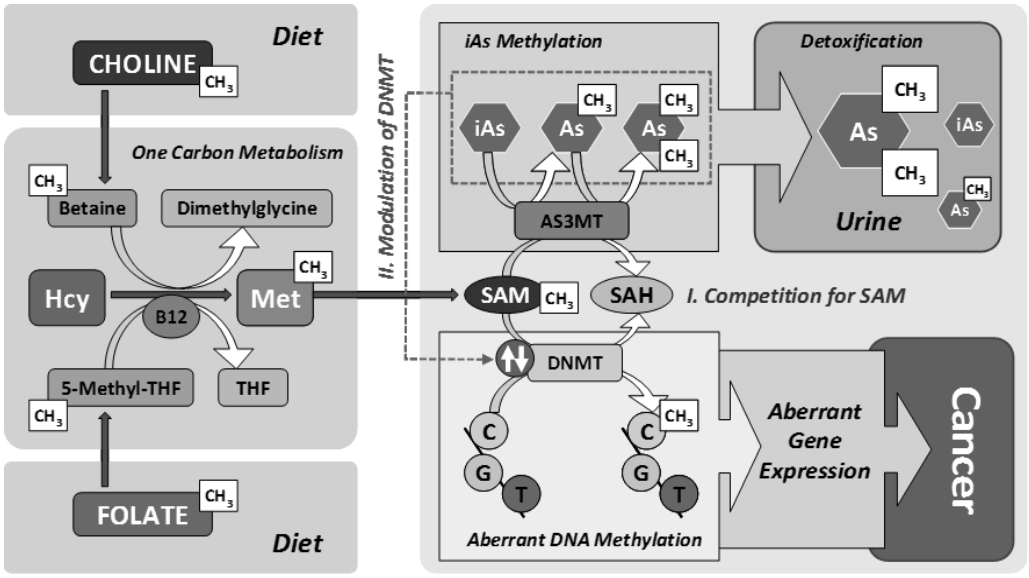


FIGURE 3. Two potential mechanisms underlying the effects of iAs exposure on DNA methylation. (I) Competition between AS3MT and DNMT for SAM and (II) modulation of DNMT expression and/or activity by iAs or its metabolites retained in tissues. The left panels demonstrate the role of dietary folate and choline in modifying both these mechanisms. (Hcy, homocysteine; Met, methionine; THF, tetrahydrofolate; B12, Vitamin B12; C, cytosine; G, guanine; T, thymidine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.)

Summary

We adopted the transplacental carcinogenesis CD1 mouse model as described by Michael Waalkes' research group at NIEHS to test our hypothesis that folate supplementation would mitigate changes in one-carbon metabolism, DNA methylation, and gene expression in fetal livers following *in utero* iAs exposure. We hypothesized that the provision of extra methyl groups could reduce SAH and concomitantly increase SAM availability to support an increased methylation burden imposed by iAs exposure, or by preventing arsenic modulation of enzymes involved in one-carbon metabolism and transmethylation reactions. In the mouse model, liver cancer was observed to be a sexually dimorphic disease, occurring in male offspring exposed *in utero* to iAs (Waalkes 2004a, Nohara 2010). For this reason, the bulk of analyses in our study were conducted in the livers of male fetuses.

Briefly, we exposed timed-pregnant dams to 0 or 85ppm arsenic from gestational day (GD) 8 to 18, and to 2.2 or 11 mg of folic acid per kg of diet from GD 5 to 18 (Figure 4). We collected tissues from dams and fetuses at GD18 for analyses to identify biomarkers, hepatic DNA methylation and

gene expression profiles, and biological pathways significantly affected by these treatments, paying special attention to cancer-related pathways.

The goals of our study were to examine (1) whether there was an effect of arsenic on fetal development, iAs metabolism, DNA methylation, the one-carbon metabolism pathway and imprinted genes; (2) whether any of the observed effects were mitigated by dietary folate supplementation; and (3) whether collective methylation changes implicate potential biological pathways to explain the previously described endpoint of liver cancer in male fetuses due to *in utero* arsenic exposure.

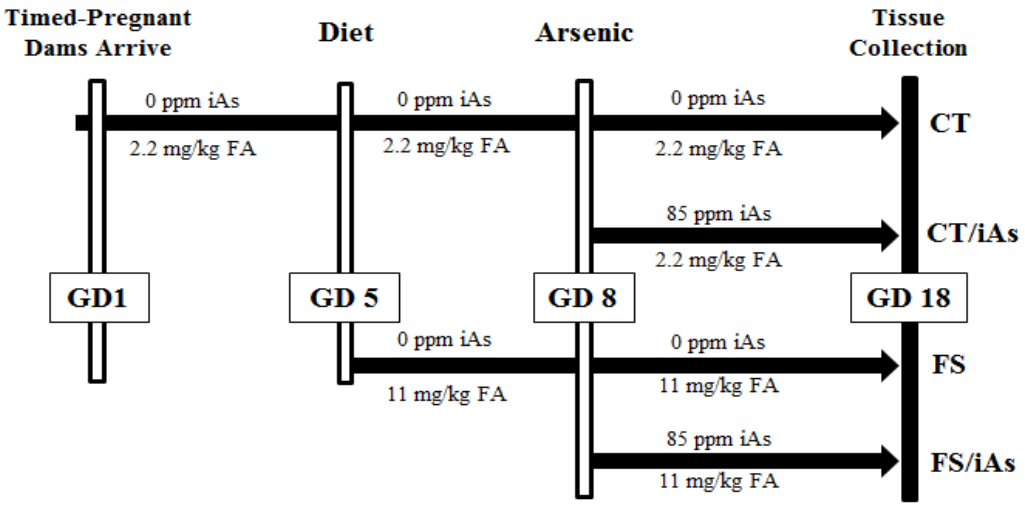


FIGURE 4. Schematic of treatment timeline. On GD1, dams were given 0 ppm iAs in drinking water and 2.2mg/kg folic acid (FA). On GD5, half the dams were given a diet of 11mg/kg FA, and on GD8, the two diet groups were each split into two smaller groups, one given 85 ppm iAs in drinking water, and the other was kept on 0 ppm iAs. Tissues were collected from the four groups at GD18.

Results

iAs consumption and folate supplementation modulate maternal and fetal health

From GD 5 to 18, timed-pregnant CD1 mice were fed purified diets containing either 2.2 (control, or CT) or 11.0 mg/kg (folate supplemented, or FS) folate. Dams drank either 0 or 85ppm iAs in deionized (DI) water from GD 8 to 18. Analyses of DNA methylation and gene expression were conducted on the GD 18 fetal tissues, primarily the liver. Our 2 (Diet: CT vs. FS) by 2 (Exposure: water vs. iAs) experimental design yielded the following four groups: CT, CT/iAs, FS and FS/iAs. Relative gene expression levels in fetal (GD18) liver for each treatment group were subjected to separate Diet (control vs. folate) x Exposure (water vs. iAs) between-subjects ANOVAs for each gene of interest (for more detail, see Statistical Analyses in the Methods section).

Prior to assessing the effects of iAs exposure and folate supplementation on mouse fetuses on a molecular level, we needed to determine the effect of treatment on maternal consumption behavior. When controlling for number of fetuses, neither Diet nor Exposure altered food consumption by dams (*p*s = n.s.; Figure 5A). Two-way ANOVA revealed a significant effect of Exposure for maternal water consumption, $F(1, 34) = 23.10$, $p < 0.001$, but no effect of Diet or Diet x Exposure interaction. Notably, water consumption was significantly lower by almost 3 grams of water per day in dams on iAs-water compared to their diet matched DI-water groups (*p*s < 0.05; Figure 5B). For maternal plasma folate levels, there was a significant effect of Diet, $F(1, 34) = 175.83$, $p < 0.001$, but no effect of Exposure or Diet x Exposure interaction. We observed an increase in plasma folate of approximately five-times in groups supplemented with folate compared to control diet, (*p*s < 0.05), (Figure 5C). Weights of fetuses at GD18 were approximately 10% lower in CT/iAs treatment group compared to CT, and almost 25% less comparing the FS with FS/iAs group (*p*s < 0.05; Figure 5D).

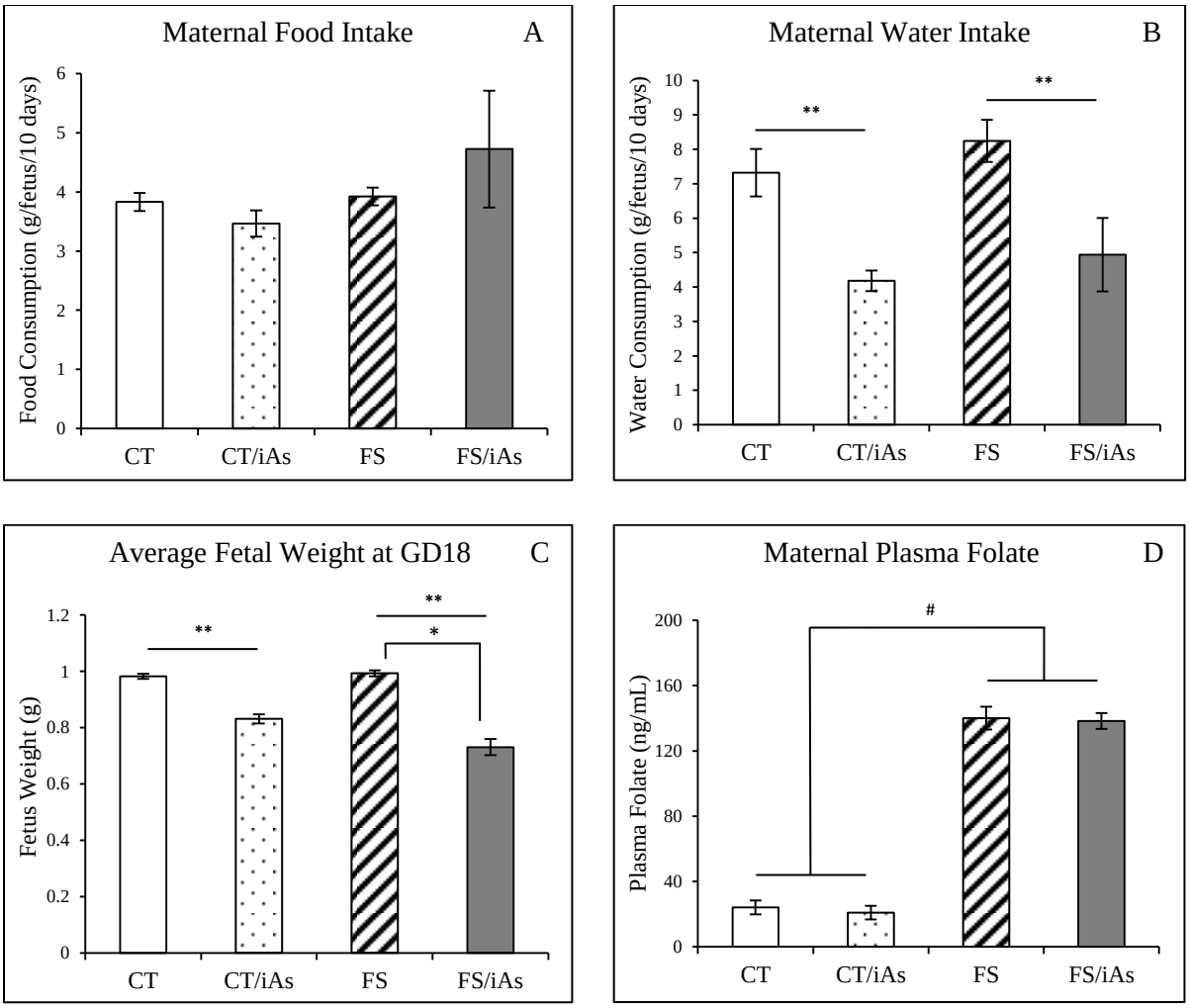


FIGURE 5. Preliminary results of animal monitoring including food consumption (A) and water intake (B), both expressed as average intake in grams per day per fetus, as well as average fetal weight (C) as measured on gestational day 18 and folate concentration in maternal blood (D). * Denotes significantly different at $P<0.05$; **, main effect of Exposure, $P<0.05$; and #, main effect of Diet, $P<0.05$, revealed by two-way ANOVA (exposure x diet). Error bars represent ±SEM.

Exposure to iAs significantly increased both SAM, $F(1, 34) = 8.61$, $p < 0.01$, and SAH, $F(1, 34) = 9.73$, $p < 0.01$, levels in livers of fetuses (Figure 6A and 6B). There was also an effect of Diet for SAM levels, $F(1, 34) = 7.61$, $p = 0.01$; folate supplemented fetuses had higher levels of SAM in the liver than those on the control diet (Figure 6A). Although levels of both SAM and SAH are higher in livers of fetuses on iAs, more important is the SAM/SAH ratio, which serves as an index of the transmethylation potential (Williams 2007). To that end, there was a strong trend for an effect of Exposure for SAM/SAH ratios, $F(1, 34) = 3.52$, $p < 0.07$, in the liver where SAM/SAH ratios

appeared lower in both groups consuming iAs (Figure 6C). Planned comparisons revealed that compared to CT fetuses, FS/iAs animals had lower SAM/SAH ratios approaching significance ($p < 0.06$; Figure 6C), suggesting poorer transmethylation potential in FS/iAs fetuses compared to CT.

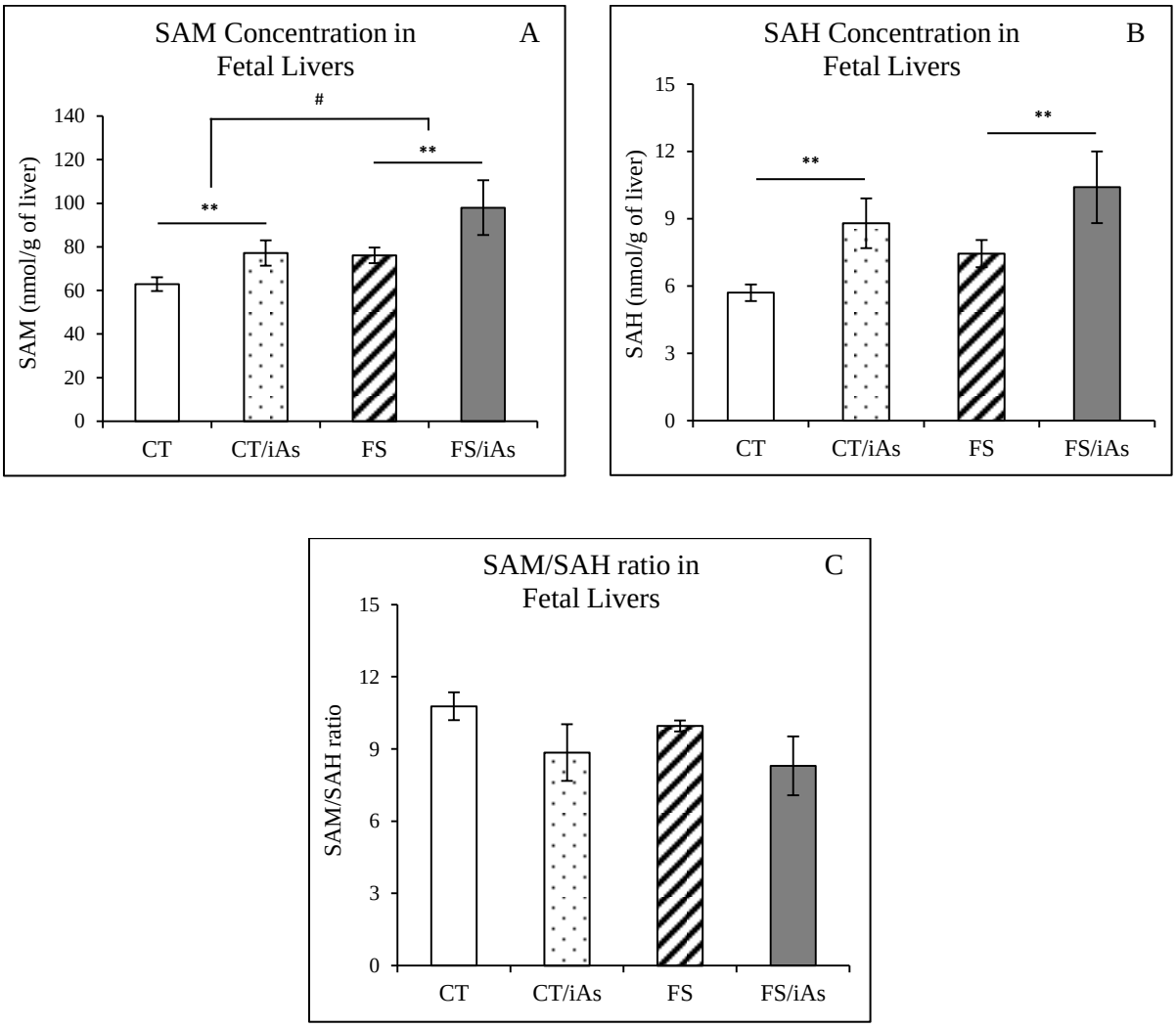


FIGURE 6. Concentration of the universal methyl donor SAM (A) and product of transmethylation reactions SAH (B), as measured in nmol/g of fetal liver tissue by HPLC. An index of transmethylation potential as measured by the SAM/SAH ratio (C). **, Main effect of Exposure, $P<0.05$; and #, main effect of Diet, $P<0.05$, revealed by a two-way ANOVA (exposure x diet). Error bars represent ±SEM.

Analyses of arsenic metabolites in the liver of dams and fetuses confirmed transport of iAs and its metabolites across the placenta, though levels in fetuses were lower than that of dams (Figure 7). Exposure significantly increased levels of iAs, $F(1,30) = 17.36$, $P<0.001$; MAs, $F(1,31) = 140.62$, $P<0.001$ and DMAs, $F(1,29) = 75.36$ $P<0.001$ in dams (Figure 7B, D and F). Interestingly, a Diet x Exposure effect was noted in dams indicating a significant decrease in iAs comparing CT/iAs to FS/iAs, $F(1,3) = 32.89$, $P<0.001$. In fetuses, iAs-exposure did not affect iAs, but did significantly increase MAs, $F(1,30) = 52.75$, $P<0.001$ and DMAs, $F(1,31) = 318.25$, $P<0.001$ in the liver (Figures 7A,C and E). Planned comparisons noted a small, but significant increase in MAs and DMAs comparing FS and CT fetuses (Figure 7C and E).

Total arsenic burden (iAs + MAs + DMAs) was higher in dams in the CT/iAs group than the FS/iAs group, though this was largely due to the significant decrease in iAs, as opposed to its metabolites (Figure 7B). Percent of total arsenic represented by the metabolites MAs and DMAs were approximately 65% in CT/iAs maternal livers, and about 78% in FS/iAs livers. Levels of metabolites (MAs + DMAs) in CT/iAs livers versus FS/iAs livers were approximately the same, at 2070 ng As/g of liver and 2083 ng As/g of liver, respectively.

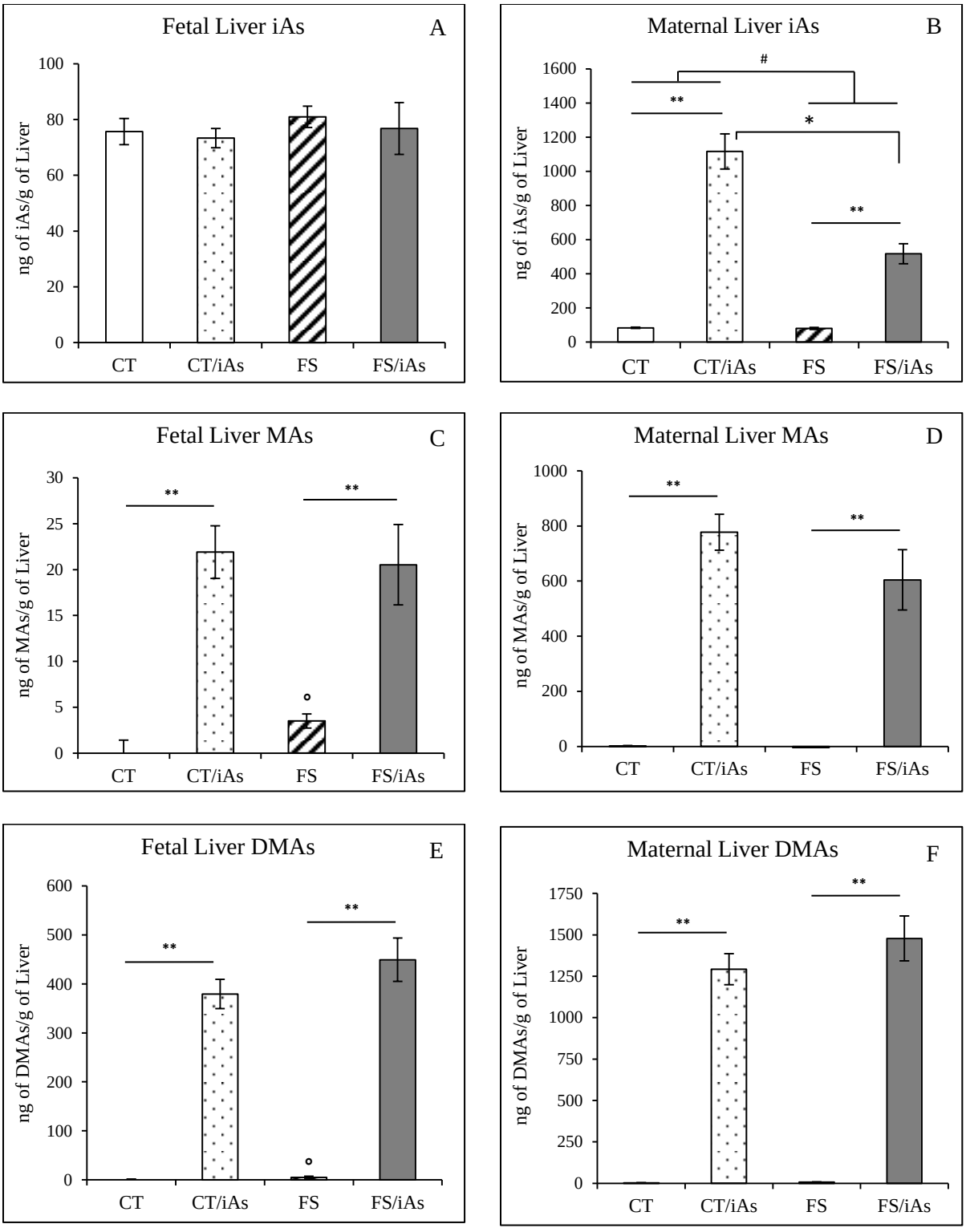


FIGURE 7. Concentration of iAs and its biological metabolites, monomethyl-arsenic (MAs) and dimethyl-arsenic (DMAs) in fetal (panels A, C, E) and maternal (panels B, D, F) livers in ng of arsenic per gram of liver. * Denotes significantly different (P<0.05); °, significantly different from CT (P<0.05); **, main effect of Exposure (P<0.05); #, main effect of Diet (P<0.05) revealed by two-way ANOVA (Exposure x Diet). Error bars represent ±SEM.

Folate supplementation with or without iAs treatment affects gene methylation in the fetal (GD18) liver

The software package Significance Analysis for Microarrays (SAM-software), embedded within the Multi-Experiment Viewer of the TM4 Microarray Software Suite (Saeed 2006) was used to analyze the DNA methylation microarrays and changes between treatment groups using a two-class unpaired response model. The SAM-software plots a visual representation of the distribution of differentially methylated probes (Figure 8). Plotted data indicated whether particular probe sequences were hyper- or hypomethylated compared to other groups; delta values (d-values) were computed by the SAM-software as a measurement of strength between extent of DNA methylation and the log-ratio of Cy3/Cy5 signal. The d-value cutoff was assigned based on a desired significance with a maximum 5% false discovery rate.

These data show that iAs exposure alone had minimal impact on DNA methylation in the fetal liver, whereas folate supplementation resulted in a larger change. The combination treatment of iAs and folate supplementation influenced gene methylation patterns to a much greater extent than either treatment alone. Specifically, folate supplementation combined with iAs consumption yielded the changes in methylation profiles of 5,357 genes, whereas folate supplementation showed changes in 253 genes compared to control. iAs consumption alone yielded only 4 differently-methylated genes compared to controls (Table 1).

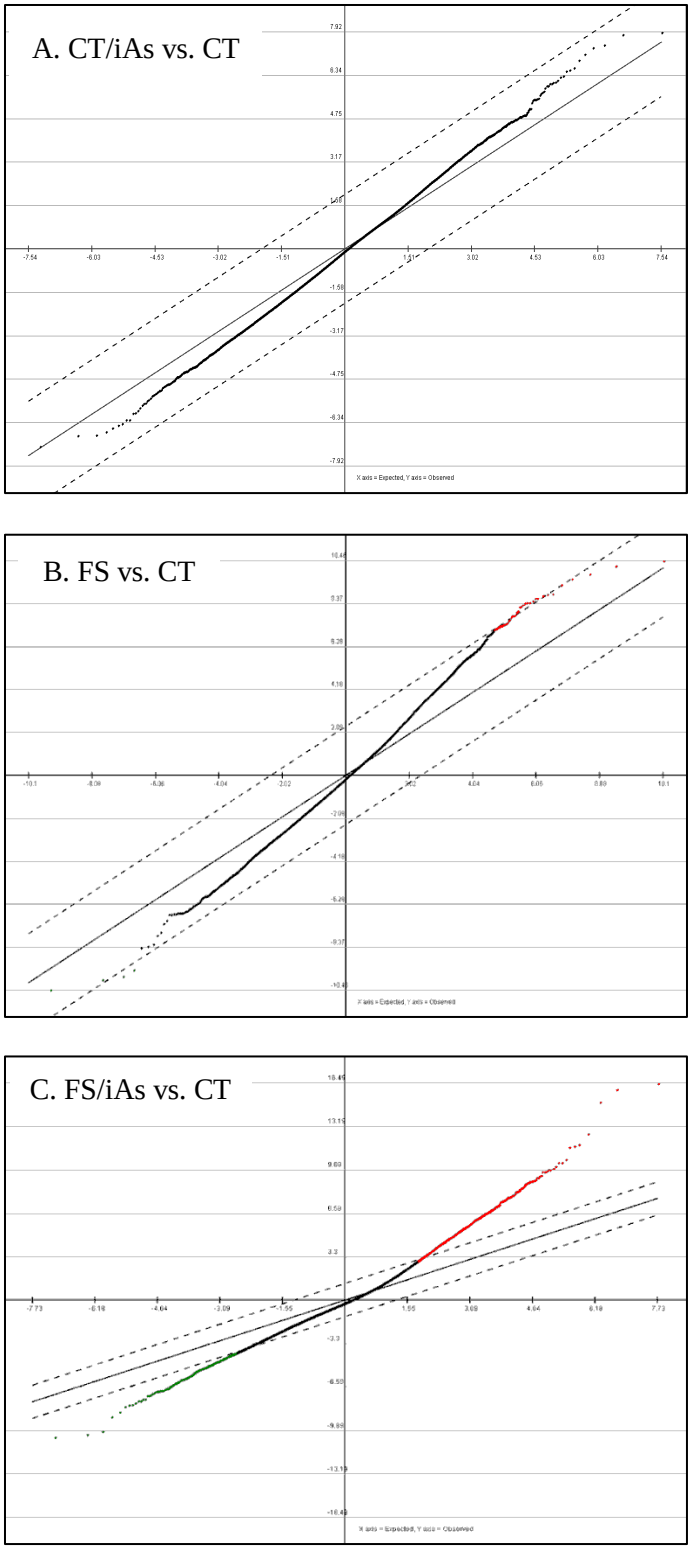


FIGURE 8. Visual plots of DNA probes comparing expected (*x*-axis) to observed (y-axis) methylation values. Points lying outside the selected delta-value (dashed lines) set at a false discovery rate (FDR) of 5% are either significantly hypermethylated (red) or hypomethylated (green) as calculated by Significance Analysis of Microarrays (SAM-software) within TIGR Multi-experiment Viewer (TMeV) in fetal liver tissue of CT/iAs (A), FS (B), and FS/iAs (C) treated fetuses compared to CT.

TABLE 1. Number of differentially methylated probes comparing across treatments

	CT	CT/iAs	FS	FS/iAs
CT		4 (4)	253 (244; 9)	5357 (4313; 1044)
CT/iAs	4 (4)		1 (1)	4 (4)
FS	253 (244; 9)	1 (1)		0
FS/iAs	5357 (4313; 1044)	4 (4)	0	

Note: Numbers in parentheses show breakdown by number of probes hypermethylated (red) and number hypomethylated (in green).

Effect of treatment on one-carbon metabolism

To determine whether changes in methylation patterns were the result of iAs or folate modulation of the one-carbon metabolism cycle which could subsequently affect the SAM/SAH ratio, we examined hepatic mRNA expression of methyltransferases and of genes associated with one-carbon metabolism. Statistical analyses of qPCR results show that there were no significant differences across treatment groups in expression of *Dnmt1,* methionine adenosyltransferase-2a (*Mat2a*), or 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*Mtrr*). However, *Dnmt3a,* Adenosylhomocysteinase (*Ahcy*), cystathionine β-synthase (*Cbs*) and arsenic-3-methyltransferase (*As3mt*) demonstrated significant changes in mRNA expression.

Maternal exposure to iAs led to an overall significant decrease in fetal mRNA expression for both *Dnmt3a, F*(1,19) = 17.09, $p = 0.001$, and *Ahcy*, *F*(1,18) = 4.79, $p < 0.05$ (Figure 9B and 10A, respectively). For *Cbs,* there was a significant Diet x Exposure interaction, *F*(1,20) = 5.98, $p< 0.05$. Mean comparisons revealed that folate supplementation significantly increased *Cbs* mRNA expression in fetuses that were not exposed to iAs ($p < 0.05$), but that this increase was abrogated by iAs exposure (Figure 10B). A similar pattern was found for *As3mt* mRNA expression levels, with a significant Diet x Exposure interaction, *F*(1,20) = 7.03, $p < 0.05$. Similar to *Cbs* mRNA, there was a strong trend for folate supplementation alone to increase *As3mt* mRNA expression levels compared to

CT (p = 0.06), but this increase was not present in the FS/iAs group (Figure 9C). In fact, compared to the FS group, the FS/iAs group had significantly lower *As3mt* mRNA expression levels (p < 0.001), which were similar to that of CT levels, suggesting that iAs exposure completely mitigated the effects of folate supplementation on *Cbs* mRNA.

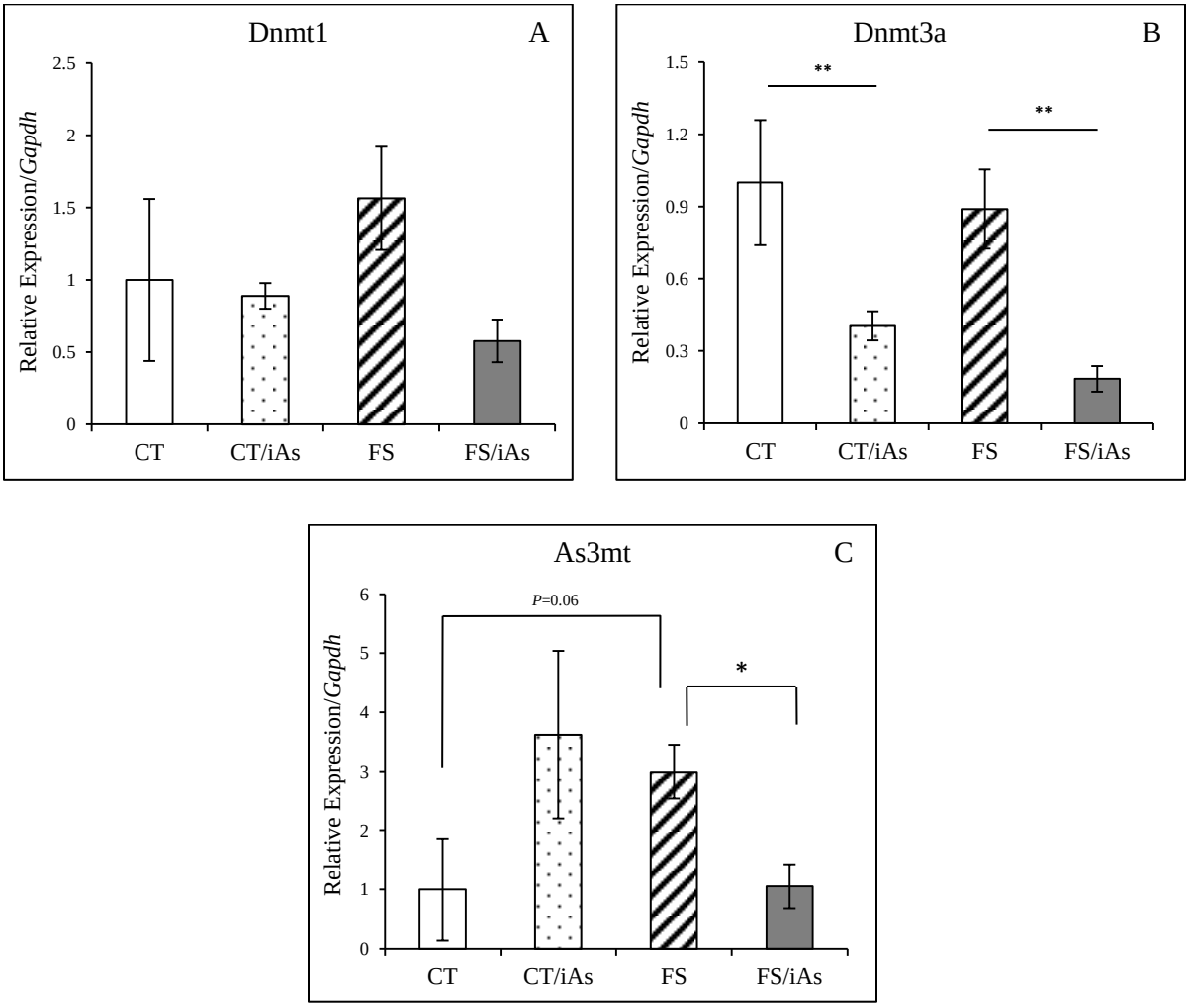


FIGURE 9. Effects of iAs-exposure and/or folate supplementation on hepatic mRNA transcripts of four methyltransferases in GD18 fetuses: Dnmt1 (A); Dnmt3a (B); and As3mt (D), as compared to controls. * Denotes significantly different at P<0.05; **, main effect of Exposure, P<0.05; and #, main effect of Diet P<0.05, revealed by two-way ANOVA (Exposure x Diet). Error bars represent ±SEM.

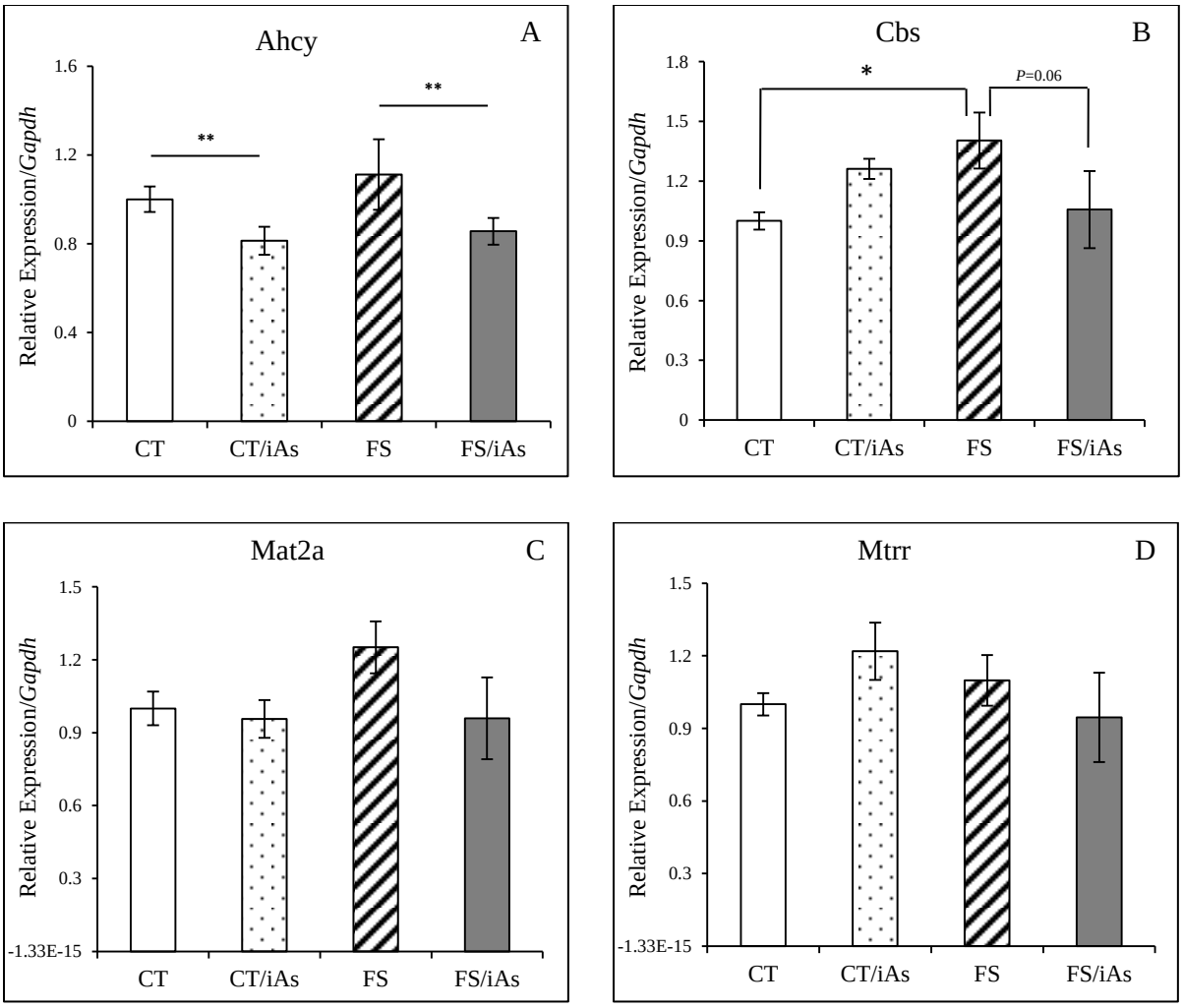


FIGURE 10. Effects of iAs exposure and/or folate supplementation on hepatic mRNA transcripts of genes involved in the one-carbon metabolism pathway of GD18 fetuses; *Ahcy* (A), *Cbs* (B), *Mat2a* (C) and *Mtrr* (D). * Denotes significantly different at $P<0.05$; **, main effect of Exposure at $P<0.05$; and #, main effect of Diet at $P<0.05$, revealed by 2-way ANOVA (Exposure x Diet). Error bars represent ±SEM.

Effect of treatment on imprinted genes

Imprinted genes are expressed in a mono-allelic, parent-of-origin manner. They are uniquely susceptible to mutations and changes in function because one of the two parental alleles is epigenetically silenced. For this reason, changes in promoter region methylation of the remaining functional allele alone could cause dysregulation and adverse health effects. In mammals, imprinted genes are silenced by a mix of epigenetic modifications, including DNA methylation. We examined expression changes of the imprinted genes *Dlk1* and *Igf2r*, identified to be significantly differentially

methylated by the SAM-software filtering of our CpG island microarrays and chosen because their dysregulation is associated with tumor progression. *Dlk1* and *Igf2r* are maternally imprinted genes (only the paternal allele is expressed).

Two-way ANOVA for *Dlk1* mRNA expression revealed a strong trend for an effect of Diet, $F(1,19) = 4.15$, $p = 0.057$, which was primarily driven by a 60% increase in mRNA transcripts in the FS/iAs group compared to the CT group, (Figure 11A). Thus, exposure to both folate supplementation and iAs, but not either one individually, led to a robust increase in *Dlk1* mRNA expression. The negative d-score from SAM-software analysis (Table 2) indicates that *Dlk1* was hypomethylated inside the gene. *Igf2r* was hypermethylated inside based on SAM-software analysis in both FS and FS/iAs groups. Methylation changes inside the gene are more complicated than that of the promoter region, and correlation to changes in expression are more difficult to establish. CT/iAs and FS/iAs showed increased Igf2r mRNA expression compared to their respective CT and FS groups, $F(1,18) = 5.17$, $p < 0.05$ (Figure 11B). FS showed a small 13% increase in expression over CT, though this was not statistically significant.

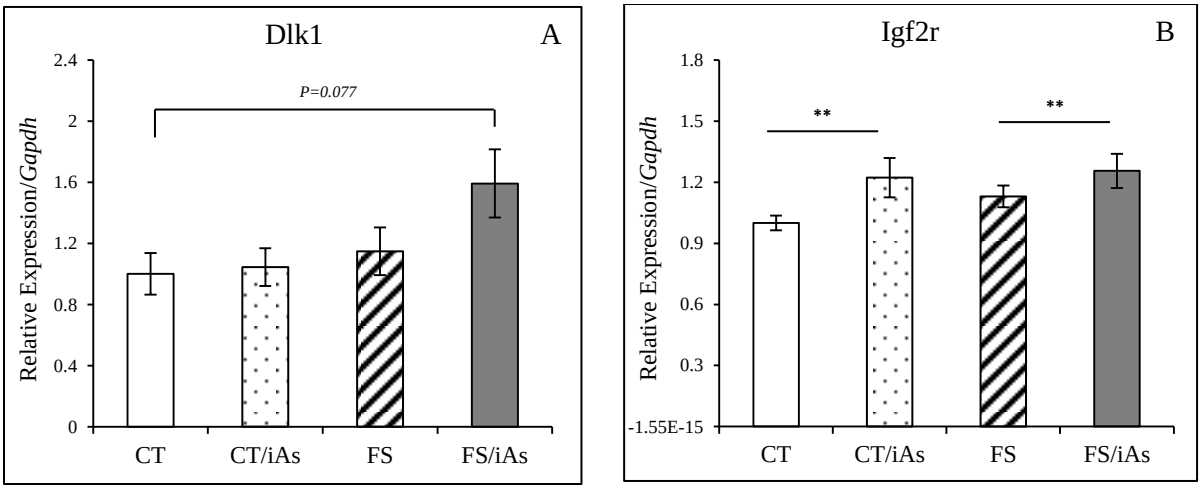


FIGURE 11. Effects of iAs exposure and/or folate supplementation on hepatic mRNA transcripts of maternally imprinted genes of GD18 fetuses; Dlk1 (A) and Igf2r (B). **, Main effect of iAs exposure at $P<0.05$, revealed by 2-way ANOVA (exposure x diet). Error bars represent ±SEM.

TABLE 2. Differentially methylated probes of imprinted, cell cycle and cancer-related genes

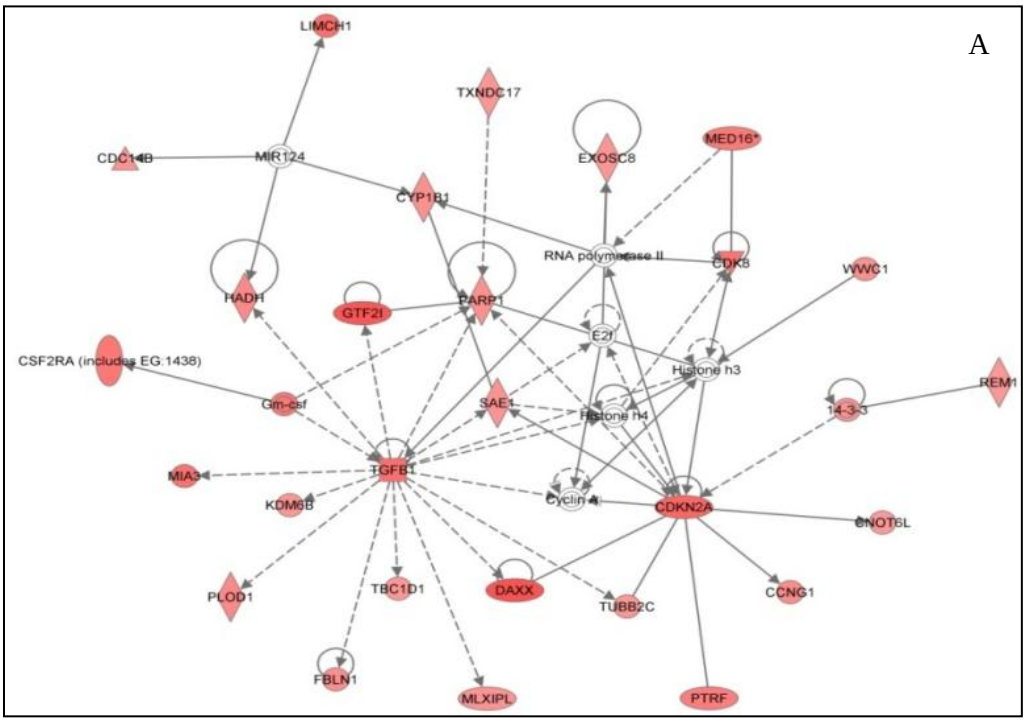
Gene	D-score (FS)	D-score (FS/iAs)	Position
Dlk1 (Paternal)	**---**	**-3.770**	**Inside**
Igf2r (Paternal)	**6.474**	**5.338**	**Inside**
P21(Cdkn1a)	**---**	**3.379**	**Inside**
Cdkn1b	**---**	**3.890**	**Downstream**
Cdkn2a	**7.799**	**8.554**	**Promoter**
Igf2bp1	**7.411**	**4.607**	**Promoter**
Daxx	**8.835**	**6.778**	**Promoter**

Effect of treatments on tumor suppressors and apoptotic genes

Genes with significant changes in methylation status were analyzed for known interactions in Ingenuity Systems Pathway Analysis (IPA) to determine their collective roles in a biological system. Network analyses indicated that biological pathways among the most changed as a result of our treatments (especially with combined folate supplementation and iAs-exposure) were related to cancer, neurological development, and cell signaling (Figure 12). Based on our initial goal to focus on arsenic carcinogenicity, we used information from these networks to select genes associated with tumorigenic pathways; these included *Daxx*, *Igf2bp1*, *Cdkn1a*, *Cdkn1b,* and *Cdkn2a*.

Daxx encodes a protein associated with regulation of apoptosis, and was found to be hypermethylated in the promoter region of FS and FS/iAs treated mice (Table 2). However these differences in methylation did not translate to changes in mRNA expression; changes in expression were negligible (*F*s < 1; Figure 13). Similarly, d-scores for *Igf2bp1*, an mRNA binding protein that regulates translation of *Igf2*, indicated that the promoter region was highly methylated in FS and FS/iAs groups. Again, differences in expression across treatment groups were not significant (*F*s – n.s.; Figure 13B). *Cdkn2a* encodes a protein that induces cell cycle arrest by stabilizing and

preventing degradation of p53, and is therefore a tumor suppressor. In FS and FS/iAs groups, *Cdkn2a* was hypermethylated in the promoter region. Analyses of *Cdkn2a* mRNA expression aligned with methylation data, as there was a significant effect of Diet, $F(1,17) = 5.22$, $p < 0.05$, where folate supplementation caused an overall decrease in mRNA expression (Figure 13E). Interestingly, despite increased methylation of its promoter due to folate supplementation, *Cdkn2a* transcript levels in FS/iAs increased nearly 40% from FS (similar to that of CT). The last genes we investigated were *Cdkn1a* and *Cdkn1b*, cyclin-dependent kinase inhibitors involved in G1 arrest. Inside and downstream analysis indicated hypermethylation, with a d-score of 3.379 and 3.89, respectively, in the FS/iAs group. However, mRNA levels were not significantly altered by Diet or Exposure (*F*s < 1; Figure 13C).



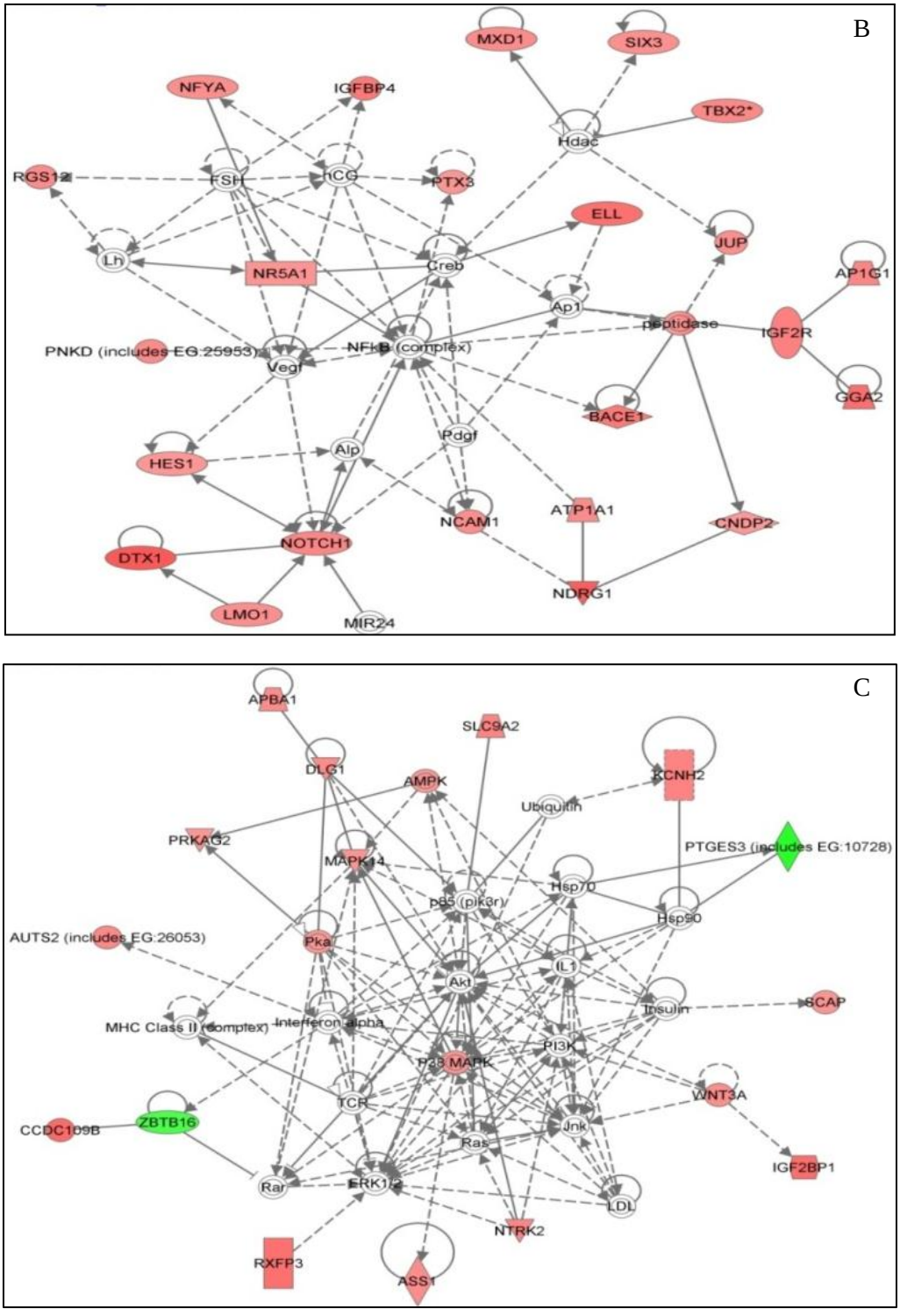


FIGURE 12. Ingenuity Pathways Analysis was used to establish biological context of genes identified as significantly differentially methylated following statistical filtering with the TM4 microarray software suite. Top candidates for further investigation included cancer (A), neurological development (B) and cell signaling networks (C). Networks were enriched for hypermethylated genes (in red) and hypomethylated genes (in green).

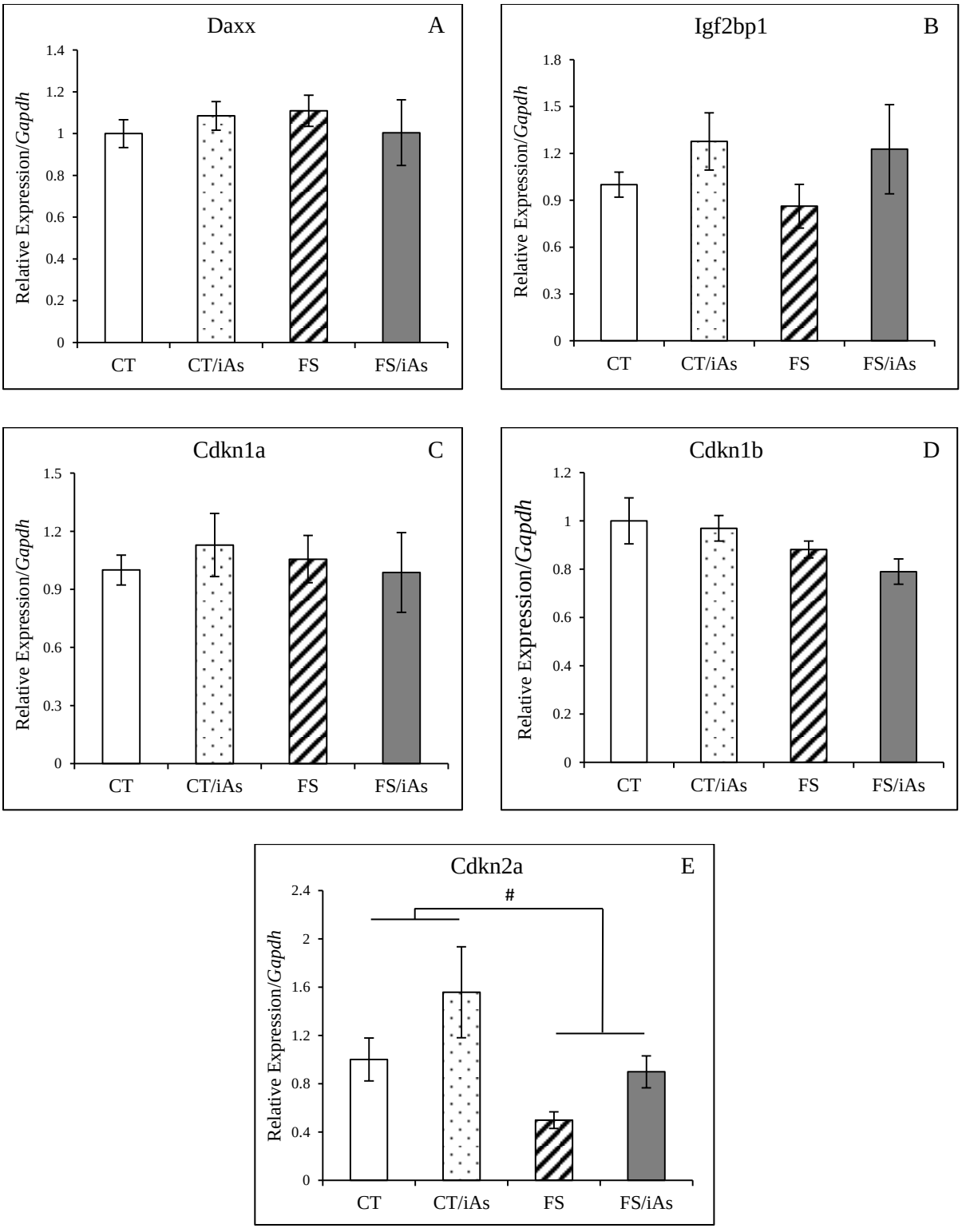


FIGURE 13. Effects of iAs exposure and/or folate supplementation on hepatic mRNA transcripts of genes involved in apoptosis regulation, *Daxx* (A); *Igf2* mRNA translation regulation, *Igf2bp1*(B); and cell cycle regulators *Cdkn1a* (C), *Cdkn1b* (D), and *Cdkn2a* (E). #, Main effect of Diet at $P<0.05$, revealed by 2-way ANOVA (Exposure x Diet). Error bars represent ±SEM.

Chapter 2 – Hepatocellular Carcinoma and the Wnt Pathway

Introduction

Wnt/β-catenin in development and cancer

The Wnt signaling pathway is primarily responsible for cell differentiation, migration and proliferation during embryogenesis. Wnt proteins are secretory glycoproteins that function as extracellular signaling molecules, and are the ligands for the cell surface Frizzled (Fzd) family of receptors. The interaction of Wnt ligands with Fzd canonically initiates cellular accumulation and translocation of the signal transducer and transcription factor β-catenin into the nucleus, where it induces transcription of target genes (Rao 2010). Because of its role in embryonic development and capacity to affect cell proliferation, survival and differentiation, the Wnt pathway is typically very tightly regulated.

Over-activation of the Wnt pathway has been linked to a variety of cancers, first elucidated by examination of adenomatous polyposis, a type of human colon cancer (Klaus and Birchmeier). It is estimated that approximately 65% of hereditary nonpolyposis colorectal cancers can be attributed to abnormal activation of β-catenin through gain of function mutation of β-catenin or loss of adenomatous polyposis coli (APC), a protein involved in the inhibition of the Wnt pathway (Miyaki 1999).

Canonical and non-canonical Wnt signaling

There are currently 19 identified members of the Wnt family, and 10 Frizzled receptors (Zeng 2007). Interactions between specific Wnts and Fzds have not yet been fully characterized, and the specific Wnt pathways that the Fzds activate, whether canonical or noncanonical, have not been identified for all Fzds.

In the first of two identified non-canonical pathways, Wnt protein binding to Fzd activates Dishevelled and results in the activation of protein kinase C via an increase in intracellular Ca2+ levels. Of the Wnt and Fzd families, this particular pathway has been associated with WNT5A, WNT11, and FZD2. In the second non-canonical pathway, Wnt activation causes Dishevelled to regulate the generation of planar cell polarity to control polarized cell movement during gastrulation and neurulation. Wnt/polar cell polarity signaling requires WNT11 and possibly WNT7a, and is potentially mediated by the FZD7 receptor (Zeng 2007).

In the canonical pathway, the absence of Wnt signaling leaves β-catenin held in complex with the tumor suppressors, APC and Axin. Also residing in the complex are two kinases, cyclin dependent kinase inhibitor-1 (CKI) and glycogen synthase kinase 3β (GSK3β), which phosphorylate specific serine and threonine residues of β-catenin. The phosphorylation event recruits an E3 ubiquitin ligase which targets β-catenin for proteasomal degradation. DNA-binding proteins TCF/LEF repress target genes by recruitment of co-repressors. On the other hand, when activated, Wnt occupation of the Frizzled receptor inhibits kinase activity via activation of an Axin-binding protein, Dishevelled, causing β-catenin to accumulate and translocate to the nucleus where it induces transcription of genes bound to the DNA-binding proteins TCF/LEF, which are briefly converted to transcriptional activators of target genes like *Ccnd1* and *Cmyc* (Reya 2005).

Altered canonical Wnt signaling in hepatocellular carcinoma (HCC)

As the effector of the Wnt signaling pathway, levels of β-catenin in the cell and particularly in the nucleus must be tightly regulated. Unlike colorectal cancers associated with APC mutations, hepatocellular carcinoma are highly associated with mutations in the *Ctnnb1* gene, which codes for β-catenin (Polakis 1999). The mutated β-catenin protein is often truncated, affecting serine/threonine residues in the regulatory sequence required for the targeted degradation of β-catenin. In response to this mutation, downstream targets *Cmyc* and *Ccnd1* are upregulated, resulting in HCC.

In addition to genetic mutations, activation of the Wnt/β-catenin pathway can be affected by epigenetic changes. Currently, little information is available concerning the effect of epigenetic

changes on gene expression of the Wnt pathway components like the ligands or receptors; instead, a greater focus is placed on proteins that inhibit or activate these Wnt pathway proteins. For example, aberrant promoter methylation of canonical Wnt pathway antagonists has been identified in human acute lymphoblastic leukemia (ALL) patients (Martin 2008). These include secreted frizzled-related proteins (SFRPs), Wnt inhibitory factor -1 (WIF1), Dickkopf-3 (DKK3) and dapper homologue-1 (HDPR1), which are all Wnt inhibitors. Specifically, ALL patients whose Wnt antagonist genes were methylated in the promoter regions had consistently lower rates of disease free survival at multiple time points and lower rates of complete remission compared to patients in the non-methylated group. Multivariate analysis demonstrated that promoter hypermethylation was an independent predictive factor of disease-free survival. Similar epigenetic gene silencing of some of the same Wnt inhibitors including SFRPs, HDPR1, WIF1 and DKK3 via promoter hypermethylation has been characterized in HCC (Yau 2005, Suzuki 2004, Shih 2006, Yang 2010 and Ding 2009).

Notably absent from the discussion of epigenetic influence on the Wnt pathway in HCC and other cancers are the influences of DNA methylation on promoter regions of *Wnt*s, *Fzd*s, and *Ctnnb1*. It has been established that Wnt pathway overexpression is often found in cancerous cells. However, assigning responsibility to specific WNTs and FZDs has been difficult. Until recently, localization of the 19 identified WNTs and 10 FZD receptors, much less their functions and interactions, had been unknown (Zeng 2007). Information remains scarce, but some recent evidence suggests that interaction between WNT3 and FZD7 proteins leads to activation of the Wnt/β-catenin pathway in HCC (Kim 2008). Through a series of knockdown experiments conducted in HCCs, Kim and colleagues demonstrated that the over-expression of *Fzd7* was an early tumorigenic event, and that binding of the concomitantly upregulated ligand *Wnt3* activated the canonical Wnt/β-catenin cascade in the progression of HCC. Other work confirms the involvement of *Fzd7* in HCC (Merle 2004, Nambotin 2011).

Folate supplementation attenuates Wnt signaling

While most epidemiologic studies regarding dietary folate studies are concerned with prevention of neural tube defects, a growing body of literature addresses the role of folate in carcinogenesis. Specifically, these studies suggest that diminished folate status increases risk of colorectal cancer (Giovannucci 2002), though the exact mechanism has not yet been fully characterized.

Some recent evidence indicates that in the context of carcinogenesis, folate status affects canonical Wnt signaling in mediating some of the cellular changes associated with carcinogenesis. Jaszewski and colleagues demonstrated in human patients with colorectal adenomas that dietary supplementation of 5 mg/day folic acid significantly reduced nuclear expression of β-catenin in rectal mucosal crypts compared to those given a placebo treatment (Jaszewski 2004). In addition, animal studies have supported the epigenetic role of folate as an essential cofactor in enzymatic biomethylation in affecting the Wnt pathway. Mild folate depletion alone in rats did not induce significant changes in components of the Wnt signaling pathway. Instead, the additive effects of reduced intake of B-vitamins associated with 1-carbon metabolism had a substantial impact on Wnt signaling and affected expression of downstream gene targets of β-catenin (Liu 2007). In a study of LRP6, a co-receptor for canonical Wnt signaling, researchers demonstrated that folate supplementation impacted transcriptional activation via the Wnt pathway (Carter 2005).

Summary

Based on results from CpG-island microarrays, we identified the Wnt/β-catenin pathway as a potential biological pathway affected by iAs consumption, especially given its role in hepatocarcinogenesis as described in the introduction. We examined (1) whether there were any treatment differences in methylation of genes related to the Wnt pathway; (2) if any observed methylation differences corresponded to changes in mRNA and protein expression; and (3) whether downstream transcription targets of the Wnt pathway were up-regulated in the liver as a result.

Results

Effect of treatment on transcription of Wnt pathway components

Among the differentially methylated genes identified by the CpG island microarray were elements of the Wnt pathway, including *Wnt3, Fzd*8, and *Fzd10* (Table 3). To that end, we noted that *Fzd8* and *Fzd10* were both hypermethylated with a d-score of 5.003 and 3.909, respectively, *Wnt3* was hypomethylated with a d-score of -3.814, and the gene encoding the effector molecule, β-catenin (*Ctnnb1*) was hypermethylated with a d-score of 3.670.

We conducted follow-up analyses of gene expression for specific ligands and receptors based on probe position and relevance to hepatic carcinogenesis as described in literature. Relative gene expression levels in fetal (GD18) liver for each treatment group were subjected to separate Diet (control vs. folate) x Exposure (water vs. iAs) between-subjects ANOVAs for each gene of interest. Analysis of Fzd8 mRNA expression did not reveal any main effects or Diet x Exposure interaction (Fs < 1; Figure 14B). Although there appeared to be a large difference in Fzd10 between control and each of the other treatment groups, inter-sample variability was too high to attain statistically significant group differences. For the Wnt3 ligand, there was an effect of Exposure, $F(1,18) = 5.73$, $p < 0.05$), but no effect of Diet or Diet x Exposure interaction. Arsenic treatment led to a significant overall increase in Wnt3 transcript levels, regardless of folate status (Figure 14A). CT/iAs fetal livers exhibited nearly five-times the mRNA transcript level of CT animals, and FS/iAs fetal livers showed transcript levels of Wnt3 about twelve times that of control. Lastly, we examined transcript levels of the transcription factor β-catenin due to its importance in the canonical Wnt signaling pathway. The ANOVA revealed an effect of Diet, $F(1,18) = 7.10$, $p < 0.05$, but no effect of Exposure or Diet x Exposure interaction. Folate supplementation elicited an overall decrease in *Ctnnb1* mRNA (Figure 14D). In FS and FS/iAs groups, transcription of *Ctnnb1* was reduced approximately by 40% in FS, and 20% in FS/iAs (Figure 14D). Importantly, signaling by β-catenin is not regulated at the transcriptional level, but by its post-translational phosphorylation status.

TABLE 3. Differentially methylated probes of Wnt pathway genes

Gene	D-score (FS)	D-score (FS/iAs)	Position
Wnt3	**---**	**-3.814**	**INSIDE**
Fzd8	**---**	**5.003**	**INSIDE/PROMOTER**
Fzd10	**---**	**3.909**	**PROMOTER**
Ctnnb1	**---**	**3.670**	**PROMOTER**

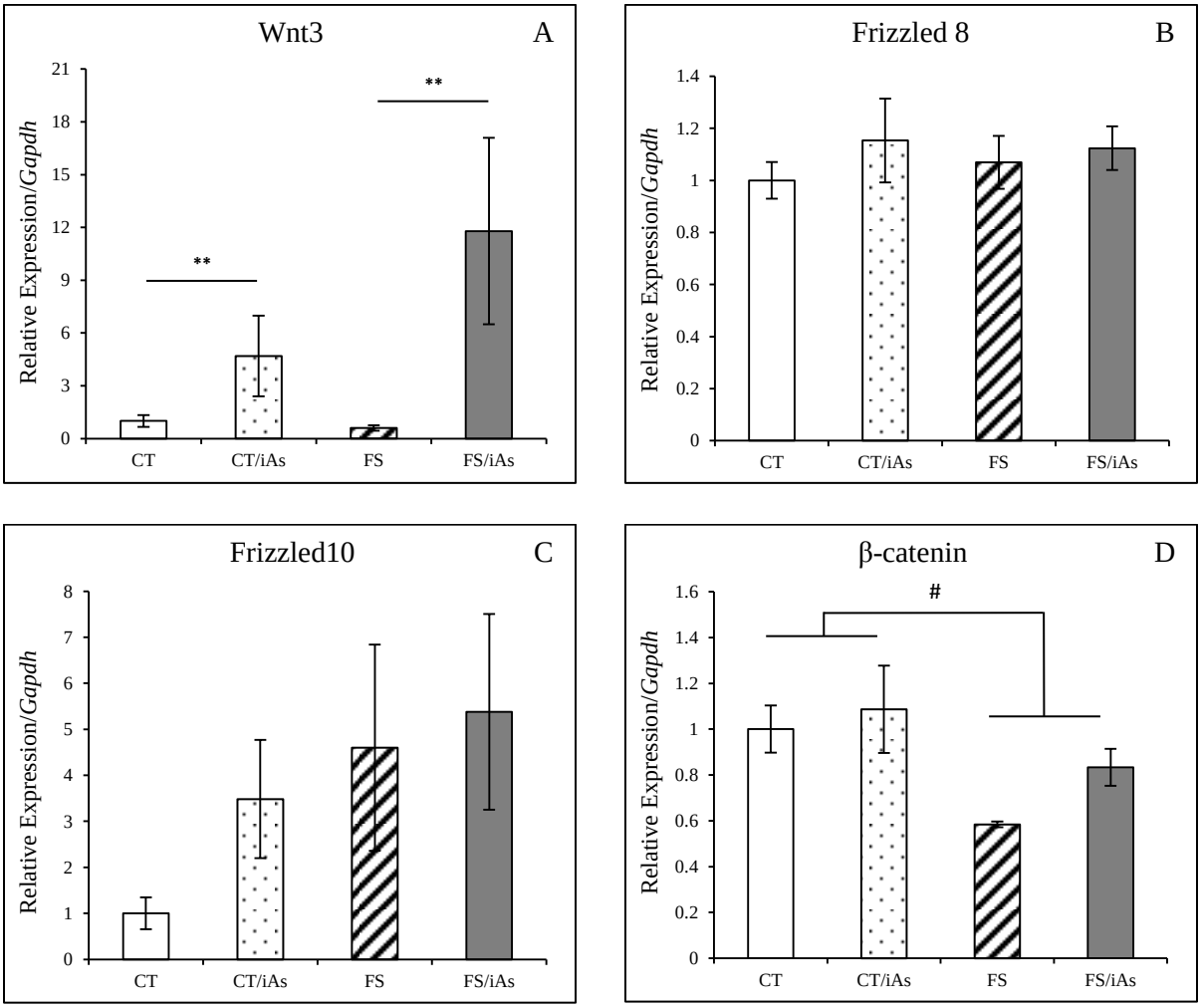


FIGURE 14. Effects of iAs exposure and/or folate supplementation on hepatic mRNA transcripts of Wnt pathway genes, including the ligand Wnt3 (A); Frizzled (Fzd) Receptors Fzd8 (B) and Fzd10 (C); and the transcription factor β-catenin (encoded by the gene *Ctnnb1*) (D). **, Main effect of Exposure at $P<0.05$; # main effect of Diet at $P<0.05$, revealed by 2-way ANOVA (Exposure x Diet). Error bars represent ±SEM.

We continued our investigation of the Wnt pathway by examining protein expression of the various components by western blot. From fetal (GD18) livers of each treatment group, we assayed Wnt3, Fzd7, Fzd10, and non-phosphorylated beta-catenin. Protein expression of Fzd7 was considered because of its association with HCC (Kim 2008). Similar to RNA expression, relative protein expression in GD18 livers for each treatment group was subjected to separate Diet (control vs. folate) x Exposure (water vs. iAs) between-subjects ANOVAs for each protein of interest.

Analysis of Wnt3 protein levels demonstrated a significant Diet x Treatment interaction $F(1,12)$, $p < 0.05$, but no effects of Diet or Treatment alone. Mean comparisons, however, only revealed a trend indicating that folate supplementation increased protein levels of Wnt3 in fetuses not exposed to iAs ($p = 0.07$); this increase was negated by iAs exposure. Similarly, a second trend indicated that Wnt3 protein levels were increased in non-folate supplemented animals ($p = 0.07$) when given iAs, but this trend was reversed in the folate-supplemented group (Figure 16A). Both Fzd7 and Fzd10 protein showed significant effects of interaction Diet x Exposure, $F(1,12) = 12.29$, $p < 0.05$ and $F(1,12) = 12.26$, $p < 0.05$, respectively. Mean comparisons for Fzd7 showed a significant increase in Fzd7 protein levels in the liver of iAs-exposed fetuses when supplemented with folate ($p < 0.05$). Additionally, mean comparisons demonstrated that FS fetuses decreased Fzd7 expression when given iAs ($p < 0.05$), but the opposite was true in their CT-diet counterparts (Figure 16B). For Fzd10, mean comparisons showed significant increases of expression comparing FS to CT ($p < 0.05$), as well as a significant decrease of expression in FS fetuses ($p < 0.05$), when exposed to iAs (Figure 16C). Lastly, non-phosphorylated β-catenin also had a significant effect of interaction Diet x Exposure, $F(1,11) = 5.703$, $p < 0.05$. Mean comparisons were not significant, but exhibited strong trends indicating that non-phosphorylated β-catenin decreased in FS/iAs in comparison to both CT/iAs and FS (Figure 16D).

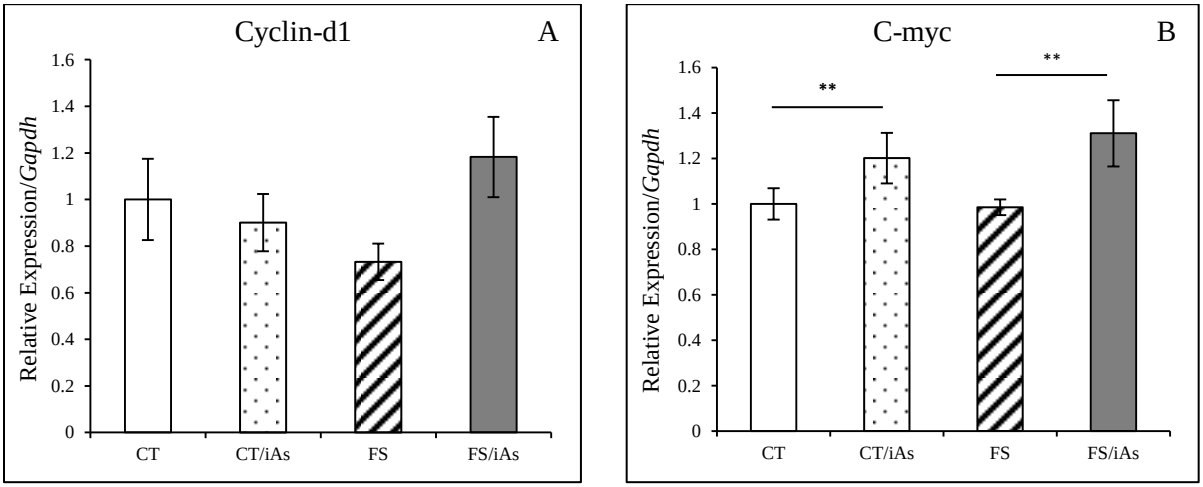


FIGURE 15. Effects of iAs exposure and/or folate supplementation on hepatic mRNA transcripts of genes upregulated by activation of the Wnt pathway, mediated by β-catenin translocation to the nucleus, Cyclin-d1 (A) and C-myc (B) **, Main effect of Exposure at $P<0.05$, revealed by 2-way ANOVA (Exposure x Diet). Error bars represent ±SEM.

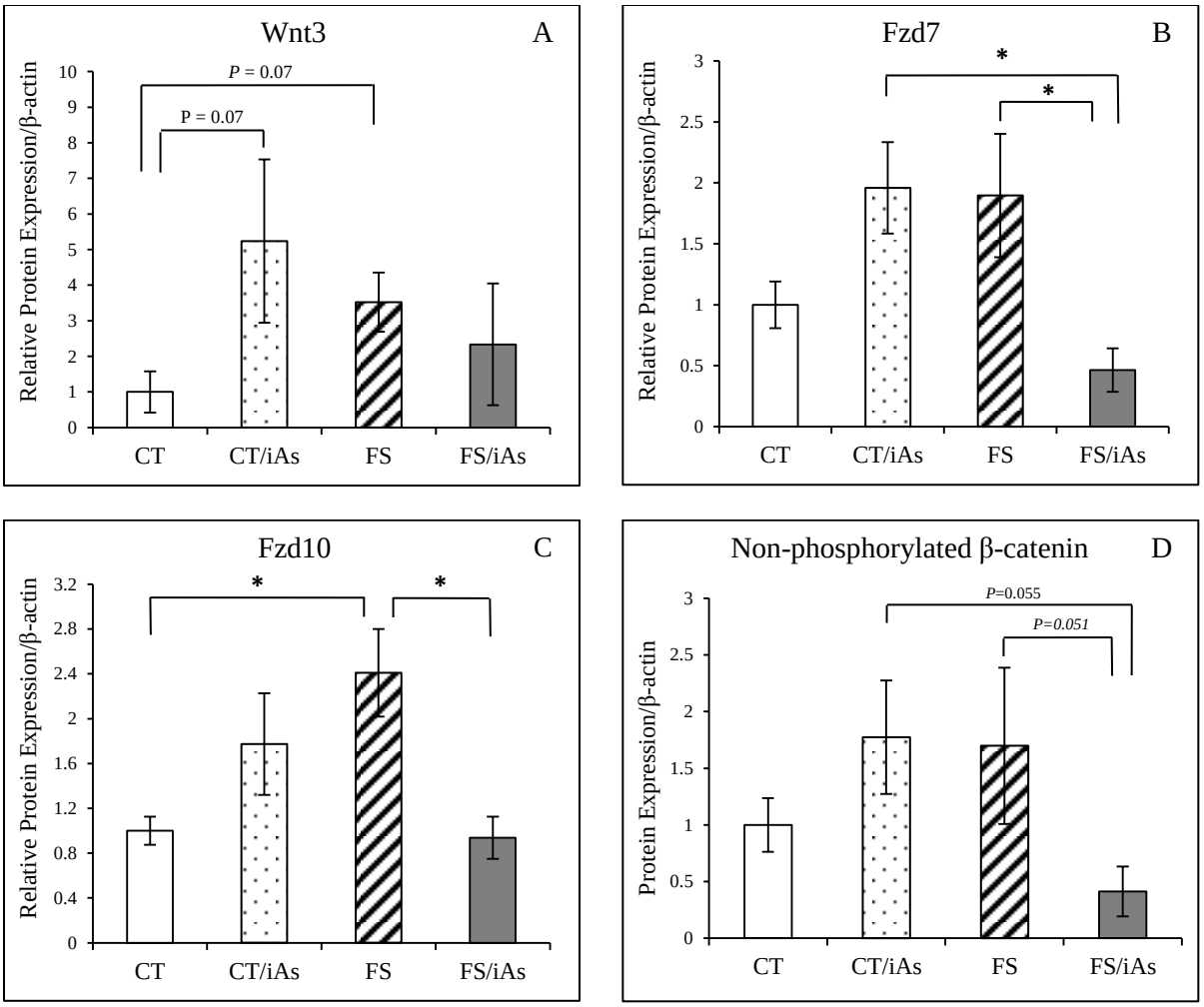


FIGURE 16. Effects of iAs exposure and/or folate supplementation on hepatic proteins associated with the Wnt pathway; Wnt3 (A), Fzd7 (B), Fzd10 (C), and non-phosphorylated β-catenin (D). Proteins derived from the cellular extract were normalized to β-actin, and proteins from the nuclear extract were normalized to Lamin-b1 (E). * Denotes significantly different, $P<0.05$. Error bars represent ±SEM.

Discussion

The absence of faithfully maintained DNA methylation is associated with abnormal gene expression and cancer (Baylin 2001). DNA methylation is a dynamic, yet tightly controlled process that regulates gene transcription. Aberrant DNA methylation can stem from a multitude of changes, but ultimately causes cancer by affecting gene expression. Arsenic is a known carcinogenic agent, and its abundance in supplies of drinking water around the world is a significant public health concern (IARC 2004). However, the mechanism by which arsenic causes cancer remains unknown, though evidence to support the hypothesis for an epigenetic mechanism of iAs carcinogenesis continues to mount; it is thought that the consumption of the universal methyl donor SAM used by AS3MT for the methylation of iAs in its excretion from the body competes with maintenance of DNA methylation by the DNMT family of enzymes and ultimately affects expression of genes important in the development of cancer (Dolinoy 2008).

Based on evidence pointing to the depletion of SAM as the manner in which iAs disrupts DNA methylation, we hypothesized that dietary supplementation of the methyl donor folate would alleviate the effects of arsenic exposure and reduce the incidence of cancer. Folate provides a methyl group to SAH, the product of transmethylation reactions catalyzed by both DNMT and AS3MT (Figure 8). With an abundance of SAM following folate supplementation, we predicted that the competition between DNA methylation and arsenic biomethylation would be lessened.

Though the effect of arsenic is well documented in humans, being able to fully understand its mechanism of action has been elusive due to the lack of appropriate animal models. Recently, studies conducted by Michael Waalkes and his colleagues at NIEHS identified a viable mouse model that closely mimicked the effect of iAs exposure to humans as observed in a multitude of epidemiological studies (Waalkes 2003, 2007). In their studies, pregnant C3H and CD1 mice were exposed to varying amounts of arsenic in drinking water, and characterized the cancers resulting in offspring. To that end, our study adapted the putative mechanism and animal model described by Waalkes and others in

hopes of alleviating the incidence of arsenic-induced liver cancer among male offspring with nutritional supplementation.

Preliminary Observations

The addition of 85ppm arsenic to drinking water negatively affected fetal and maternal health. Supplementation of folate to the diet did not improve outcomes to *in utero* arsenic exposure, and did not initially appear harmful to dams on deionized water. Based on observations during tissue collection and data collected from our experiments on fetal livers, we demonstrate that folate supplementation hypothesized to mitigate effects of *in utero* arsenic exposure was more detrimental to fetal health than either folate supplementation or arsenic treatment alone.

Initial observations over the course of treatment and tissue collection indicated that the dams exposed to iAs consumed significantly less water than their respective controls, regardless of folate supplementation status (Figure 5B). However, consumption of arsenic still resulted in large differences in hepatic levels of arsenic and its methylated metabolites (Figure 7). Furthermore, of the dams given iAs and dietary folate supplementation, two miscarried; these observations were noted on GD18 prior to euthanasia. Additionally, fetuses from one other dam were excluded from all analyses because she was extremely lethargic prior to euthanasia, and the majority of her fetuses were considerably underdeveloped and underweight (0.4g or less, data not shown). Fetuses of CT and FS dams appeared relatively healthy. In contrast, fetuses from the CT/iAs group weighed significantly less than either non-iAs-exposed group, and those supplemented with folate in addition to *in utero* exposure to iAs weighed the least of all groups (Figure 5C). These preliminary findings conflicted with previously published results that reported no differences in water consumption when given 85ppm iAs in drinking water as compared to DI-water controls (Waalkes 2003, Waalkes 2007). These prior studies also noted that weight of offspring were not significantly affected, another observation that conflicted with our own. However, it should be pointed out that offspring in studies conducted by the Waalkes research group were weighed at birth (approximately GD21), whereas our data were collected at GD18. It is possible that developmental deficits that we observed were compensated for

over the final days prior to birth. Taken together, these initial observations suggest that iAs was harmful to overall health of both dams and fetuses; folate supplementation on its own appeared innocuous, but resulted in significantly worse outcomes in combination with iAs exposure.

SAM/SAH Ratio

We determined concentrations of SAM and SAH in male fetal livers to assess methyl donor status and determine their availability for DNMT and AS3MT. Dietary supplementation of folate, a dietary methyl donor important for SAM synthesis, significantly increased levels of hepatic SAM above control, as anticipated (Figure 6A). We further expected iAs exposure to cause SAM depletion, resulting from AS3MT activity in accordance with previous published studies in HaCaT (Reichard 2007) and TRL 1215 cells (Zhao 1997). However, we observed the opposite phenomenon; iAs exposure increased SAM levels in both diets (Figure 6A). Patterns of SAH levels mirrored that of SAM. The most frequently cited measure of transmethylation potential affected by iAs and folate status is the SAM/SAH ratio (Williams 2010). Though none of the mean differences were significant, iAs negatively impacted the SAM/SAH ratio compared to their respective non-iAs-treated groups. A similar trend was observed in other papers, though reduction in SAM/SAH ratios was attributed mostly to a reduction in SAM alone and stagnant levels of SAH (Reichard 2007, Zhao 1997). In all, our SAM and SAH data suggest that iAs potentially reduces the hepatic transmethylation potential, a change that can lessen the liver's ability to maintain proper DNA methylation.

Methyltransferases and One Carbon Metabolism

We examined transcriptional changes of enzymes central to DNA and iAs methylation and one-carbon metabolism to investigate the effect of iAs and folate on the expression of genes that are key regulators of SAM production and consumption. Previous studies have shown that arsenic can inhibit expression and modulate enzyme activity of DNMT1 (Cui 2006, Shi 2004, Zhao 1997), and that folate can affect global DNA methylation and enzymes involved in one-carbon metabolism (Davis 2003), though some found no differences in *Dnmt1* expression (Jensen 2009).

Using quantitative real-time RT-PCR, we were able to compare levels of gene-specific mRNA transcripts in fetal livers from each treatment group. Transcripts for *Dnmt1*, the primary maintenance methylase, were not significantly different between groups (Figure 9A). However, a reduction in *Dnmt3a* transcripts was noted as a result of iAs-exposure. This was similar to observations in HaCaT cells (Reichard 2007). Though a significant decrease in *As3mt* expression (Figure 9C) could provide a potential explanation for the negative effects observed in FS/iAs animals, we would have expected iAs to accumulate in the liver; however, the opposite phenomenon was observed (Figure 7). We can only speculate that translation of AS3MT mRNA increased to over-compensate, or enzyme efficiency and activity increased.

Our treatments also affected genes of the one-carbon metabolism pathway responsible for removing SAH and regenerating SAM after transmethylation reactions, amongst other functions. *Ahcy*, responsible for the first step in converting SAH back to SAM, was significantly downregulated by iAs, regardless of diet (Figure 10A). Transcripts of CBS, the protein responsible for removing Hcy, were significantly increased by folate supplementation, but decreased by combined iAs and folate exposure (Figure 10B). In all, these data suggest that the combination of folate supplementation and iAs exposure leads to an accumulation of SAH and Hcy, products of transmethylation reactions recognized as risk factors for various diseases (Graham 1997, Wald 2002, Seshadri 2002). Supplementation of folate causes more SAM to be made, but also makes more available to be converted to SAH. This could result in an accumulation of SAH and Hcy as it appears iAs downregulates transcription of *Ahcy* and *Cbs*.

DNA Microarrays

We submitted DNA samples from six fetal livers in each group for microarray analysis to explore global methylation changes and examine other genes with significant changes in methylation for biological network analysis. To our knowledge, this is the first study to use DNA methylation microarrays to study the consequences of arsenical exposure in animal models; a few other studies

have examined the effect of arsenic on genome-wide methylation in immortalized human urothelial (UROtsa) cells (Jensen 2009) and on a human population with on-going exposure (Smeester 2011).

Considering the original hypothesis that arsenic exposure increases hepatic SAM consumption, we expected to see decreased availability of SAM for DNA methylation and subsequently genome-wide hypomethylation. Thus, we further hypothesized that supplementation of folate would result in genome-wide hypermethylation due to the increased availability of methyl groups. Unexpectedly, previous studies indicated that aberrantly-methylated regions of gene promoters identified in UROtsa cells and in a human population from Mexico chronically exposed to arsenic were primarily hypermethylated (Jensen 2009, Smeester 2011). Our microarray experiment reflected neither these previous results nor our original hypothesis. Filtering by SAM analysis of our microarray data indicated that iAs exposure alone had little effect on changing methylation of CpG islands; only 4 genes were considered to be significantly differentially methylated at a d-value of 1.239 (Table 2).

Folate supplementation, in contrast, yielded altered methylation of 253 probes compared to control, of which 244 were hypermethylated. A study examining the effect of dietary folate availability on the human fetal epigenome noted a positive association between methylation of long interspersed nuclear element 1 (LINE-1), which covers approximately 17% of the genome, and serum folate in cord blood (Fryer 2011). A different study conducted in pregnant Wistar rats, found that high-dose (40mg/kg) dietary supplementation of folic acid did not change in hepatic DNA methylation, in spite of significantly increased levels of SAM (Achon 2000), while yet another study reported that 25 mg/kg of folic acid delivered by oral gavage in mice induced global hypomethylation; the investigators hypothesized that this effect resulted from folic acid inhibition of glycine hydroxymethyltransferase, reducing production of the 5,10 methyltetrahydrofolate precursor required for SAM production (Finnell 2002).

These conflicting effects of folate supplementation on DNA methylation are due to a possible U-shaped curve of benefits derived from folate consumption. Recent reviews relating folate

supplementation to clinical outcomes like neural tube defects and cancer caution the scientific community against drawing conclusions about folate based on individual studies, specifically those employing high-doses of folate in their experimental design (Kim 2003, Ulrich 2006). This fact is especially problematic given that folate supplementation is generally well-tolerated and does not cause obvious symptoms of disease. The cited studies suggest that benefits realized from increased folate are highly dependent on timing of supplementation and dosage provided.

Our study design incorporated supplementation of folate at five times the normal amount found in rodent diets (11 mg/kg versus 2.2 mg/kg). We did not observe any outward detrimental health effects of folate to either dams or fetuses, though results from our SAM analysis indicate that folate supplementation alone increased changes in DNA methylation over control. Dose-dependent studies of folate supplementation are needed in order to outline a potential U-shaped DNA methylation curve; this information could have important implications for our data.

A few previous studies have delved into using folate supplementation to ameliorate symptoms of arsenic exposure; Mary Gamble and her collaborators used folic acid supplementation in a double-blinded, placebo-controlled trial in Bangladesh, a country with notoriously high levels of arsenic in drinking water, and reported that their treatments increased proportions of DMAs in urine by about 8% and decreased total blood arsenic by approximately 14%, suggesting that increased dietary folate improved the ability of individuals to methylate and excrete arsenic. Interestingly, their placebo control also significantly decreased arsenic in blood, but by just over 1% (Gamble 2006, 2007, Heck 2007). Lisa Gefrides and her colleagues administered 25mg/kg folate and folinic acid during neurulation to reduce the increased incidence of neural tube defects in heterozygous CXL-*Splotch* mice exposed *in utero* to arsenic, but were unsuccessful; instead, they found injection with 40mg/kg arsenic and 25mg/kg folate to be maternally lethal (Gefrides 2002).

Again, to our knowledge, this study appears to be the first to consider gene-specific DNA methylation in a combined exposure setting of arsenic and folate. According to our proposed mechanism, we expected that at worst, the FS/iAs group would not show any improvement compared

to the CT/iAs animals. At best, we hoped for complete reversal of effects brought on by iAs exposure. Unfortunately, our findings were comparable to those reported by Gefrides. Contrary to our expectations, folate supplementation intended to lessen the changes induced by arsenic exposure exacerbated changes by either folate supplementation or arsenic exposure alone. SAM-software analysis revealed that in comparison to the CT group, livers of FS/iAs fetuses had over 5300 significantly changed probes at a d-value of 1.219, of which 4300 were hypermethylated (Table 1).

Previous findings from our laboratory demonstrated that iAs metabolites formed in the *de novo* methylation/detoxification pathway are more cytotoxic than iAs itself, and are more potent inhibitors of enzyme activity (Styblo 2000, Thomas 2001). The profile of iAs and its metabolites found in maternal liver at GD18 in our experiment indicate that iAs composed about 35% of total arsenic (iAs + MMA + DMAs) in CT/iAs animals, and only about 20% of total arsenic in FS/iAs (Figure 7). Metabolite profiles in fetal livers were similar between the two iAs-exposed groups, though little is known about the fetal liver's ability to methylate arsenic at GD18. Our data and other research support previous observations that increased levels of folate and SAM preferentially help methylate iAs and facilitate arsenic elimination (Gamble 2006, 2007, Heck 2007).

While total arsenic burden was lowered by folate supplementation, the increased methylation of iAs for excretion did not reduce absolute levels of MAs or DMAs in the maternal liver (Figure 7). The data suggest maternal livers of the FS/iAs group methylated more iAs for elimination, and thus may have been exposed to more of the toxic iAs metabolites MAs and DMAs than dams of the CT/iAs group over the 10-day course of iAs-exposure from GD8 to 18. Because we only observed a single time point, these conclusions are difficult to verify. To our knowledge, there are no studies that correlate effects of higher rates of iAs methylation with health outcomes. However, in this way, it is possible that folate supplementation would cause synergistically worse outcomes with iAs instead of reversing negative effects by affecting maternal health, and resulting in more significantly altered DNA methylation profiles in fetuses (Figure 8).

Effect of Treatment on Imprinted Genes

Aberrant DNA methylation patterns are often associated with cancer; perhaps the genes whose expression is most prone to being affected by these changes are imprinted genes (for a review, see Falls 1999). Because of their unique susceptibility to changes in DNA methylation, we searched for imprinted genes listed among those considered significantly hyper- or hypomethylated. We identified two maternally imprinted genes, *Dlk1* and *Igf2r*. *Dlk1* was hypomethylated inside the gene in FS/iAs animals versus CT, while *Igf2r* was hypermethylated, also inside the gene for both FS and FS/iAs groups versus CT (Table 2). We used quantitative real-time RT-PCR to compare amounts of mRNA transcripts in six fetal livers from each treatment group. *Dlk1*, or Delta-like 1, is highly expressed in mouse embryos and the placenta during development, and it is suggested that *Dlk1*functions as a growth factor responsible for maintaining cell proliferation prior to differentiation (Yevtodiyenko 2006). Transcripts of *Dlk1* mRNA were unchanged in CT/iAs and FS compared to control, but exhibited a near-significant ($p=0.07$), 50% increase in FS/iAs animals versus CT. This result is in line with previous work suggesting that upregulation of *Dlk1* due to epigenetic events can contribute to human hepatocellular carcinoma (Huang 2007), an outcome of interest because liver cancer is one of the endpoints of chronic iAs exposure. A significant main effect of iAs exposure was noted to increase *Igf2r* expression, but this did not agree with methylation data suggesting that the gene was hypermethylated. However, this finding suggests that iAs can result in growth factor transcription and overexpression, which is associated with various cancers (Furstenberger 2002).

Effect of Treatment on One Carbon Metabolism and Cell Cycle

Other differentially methylated probes not associated with imprinted genes or arsenic and folate metabolism were subjected to network analysis to identify biological pathways that exhibited significant changes in DNA methylation amongst its member genes using IPA. The most prominent networks identified included carcinogenesis, neural tube defects, and cell-cycle regulation (Figure 12). Because cancer was our primary endpoint of interest, we focused on carcinogenesis and cell-cycle regulatory networks. We performed additional real-time RT-PCR assays for the apoptosis-

mediator death-domain associated protein (*Daxx*), insulin-like growth factor 2 binding protein 1 (*Igf2bp1*), and tumor suppressors, cyclin-dependent kinase inhibitors 1a, 1b, and 2a (*Cdkn1a*, *Cdkn1b*, *Cdkn2a*).

We observed no significant differences between groups in *Daxx*, *Igf2bp1*, *Cdkn1a*, and *Cdkn1b* (Figures 13A-D). A significant decrease in expression due to folate supplementation was noted for *Cdkn2a*. Also, a non-significant increase in expression was noted due to iAs-exposure. This was consistent with previous data in iAs-exposed tissues analyzed by gene expression microarray (Liu 2006). Interestingly, overexpression of *Cdkn2a* has been associated with late-stage ovarian cancer, perhaps an attempt by cells to control proliferation (Dong 1997, Fujita 1997, Niederacher 1999). In our study, overexpression was possibly due to DNA methyltransferase inhibition by arsenic, a theory consistent with previous findings that detail the use of arsenic trioxide to inhibit DNMT activity and restore epigenetically-silenced *Cdkn2a* in human hematologic malignant cells to control proliferation (Fu 2010). Folate, on the other hand, suppressed expression of *Cdkn2a* in both arsenic-exposed and control groups by increasing methylation at the *Cdkn2a* promoter. Downregulation of a tumor suppressor like *Cdkn2a* would be promote the proliferation of an existing cancer by preventing cell cycle control, and is another argument for why folate supplementation would not be beneficial in mitigating the cancers caused by iAs.

Effect of Treatment on Wnt Pathway

We further used Ingenuity Systems Pathways Analysis to cross-reference our list of probes differentially methylated with genes associated with liver cancer based on previously published data. We noted that methylation for many genes coding for proteins in the Wnt pathway were changed due to treatment. The Wnt pathway, as stated in the introduction of Chapter 2, is responsible for cell differentiation, migration, and proliferation during embryogenesis. Though normally tightly regulated, upregulation of the canonical pathway mediated by β-catenin is associated with a variety of cancers, and notably, HCC. Of the 19 Wnt ligands and 10 Fzd receptors that have been identified, we noted that a few that were hypomethylated as a result of our treatment (Table 3). With few

exceptions, relatively little is known about interactions between specific Wnts and Fzds, as well as the different cascades and effects initiated by activation. Briefly, activation of the canonical pathway involves one of the Wnt ligand binding to a Fzd receptor located on the cell surface. This activates Dishevelled, an inhibitor of a kinase complex that, in the absence of Wnt signaling, phosphorylates the transcription factor β-catenin. If phosphorylated, subsequent ubiquitination targets β-catenin for proteasomal degradation. However, if left un-phosphorylated, β-catenin translocates to the nucleus and stimulates transcription of *Tcf/Lef* genes, which have been implicated in HCC. Because hypomethylation of the Wnt ligand would suggest subsequent increased expression and activation of the pathway, we decided to investigate the potential role of the Wnt signaling in our model of iAs-mediated liver cancer.

Few published studies of iAs-induced liver carcinogenesis have considered changes to the Wnt pathway as a potential signaling cascade affected by iAs exposure (Liu 2006, Cui 2004). We performed real-time RT-PCR assays on Wnt pathway genes selected for previously cited relevance to hepatocellular carcinoma (HCC) (Kim 2008) or observed methylation changes in promoter regions. *C-myc*, a downstream effector of canonical Wnt signaling has a central role in iAs-induced cancer; Liu 2006 demonstrated cellular beta-catenin levels increased 7-fold in C3H offspring with iAs-induced HCC compared to those unexposed to arsenic. However, despite these implications, the Wnt pathway ligands and receptors were not explored further in these studies as potential upstream targets of the *C-myc* and *Ctnnb1* expression.

To address these deficiencies in the literature, we followed results from SAM analysis indicating that methylation of many Wnt pathway genes were changed, particularly in the FS/iAs group. In addition, we examined levels of mRNA transcripts for genes known to be targets of Wnt signaling. In all, we considered mRNA levels of *Wnt3, Fzd8, Fzd10, Ctnnb1*, and downstream transcriptional targets *C-myc* and *Cyclin-d1*.

Overexpression of the Wnt3 ligand and its interaction with the Fzd7 receptor has been documented in HCC (Kim 2008). We observed significant increases in expression of *Wnt3* as a result

of iAs exposure (Figure 14A). At least in the FS/iAs group, this overexpression was compatible with SAM analysis, indicating that the gene was hypomethylated. The Fzd receptors did not exhibit significant differences between groups (Figures 14B, C), though receptor expression affects pathway activation less than increases in ligand overexpression. We noted a significant decrease in β-catenin expression resulting from folate supplementation, but transcriptional activation of Wnt pathway targets is primarily dependent on non-phosphorylated β-catenin translocation to the nucleus and not necessarily transcription of *Ctnnb1*.

In the nuclear extract, β-catenin did not show any significant differences amongst groups, though the overall pattern of activation correlated to levels of Wnt3 and Fzd7 protein (Figure 16) that were detected in the cytoplasmic extract. However, there were strong trends indicating that FS/iAs animals expressed less non-phosphorylated β-catenin in the nuclear extract than controls. In all, our Wnt pathway data indicate that folate supplementation and iAs exposure decreases activation of the canonical pathway, and would possibly reduce carcinogenesis. However, we were ultimately concerned with downstream targets of the Wnt pathway, and taken together, results of *Cmyc* expression indicated that β-catenin may not be responsible for the increase in *Cmyc* (Figure 15), previously noted to be upregulated in HCC (Liu 2006).

Conclusions and Future Directions

In light of the results from our study, we would not suggest that folate supplementation should be used as a dietary intervention to mitigate iAs exposure in countries lacking a safe supply of drinking water. In fact, based on our findings, I would most likely recommend avoidance of folate supplementation far above the recommended intake. Our study noted several different factors that warrant caution. First, iAs metabolites were increased in the liver as a result of folate supplementation. While it can be argued that this is beneficial for subsequent elimination of iAs from the body, constant exposure to higher levels of metabolites due to the increased methylation of iAs could be harmful. Secondly, folate supplementation can unfortunately decrease expression of tumor suppressors such as *Cdkn2a*, resulting in an increased risk of tumorigenesis. Furthermore, our study

indicates that increased folate intake results in higher SAM levels, but also higher SAH levels. Because we noted decreased transcription of *Ahcy*, this would suggest that SAH levels remained higher, perhaps even more so than without folate supplementation.

Overall, more preliminary studies are required to assess the validity of findings contained within this thesis. High variance noted in our results may result from the use of CD1 mice. Our decision to use this strain was based on previous studies adopting the transplacental model of iAs carcinogenesis as described by Dr. Michael Waalkes and colleagues at the NIEHS. The number of animals selected per treatment group was also determined based on these studies. CD1 mice are not inbred, and thus possess more genetic diversity than other strains. Additionally, it must be noted that in a study examining cancers resulting from iAs exposure, Dr. Waalkes noted that while incidence of liver cancer increased in a dose response manner, only 38% of adult male offspring developed cancerous tumors (Waalkes 2003). Therefore, in an experiment examining 10 mice per group, we would statistically expect only 4 to develop cancer over their lifetime; it is likely that our results were diluted by mice that would not develop liver cancer. Furthermore, some of the tools we used to examine the effects of treatment, such as the Agilent CpG island microarray, were most likely designed around the genome of an inbred mouse strain, like C57BL/6J mice. With an inbred mouse strain, it is likely that variance in our experiments would be smaller. However, it should be noted that the genetic diversity in CD1 mice provides a more accurate representation of the human population due to this diversity.

This study raises interesting questions concerning combinations of folate and iAs exposure. Our data only report results of a single time point, GD18. Especially with regard to epigenetic changes, development is a particularly tumultuous period with significantly different gene expression profiles than any other; measuring changes in gene transcription and protein expression at identical stages of development is difficult, even with timed-pregnant mice, since so many programming changes are made within a fairly small window of time. A preliminary study mapping fetal changes during gestation in response to iAs would allow us to choose a better time point to focus for our

genetic studies. Little is known about the ability of fetal livers at this age to detoxify iAs. Our iAs-metabolite results (Figure 5) do not show any differences between groups of fetuses exposed to different diets. Whether the fetuses were exposed only to iAs metabolites delivered from their mothers is unknown.

As a result of analyzing tissue from a single time point, we cannot definitively say that methyl donor status (the SAM/SAH ratio) was changed by our treatment. In order to do so, a paired test is required with samples prior to treatment, to establish a baseline measure of comparison.

Our treatments were made based on previous recommendations. The amounts of folate and arsenic provided for the animals were considered high, though both have been individually reported to be well tolerated. While we exposed our animals to 85ppm iAs in water (equivalent to 85 μg/ml), the WHO limit for human consumption is currently set at 10ppb. This discrepancy results from the superior ability of rodents to methylate and detoxify arsenic without apparent health defect (Vahter 1999). Though some studies examined varying concentrations of iAs exposure to mice, our particular model warrants further investigation and biological relevance of the amount of iAs given. In a similar vein, the amount of folate supplemented in the diet was also a very high concentration relative to daily human intake. The recommended daily intake for normal adults is 400μg per day, or about 5.7μg of folic acid per day per kg of body weight, assuming a 70kg person. At 11mg folic acid/kg of diet, our mice consumed approximately 44μg of folic acid per day, or about 1100μg of folic acid per kg of body weight. Even our control diets, at 2.2 mg/kg of folic acid per kilogram, correspond to 220μg of folic acid per kg of body weight. Though higher concentrations of folic acid in diet have been used without detriment, it is difficult to say what effects this may have had in our combined experimental design.

Another element of concern about our diet was the addition of sulfathiazole, an antibiotic intended to eliminate intestinal microflora in dams and prevent endogenous production of folate. By doing so, we were able to limit the amount of folate that the mice received to dietary sources. This may be of concern since gut bacteria play significant roles in digestion and production of other

important nutrients, like vitamin K and others. Though the dams and fetuses from the CT group appeared healthy, little is known about the contribution that gut bacteria play in the context of iAs exposure and the response they would have to these insults that could potentially mitigate or supplement the effects. An experiment in Wistar rats neutralized gut contents by sterilization and reported that arsenic was capable of being metabolized to DMAs by bacteria, though the fate of DMAs was not considered (Rowland 1981); implications for our study indicate that the amount of iAs given to dams potentially resulted in higher liver concentrations in the absence of gut bacteria and thus realized more detrimental effects than were observed in previous transplacental studies.

These detractions and need for more preliminary studies notwithstanding, observations recorded during sacrifice and CpG island methylation data demonstrate that folate supplementation appears to have a negative synergistic effect when combined with iAs. Further studies are warranted to elucidate the interaction between folate and arsenic; there are more metabolic scenarios to consider other than what our original hypothesis sought to accomplish.

Materials and Methods

Animals and Treatment

42 timed pregnant CD1 mice (Charles River Laboratory, Raleigh, NC) were housed individually in a room with a 12 h light-dark cycle at 20-22°C and 50% relative humidity. From gestational day (GD) 5 to 18, the mice were provided *ad libitum* modified AIN-76A purified rodent diets obtain from Dyets (Bethlehem, PA) containing either 2.2 mg/kg folic acid or 11 mg/kg folic acid and from GD 8 to 18, either 0 or 85 mg arsenic/L (85 ppm) in the form of sodium arsenite ($NaAsO_2$, Sigma) in deionized water (see Figure 4).

On GD18, blood from dams was collected by submandibular bleeding, and dams were euthanized by cervical dislocation. Fetuses were dissected out of the uterine horn and maternal livers were removed and snap-frozen. From each fetus, liver was dissected out and snap-frozen in liquid nitrogen and stored in a cell freezer; tails were clipped for sexing. Remaining fetal carcasses were snap-frozen in liquid nitrogen and kept at -80°C.

In all, 10 dams were given 0 ppm arsenic and 2.2 mg/kg folic acid (CT), 11 dams given 85 ppm arsenic and 2.2 mg/kg folic acid (CT/iAs), 9 dams given 0 ppm arsenic and 11 mg/kg folic acid (FS), and 8 dams given 85 ppm arsenic and 11 mg/kg folic acid (FS/iAs). Number of dams per group was unequal as not all were pregnant when received from Charles River.

Follow-up analyses were conducted only in males due to sex differences in development of liver cancer (Waalkes 2003). Tail clippings were digested overnight at 55°C in 2mL lysis buffer containing 10µL Proteinase K (100 mg/mL Qiagen). 750µL of the mix was combined with 0.5mL phenol:chloroform:isoamylalcohol, centrifuged, and followed by ice-cold ethanol precipitation. Pellets were air-dried and resuspended in 50µL TE buffer. Sex determination was performed by multiplex polymerase chain reaction (PCR) of gDNA using primers for the genes *IL3* and *Sry* (for a detailed protocol, see Lambert , 2000). Products were electrophoresed and visualized by UV trans-

illumination on a 1% agarose gel containing ethidium bromide (MP Biomedicals, Solon, OH). One or two male fetuses per dam were used for each follow up experiment.

Folate, SAM and SAH assays

Plasma folate levels in dams were determined by microbiological assay using *L.casei* as outlined in Horne and Patterson, 1988, by the UNC-Nutrition and Obesity Research Center (NORC) to establish the efficacy of our dietary treatment on dams. Levels of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) were determined by high-performance liquid chromatography (HPLC) (She 1994) at UNC-NORC.

iAs metabolite assay

iAs and its various metabolites were profiled by hydride generation-cryotrapping-atomic absorption spectrometry (HG-CT-AAS) using an AAnalyst 800 atomic absorption spectrometer (Perkin Elmer, Shelton, CT) equipped with a custom-designed multiatomizer, HG, and CT units controlled by a Perkin Elmer FIAS-400 flow injection accessory as described elsewhere (Hughes 2010).

Two-color microarray preparation and network analyses

Six fetuses per group were selected for CpG island microarray analysis. DNA was extracted from livers by phenol:chloroform:isopropylalcohol separation as described for sex determination and purified on columns according to the QIAmp DNA Mini Kit (Qiagen). DNA concentration was determined using a Biospectrophotometer (Eppendorf). 4µg of DNA from each sample was then subjected to overnight DNA digestion by MSEI at 37˚C (New England BioLabs, Ipswich, MA), purified using QIAquick PCR purification kits (Qiagen) and vacuum-dried. DNA fragments were then ligated to H12 and H24 oligonucleotide linkers (H12: 5'- TAATCCTCCCTCGAA-3', H14: 5'-AGGCAACTGTGCTATCCGAGGGAT-3'). Equal amounts of both H12 and H24 oligonucleotides were added to a tube and annealed by heating to 55˚C and stepping down 5˚C after every 10 minutes to 20˚C. Ligation was carried out using the Fast-Link DNA Ligation Kit (Epicentre Biotechnologies) at room temperature for 30 minutes; the reaction was stopped at 70˚C for 5 minutes. PCR was carried

out under the following conditions, 72˚C for 5 minutes followed by 25 cycles of 97˚C for 1 minute, 65˚C for 30 seconds, and 72˚C for 3 minutes, followed by 72˚C hold step for 10 minutes. The product was electrophoresed through a 1% agarose gel with ethidium bromide. The remainder of the ligated DNA was purified through QIAquick columns and dried before methylation sensitive digestion.

DNA was digested overnight at 60˚C by BstUI, purified on QIAquick columns and dried, re-dissolved in DNase-free water and subjected to HpaII digestion overnight at 37˚C, which was directly used for PCR without further purification. PCR was carried out with DeepVent(exo-) DNA polymerase (New England Biolabs) under the same conditions used following ligation. After PCR, samples were cleaned through QIAquick columns, DNA concentration was ascertained with the Biospectrophotometer. 4µg of each sample was submitted to the University Health Network Microarray Center (UHN, Toronto, CN) for microarray analysis (Agilent).

Microarray methylation data received from UHN was filtered using Significance Analysis of Microarrays (see Statistical Analyses), part of the TMEV multiple array software. The list of significant differentially methylated genes found following software analysis was imported into Ingenuity Pathways Analysis to investigate relevance to various biological networks.

Messenger RNA Transcript Analysis

To determine the effect of methylation changes on gene expression, we extracted RNA from 6 fetal livers of each treatment group for a total of 24 samples. Each liver was transferred from liquid nitrogen and homogenized in 0.5mL TriZol Reagent (Invitrogen). 0.2 mL chloroform was added and centrifuged at 12,000rpm for 15 minutes at 4°C. The clear phase was pipetted into a new tube where we added 0.5mL isopropanol to precipitate RNA. Samples were allowed to sit at room temperature for 15 minutes, and centrifuged again at 12,000 rpm for 15 minutes at 4°C. The pellet was washed twice with ice cold 75% ethanol. After the last wash step, each RNA pellet was allowed to dry at room temperature and dissolved in 50µL of RNase-free water. Each sample was purified using an RNeasy Kit (Qiagen).

Complementary DNA (cDNA) was synthesized by adding 2µg of sample RNA to 2µL of random primer at 0.3µg/µL. RNase free water was added to bring the total volume up to 24µL and heated to 65˚C for 5 minutes. A mix of 8µL 5X first-strand buffer, 4µL 0.1nmol DTT and 2µL dNTPs was added prior to incubation at 42˚C for 2 minutes. 2µL SuperScript Reverse-Transcriptase II (Invitrogen) was added and incubated for 25˚C for 10 minutes, 42˚C for 50 minutes, and 70˚C for 15 minutes. 2µL RNase H was added for 20 minutes at 37˚C to remove remaining RNA.

We employed Taqman Gene Expression Assays (Applied Biosystems) to examine changes in gene expression for each treatment group relative to control. A list of genes and their context sequences can be found in Table 4; primer sequences are not provided. Each PCR reaction contained 2µL cDNA (equivalent to 100ng total RNA), 6.67µL GeneAssay mix (containing buffers and Taq polymerase), 0.67µL primer at 10 ng/µL, and 4.66µL water for a total reaction volume of 14µL. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was carried out under the following conditions for all genes on a Roche LightCycler 480 (Roche Diagnostics, Indianapolis, IN): Taq polymerase activation for 15 minutes at 95°C, followed by 40 cycles of 2-minute denaturation at 95°C, 1-minute of primer annealing at 60°C, and 30 seconds of synthesis/extension at 72°C. Samples were loaded onto a 96-well plate in duplicate, normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Transcripts of methyltransferases *Dnmt1*, *Dnmt3a*, and *As3mt* were analyzed by primer compatible with Universal Probe Library (UPL) probes (Roche Diagnostics). Primer sequences and probes for these genes are listed in Table 5.

Results were analyzed in LightCycler 480 Software (Roche Diagnostics), using Roche's C_p analytical method "Advanced Relative Quantification" similar to the method described elsewhere (Pfaffl 2001).

Protein Analysis

Protein analysis was performed by western blot. Four livers per group, siblings of those used in gene transcript analysis, were homogenized and split into cellular and nuclear extracts for a total of 16 samples. Cytoplasmic extract (CE) was obtained by homogenizing tissue on ice in PBS with 1mM

DTT, 1mM PMSF, and the protease inhibitors aprotinin, leupeptin and pestatin at 5µg/mL, 2.5µ/mL and 2.5µg/mL, respectively. CE buffer, containing 10mM Tris-HCl (pH 7.6), 60mM KCl, 1mM EDTA in addition to the protease inhibitors listed for PBS was added at 1mL per 10mg of tissue. 10µL of 10% NP-40 was added to each tube and vortexed briefly. Tubes were centrifuged for 5 minutes at 12,000 rpm and 4˚C, supernatant is the CE. Remaining sediment (nuclei) were washed with 500µL CE buffer lacking NP-40, spun at 1,500rpm for 5min and 4˚C. 100µL of nuclear extract (NE) buffer was added containing inhibitors in PBS as well as 20mM TrisHCl (pH 8.0), 400mM NaCl, 1.5 mM $MgCl_2$ and 25% glycerol. The solution was set on ice for 1 hour, with periodic vortexing. NE samples were spun at 12,000rpm for 5 minutes at 4˚C to pellet nuclei; the supernatant is the nuclear fraction. Both CE and NE were stored at -80˚C.

Protein concentration was determined by bicinchoninic acid (BCA) assay. With the exception of the gel run for β-catenin, 500 ng of cellular extracts were loaded directly onto a 10% acrylamide gel and electrophoresed for 25 minutes at 100V and 175 volts for 6 hours. Proteins were transferred using a Trans-blot cell (BioRad) overnight at 4°C under 250 milliamps of current onto a PVDF membrane. Transfer efficiency was estimated by Ponceau staining.

The membrane was blocked with 5% milk in Tris-buffered saline (TBS) for 1 hour prior to overnight incubation with primary antibody (for concentrations and predicted size, see table 6). Following primary incubation, membranes were washed three times for five minutes with 0.1% Tween-TBS (TBS-T) and incubated with their respective secondaries at a 1:2000 concentration for two hours. Membranes were then washed three times in TBS-T, incubated in ECL (Thermo Scientific) for 5 minutes and exposed to Biofilm (Kodak). Optimal exposure varied according to the times indicated in Table 5. Semi-quantitative analysis was carried out using Photoshop 7.0 (Adobe) and normalized to β-actin for cellular extracts, or nuclear lamina for the nuclear extract. Following each exposure to film, the membrane was re-blocked for an hour with 5% milk in TBS and placed in the next primary.

Protein analysis for β-catenin required immunoprecipitation (IP) of the protein from the nuclear extract. 500ng of nuclear extract from each sample was added to 1mL RIPA buffer containing aprotinin, leupeptin and pepstatin, as well as 1nmol PMSF. Primary antibody to non-phospho-β-catenin (Santa Cruz Biotechnology) was added and mixed for 1 hour. 20μL of agarose beads were added and allowed to mix overnight at 4°C. The agarose beads were pulled down via centrifugation at 1000xg for 5 minutes and the supernatant was placed into new tubes. Proteins were denatured and released from the beads by heating up to 95°C for 5 minutes in a dry bath. Samples were run on a 10% acrylamide gel and transferred according to conditions as reported for other proteins.

The supernatant after IP was retained to run a separate western blot to determine loading control for β-catenin, nuclear lamin-b1 (Abcam). 30μg of nuclear extract was electrophoresed similar to other proteins.

Statistical Analyses

Microarray data was analyzed with Significance Analysis of Microarrays software (SAM software, Tusher 2001) embedded within the TM4 Microarray Software Suite (Saeed 2006), using a two-class unpaired response model of log ratios of the Cy3 to Cy5 signal. Number of permutations was set to 210 and S_0 values were determined using the minimum S value. Delta-values were computed by the SAM-software as a measurement of strength between extent of DNA methylation and the log-ratio, and selected based on a 5% false-discovery-rate.

For gene expression and protein data, values were averaged and checked for conformity by Chauvenet's criterion. For each measure, data were subjected to two-way ANOVA with Diet (CT vs. FS) and Exposure (DI-water vs. DI-water + iAs) as between-subjects factors. Significant interactions were followed up by post-hoc tests. Planned comparisons were made where appropriate. *P*-value for α was set to 0.05.

TABLE 4. List of genes run by Taqman Gene Assay and their context sequences

Group	Gene Name	Context Sequence, 5' → 3'
One Carbon and iAs Metabolism	*Ahcy*	TAGCCTGGACAGTGCTTCTCCCACA
	Mat2a	CCTCAGATGGCAGCTTTTAAAAGAT
	Cbs	CACACAGTGCTGACCAAATCCCCCA
	Mtrr	AATATGGATTATTGGGCTTGGGTGA
Cell Cycle and Apoptosis	*Cdkn2a*	CTCAAGCACGCCCAGGGCCCTGGAA
	Cdkn1a	GCAGACCAGCCTGACAGATTTCTAT
Imprinted and Associated Genes	*Dlk1*	TCTGCGAAATAGACGTTCGGGCTTG
	Igf2r	TGGCTCGTCACTCAGAATCAGAACA
	Igf2bp1	TGCCAGCCAGATGGCTCAGCGGAAG
Wnt Pathway	*Wnt3*	AGTAGTGAGCCAGGGCACTGGGAAG
	Fzd8	CCTGTGGTCGGTGCTCTGCTTCGTC
	Fzd10	CCTTCATCCTGTCCGGCTTTGTGGC
	Ctnnb1	AATGAGACTGCAGATCTTGGACTGG
	Cnnd1	TGTGCCACAGATGTGAAGTTCATTT
	C-myc	TTGGAAACCCCGCAGACAGCCACGA

TABLE 5. UPL Primers and Probes

	Forward Primer	Reverse Primer	UPL Probe
Dnmt1	gagccagcccagagatcc	cgtctctgtcctcgggagt	#38; ggaagcag
Dnmt3a	acacagggcccgttacttct	tcacagtggatgccaaagg	#72; gccaggaa
As3mt	tgcagaatgtacacgaagacg	cagccgctcaggaacagt	#76; tggctgtg

TABLE 6. Antibody concentration and exposure time for western blot

	Primary Concentration	Exposure Time	Manufacturer	Predicted Size
Wnt3	1:1000	5 minutes	Abcam	37 kDa
Fzd7	1 μg/mL	5 minutes	Abcam	63 kDa
Fzd10	1:2000	10 seconds	Abcam	65 kDa
β-catenin	1:500	10 minutes	Cell Signaling	92 kDa

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