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DNA methylation and gene expression in the runt and methyl-deprived pig

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LIST OF ABBREVIATIONS

ARS	aberrant RNA species
BHMT	betaine-homocysteine methyltransferase
cDNA	complementary DNA
CGI	CpG islands
ChIP-Seq	Chromatin immunoprecipitation sequencing
CTH	cystathionase
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DPM	disintegrations per minute
FASN	fatty acid synthase
GAMT	guanidinoacetate N-methyltransferase
GLUT4	glucose transporter type 4
GNMT	glycine N-methyltransferase
H	histone
HDAC	histone deacetylase
HEV	Hepatitis E virus
IGF-1	insulin-like growth factor 1
IUGR	intrauterine growth-restricted
K	lysine
kg	kilograms
MAGE-1	melanoma antigen family A1
MAT	methionine adenosyltransferase
MeCP2	methyl CpG binding protein 2
mg	milligrams
ml	milliliters

MS	methionine synthase
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase
MTHFD	methylenetetrahydrofolate dehydrogenase
N/A	not applicable
ng	nanograms
NR3C1	nuclear receptor subfamily 3 group C member 1
NW	normal weight
Pdx-1	pancreatic and dueodenal homeobox 1
PCR	polymerase chain reaction
PEMT	phosphatidylethanolamine methyltransferase
qPCR	real-time quantitative polymerase chain reaction
RNA	ribonucleic acid
RT-qPCR	quantitative reverse transcription polymerase chain reaction
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SAHH	S-adenosylhomocysteine hydrolase
SHMT	serine hydroxymethyltransferase
SD	standard deviation
WTSS	whole-transcriptome shotgun sequencing
μg	microgram
μl	microliter

ABSTRACT

This research focuses on studying the effect of reduced maternal methyl donor supply as well as intrauterine growth restriction (IUGR) on DNA methylation and on gene expression in pigs. Two separate techniques were used to determine global DNA methylation levels (cytosine extension and methyl incorporation assays) but no differences at the genomic level were identified for either experimental group using either technique. The expression levels of numerous genes involved in methylation directly or whose expression might be expected to change under these experimental conditions were also investigated. The expression of DNMT3a2 and BHMT were significantly lower in B_6-deprived pigs but no differences were observed in folate-deprived or runt pigs compared to relevant controls. Further, sex differences were observed, indicating that sex should be considered for future studies regarding methyl-donor or runt-large studies in pigs. Lastly, we discovered an aberrant RNA species (ARS) in numerous pigs in both of our studies, from both Newfoundland and Manitoba research pig populations, and this ARS significantly affected the expression of numerous genes in both control and treatment groups.

LITERATURE REVIEW AND INTRODUCTION

Developmental origins of adult disease

David Barker and colleagues first introduced the idea of fetal origins of adult disease in 1995, proposing that the development of disease later in life is influenced by the *in utero* environment of the fetus (Barker, 1995). However, research supporting this hypothesis goes back as early as 1934 when Kemack et al. (1934) examined populations in the UK and Sweden and identified a relationship between death rates and living conditions. Since that time other studies have come to similar conclusions. In 1986 a geographical relation between neonatal mortality in 1921-25 was found with cardiovascular and stroke death in 1968-78 (Barker and Osmond, 1986). This study led the authors to the conclusion that poor maternal health was a risk indicator for stroke in offspring later in life. This link was further supported with findings in 1993 that cardiovascular disease correlates with low birth weight (Osmond et al., 1993). Not only do these studies establish a link between maternal health and that of the offspring, but they also demonstrate that the effects did not manifest until much later in life, suggesting a possible epigenetic mechanism rather than a simple perturbation of development. Further, human epidemiological studies linked small birth weight and increased postnatal growth to several chronic adult diseases such as obesity, hypertension, coronary heart disease, and diabetes (McMillen and Robinson, 2005). Clearly a strong correlation between early development and adult disease has been established, but the biochemical mechanisms involved remain to be elucidated.

Swine models

Pigs are an excellent animal model for the study of human health for a number of reasons. In contrast to rodent models, pigs are relatively mature at birth (Vuguin, 2007) and are similar to humans physiologically. For example, pigs and humans share a similar metabolism, lipoprotein profile, and cardiovascular system. Further, their omnivorous diet, eating habits and nutrient requirements are also similar (Bellinger et al., 2006).

Unlike humans, pigs are litter-bearing animals. Interestingly, piglets show natural variation in birth weight due to variation in fetal nutrient supply, resulting in large as well as runt offspring (Bertram and Hanson, 2001). This is advantageous when studying intrauterine growth restriction (IUGR) as direct comparisons between genetically similar large and runt littermates are possible (Poore and Fowden, 2002). Moreover, runt piglets often experience catch-up growth postnatally (Ritacco et al., 2007).

Our laboratory is particularly interested in developing the Yucatan miniature pig as a model of the developmental origins of adult diseases. This strain of pigs has all of the advantages of the farm breeds for the study of human diseases with the added advantages of smaller size, slower growth, propensity to obesity and easier experimental manipulation. For this reason, they are widely used for longer-term studies (Swindle et al., 1994).

Intrauterine growth restriction (IUGR)

IUGR refers to a condition in which offspring are born smaller than normal due to a reduced growth rate inside the womb. IUGR is a useful model to examine the developmental origins hypothesis. The three ways IUGR may present include perturbations in placental blood flow, poor maternal nutrition, or maternal exposure to toxins (reviewed in Hendrix and

Berghella, 2008). The effects of IUGR have been difficult to map as mammalian metabolic pathways are highly interrelated and IUGR has been shown to affect many of these pathways (reviewed in Joss-Morre and Lane, 2009). Moreover, the effects of IUGR are dependent on sex, the specific nature of the insult, gestational timing of the insult, and the rate of postnatal growth (Josse-Moore and Lane, 2009), making it all the more difficult to pinpoint the cause of adult morbidity generated by IUGR.

The Dutch famine of the Second World War, where a Nazi blockade severely restricted access to food in western Holland for a period of approximately 6 months, provides some of the strongest evidence for the impact of maternal nutrition on disease incidence occurring later in life in offspring. Extensive research of these individuals showed that maternal undernutrition resulted in offspring at increased risk for hypertension, coronary heart disease, renal dysfunction, obesity and glucose intolerance (Ravelli et al., 1976, Roseboom et al., 2001, and Painter et al., 2005). Hales and Barker proposed the thrifty phenotype hypothesis to explain why pre-natal nutrition resulted in poor health outcomes later in life (Hales and Barker, 2001). This hypothesis argues that fetal nutrition results in developmental adaptation to the predicted post-natal environment. When the post-natal environment does not match the predicted environment, as is the case with poor fetal nutrition meeting the adult environment of nutrition abundance, negative health outcomes result. This hypothesis was later expanded to include the effects of early nutrition and post-natal growth rates and has since been termed developmental origins of adult disease (McMillen and Robinson, 2005).

Much of the research regarding IUGR individuals focus on adult-onset metabolic disease. Numerous cohort studies show that individuals underweight at birth have impaired glucose tolerance in childhood and are at increased risk to develop type 2 diabetes later in life (Philips et

al., 1994, Law et al., 1995, Godfrey et al., 1996, and Yajnik et al., 2003). IUGR has been shown to affect hepatic glucose production, beta-cell function, and insulin sensitivity (Ross and Beall, 2008, Reusens et al., 2007, Simmons, 2007, and Devaskar and Thamotharan, 2007). Moreover, IUGR also alters glucocorticoid signalling molecules (Eriksson et al., 2002), which can cause a myriad of metabolic disturbances and affect a multitude of biochemical pathways and the expression of numerous genes (reviewed in Langley-Evans and McMullen, 2010). Metabolic disturbances in IUGR individuals occur in adipose tissue, liver, pancreatic β-cells, and the vascular system (Josse-Moore and Lane, 2009). Thus, it is not surprising that obesity and metabolic disease are typical outcomes of IUGR (Ross and Beall, 2008, Cottrell and Ozanne, 2008, and Wells et al., 2007). Indeed, low birth weight in sheep results in increased levels of pro-adipogenic transcription factors such as peroxisome proliferator-activated receptor gamma (PPARγ) (Duffield et al., 2008). Interestingly, this was true whether low birth weight was a result of IUGR or natural variation in birth weight. Further, male IUGR rats show increased expression of hepatic genes involved in lipogenesis as well as increased levels of inflammatory markers such as C-reactive protein (Choi et al., 2007, and Magee et al., 2008). Notably, many of the changes in gene expression that are observed in the neonatal period persist into adulthood leading investigators to deduce that epigenetics is an underlying mechanism in the developmental origins of disease.

Catch-up growth

In mammals, there exists a period of rapid, linear catch-up growth following a period of growth inhibition involving increases in height, attributed to growth at the growth plates, and also in terms of organ size (reviewed in Finkielstain et al., 2013). As such, it is difficult to delineate the effects of poor fetal nutrition from that of catch-up growth when addressing disease

that does not appear until adulthood and is why IUGR and catch-up growth may best be studied in tandem. However, numerous studies have found that catch-up growth often involves overcompensation, and the organism commonly accumulates excessive adipose tissue and exceeds normal weight (Ong et al., 2000, Cianfarani et al., 1999, and Luo et al., 2006). This may be partly attributable to subsequent development of adult disease (such as adult obesity, insulin resistance and type 2 diabetes) independent of birth weight. Barker found that risk of adult disease increases with catch-up growth above that of low birth weight alone (Barker, 2004). A large cohort study by Eriksson et al. also reported that individuals who had low birth weight and subsequently underwent rapid weight gain had higher risk of developing impaired glucose tolerance and type 2 diabetes than individuals who had low birth weight but did not experience rapid growth (Eriksson et al., 2006).

Children who experienced catch-up growth between 0 and 2 years of age were heavier, taller, and had a higher body mass index, weight circumference and adiposity at 5 years of age than their peers (Barker and Osmond, 1988). Moreover, a study of more than 1400 adults from India found that thinness in infancy correlated with impaired glucose tolerance and diabetes in young adulthood (Leger et al., 1996). Although those affected were more overweight in adulthood, it is noteworthy that these individuals had a higher rate of increase in body mass after the age of two years and they were not overweight during childhood. Alternatively, pre-term babies fed a low caloric diet experienced reduced early growth which was associated with increased insulin sensitivity during adolescence (Singhal et al., 2003). These studies suggest a causative relationship between catch-up growth and development of metabolic disease later in life.

It has been proposed that the mechanism relating catch-up growth to metabolic disease in adult life is related to compensatory increases in insulin and insulin-like growth factors. IUGR infants have low levels of serum insulin and IGF-1 at birth (Leger et al., 1996, and Cianfarani et al., 1998) and, as levels normalize in the postnatal period, insulin resistance may occur in response to the rapid increase in these hormones. Other proposed mechanisms include structural changes in developing organs (Law et al., 1992) and reduced cell number in specific organs (Bol et al., 2008). Further, as is also the case with IUGR, the changes that occur with catch-up growth may originate from alterations to the epigenome.

Epigenetics

In mammals, epigenetics refer to enzymatic modifications to cytosine bases and histone proteins in the nucleosome that do not affect the nucleotide sequence of the DNA (Kim et al., 2009). Of these modifications, cytosine methylation at CpG dinucleotides has received much attention in the literature as it relates to gene expression. DNA methylation is considered an epigenetic mechanism because it can alter gene expression without changing the structure of DNA (Waterland and Michels, 2007). Methylation occurs on the fifth carbon of the cytosine base by the covalent addition of a methyl (CH_3) group (Langley-Evans, 2006). Cytosine methylation is maintained through cell divisions by DNA (cytosine-5) methyltransferases (DNMTs). DNMT1 is thought to act primarily as a maintenance methyltransferase as it has a strong preference for hemimethylated DNA *in vitro* (Pradhan et al., 1999). It is thought that DNMT1 functions by copying the methylation pattern of the parental strand (Bird, 2002). DNMT3a and DNMT3b are the de novo methyltransferases and are responsible for establishing new CpG methylation at unmethylated DNA (Van den Veyver, 2002).

In mammals, approximately 70% of CpGs are typically methylated. However, CpG dinucleotides are often concentrated in 'islands' in the 5' regulatory region of genes (Rees, 2002; Waterland and Jirtle, 2004) and it is through methylation of these CpG islands (CGIs) that DNA methylation is thought to control gene expression. In general, hypermethylation of CGIs upstream of a gene has a silencing effect while hypomethylation allows overexpression. For example, normally unmethylated CGIs upstream of tumour suppressor genes in cancer cells are often hypermethylated during neoplasia, reducing their expression (Feinberg and Vogelstein, 1983; Issa and Baylin, 1996). Similarly, demethylation of normally methylated CGIs can lead to activation of silenced genes, as was first shown with melanoma antigen family A1 (MAGE-1) which is abnormally expressed in human tumors (De Smet et al., 1996). However, new research suggests that it is the methylation status of CpG island shores, up to 2 kb away from the expressed gene, whose methylation status more commonly controls gene expression (reviewed in Irizarry et al., 2009).

Epigenetics and methyl nutrients

DNA methylation, especially in early development, is known to be affected by manipulation of supply of methyl donors, such as methionine, betaine and choline, as well as by other components of methylation pathways, such as folate and vitamin B12. As appropriate DNA methylation is essential for early fetal development (Shames et al., 2007), the study of how early nutrition affects epigenetics is a worthwhile endeavour.

Perhaps the most convincing data relating nutrition to DNA methylation and phenotypic changes come from the yellow agouti (A^{vy}) mouse. In this mouse model, the origin of the A^{vy} allele is unknown, but thought to behave similarly to transposable elements (Waterland and

Jirtle, 2004), which are typically silenced by DNA methylation. Interestingly, this allele is responsive to dietary manipulation of methyl donors and provides an observable phenotype whereby hypomethylation of the allele leads to obesity and a yellow coat color. Notably, maternal methyl supplements have been shown to increase methylation of this allele, resulting in obesity later in life (Cooney et al., 2002). However, contention over the applicability of the agouti mouse exists because control over methylation of the A^{vy} epiallele may resemble that of transposable elements rather than typical genes. Nevertheless, sheep exposed to a methyl-deficient diet early in development show perturbations in hepatic DNA methylation and have increased risk for developing obesity, insulin resistance, and elevated blood pressure (Sinclair et al., 2007). Specifically, 4% of the 1400 CpG islands investigated had altered methylation status and 88% of these were hypomethylated.

Epigenetics and IUGR

DNA methylation is known to have an important role in gene control and several recent studies have determined that alterations in methyl supply during embryogenesis can alter gene-specific methylation as well as gene expression (Waterland and Jirtle, 2003; Waterland and Dolinoy, 2006). Over the short term, gene expression can be regulated by signal transduction and transcription factor activation but the persistent long term alterations in gene expression of specific genes observed in IUGR are best explained by epigenetic mechanisms (Josse-Moore and Lane, 2009). These mechanisms are thought to include blocking transcription factors, recruiting proteins that interfere with transcription, modifying chromatin structure, or recruiting proteins that modify chromatin structure (Van den Veyver, 2002).

Waterland and Michels (2007) contend that altered gene expression caused by pre- and post-natal diet as well as compensatory growth are mediated by changes in DNA methylation. IUGR is associated with epigenetic modifications of skeletal muscle glucose transporter type 4 (GLUT4) and with pancreatic beta cell pancreatic and dueodenal homeobox 1(Pdx-1) (Raychaudhuri et al., 2008, and Park et al., 2008). Decreased global methylation and increased histone 3 (H3) acetylation on lysine 9 (K9) and K14 in brain are also observed in the IUGR rat, along with decreased levels of epigenetic enzymes DNA methyltransferase 1 (DNMT1), methyl CpG binding protein 2 (MeCP2), and histone deacetylase 1 (HDAC1) (Ke et al., 2006).

Overview

The overall goal of my research is to evaluate the effect of reduced methyl donor supply on DNA methylation and gene expression and the potential implications of those changes on long-term health. It is clear from a variety of epidemiological as well as other types of studies that nutritional insults causing impaired intrauterine growth can be associated with an increased incidence of cardiovascular, metabolic and other diseases later in life (Barker, 1995). What is not so clear is the precise mechanism by which this is occurring. One prevalent theory is that epigenetic programming (*i.e.* lineage-specific gene control) is affected due to a shortage of the necessary components at the critical developmental time points when this programming is being established. Some of the better known and better understood epigenetic mechanisms include the addition of a methyl group to cytosine in a CpG dinucleotide arrangement and the post-translational modification of the histone tails, particularly on H3 and H4. Of these epigenetic mechanisms, DNA methylation is the more easily analyzed as methyl supply can be manipulated in the diet of the mother and/or offspring. And there is proof in principle that the epigenetic state

of DNA sequences can be affected by altering the methyl groups available in the maternal diet (Waterland and Jirtle, 2003; O'Neill et al., 2014; reviewed in Choi and Friso, 2010).

I have used two different approaches to investigate the possibility that DNA methylation changes can be induced during development due to limited methyl supply. First, I looked for changes in methylation in the genome of folate- and vitamin B_6-deprived pigs (*Sus scrofa*) as well as changes in the expression of a number of methylation related genes, including those associated with DNA methylation but also other types of methyltransferases and related genes. Analyzed genes involved in methylation pathways included methionine synthase (MTR), betaine-homocysteine methyltransferase (BHMT), cystathionase (CTH), DNA methyltransferase 1 (DNMT1), guanidinoacetate N-methyltransferase (GAMT), glycine N-methyltransferase (GNMT), phosphatidylethanolamine methyltransferase (PEMT), methylenetetrahydrofolate dehydrogenase (MTHFD), and DNA methyltransferase 3a2 (DNMT3a2). Fatty acid synthase (FASN), and glucocorticoid receptor (NR3C1) were also analyzed for methylation status as these genes were found in other studies to have altered methylation status and expression due to nutritional insults in rats (Lillycrop et al., 2005, Lillycrop et al., 2007, and Lomba et al., 2010). I then did similar analyses comparing methylation and gene expression in runt and normal weight piglets from the same litters; runt pigs were used as a model for IUGR as runts are typically deprived of total nutrients during *in utero* development.

Previous research

<u>Runt/large littermate studies using Yucatan miniature pigs:</u>

Previous research in our laboratory on Yucatan miniature pigs used in this study found that IUGR pigs undergo compensatory growth and develop greater subcutaneous fat depth. As

expected, birth weight negatively correlated with visceral fat content and females developed more visceral and subcutaneous adiposity (McKnight et al., 2012). Moreover, IUGR pigs showed higher feed intake which explained catch-up growth and increased adiposity. Notably, IUGR pigs at 10 months old (early adulthood) displayed higher diastolic blood pressure and had 26-34% fewer nephrons (Myrie et al., 2012).

B_6–deficiency study using domestic pigs:

Tissues from vitamin B_6-deficient domestic pigs used in this study were generously donated by Dr. Jim House at the University of Manitoba. Previous research in Dr. House's laboratory found that piglets consuming B_6-deficient diets had reduced average daily gains during the fourth week and had reduced activity of certain hepatic enzymes. Specifically, cystathionine β synthase (CBS), cystathionine γ lyase (also known as cystathionase, CTH) and serine hydroxymethyltransferase (SHMT) activities were reduced in B_6-deficient pigs. Plasma methionine and serine levels were found to be elevated while plasma glycine had decreased (Zhang et al., 2009).

Folate-deficiency study using domestic pigs:

Tissues from folate-deficient domestic pigs used in this study were also generously donated by Dr. Jim House at the University of Manitoba. These pigs were previously used in a study on the influence of folate deficiency on formate pharmacokinetics, as it relates to the pathophysiology of alcoholism. Folate-deficient pigs were found to have impaired formate elimination following formate infusion (Sokoro et al., 2008), but no data were collected on methylation pathways.

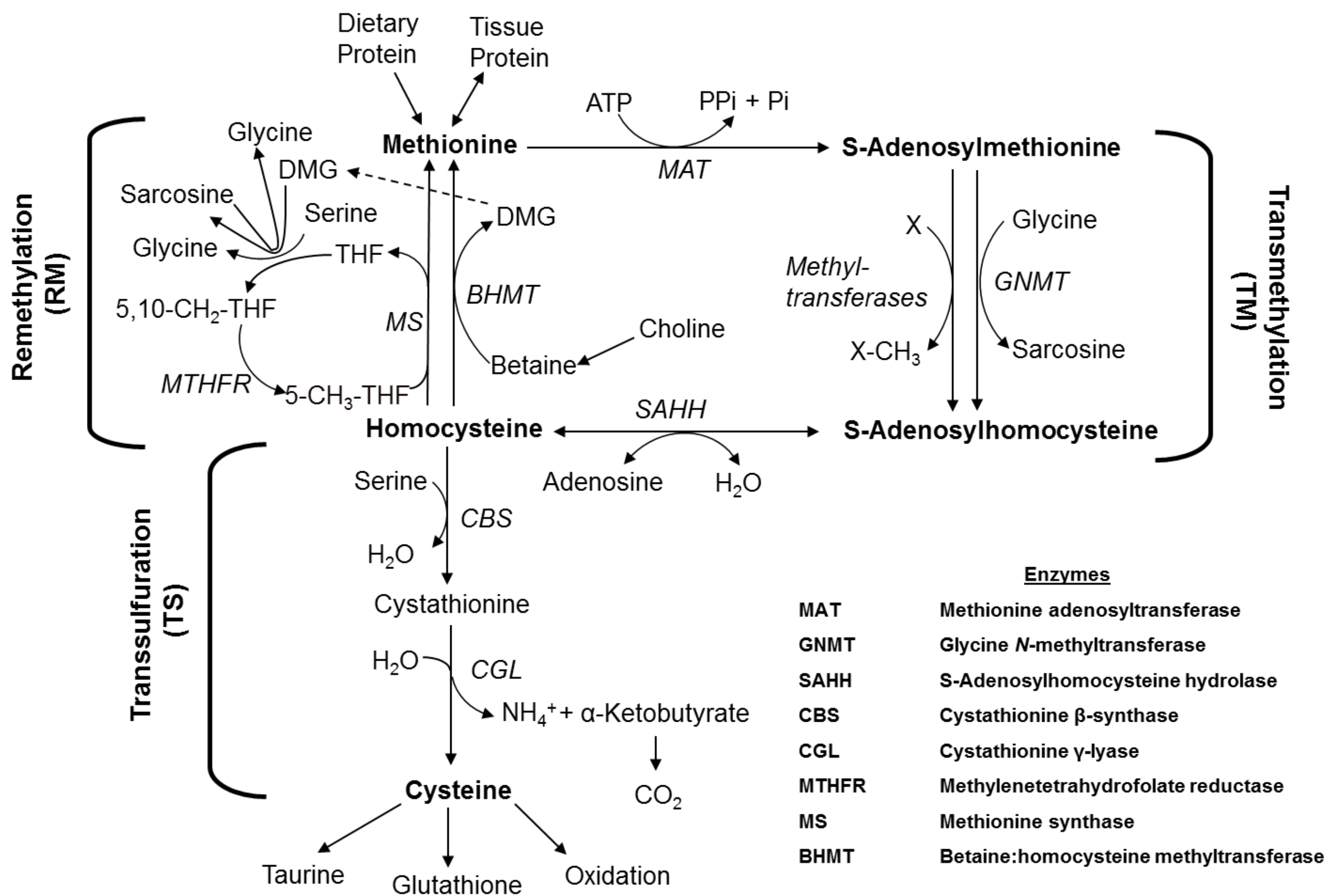

Figure 1: One-carbon metabolism in mammals

Methylation pathways

Major dietary sources of methyl donors include methionine, serine (via folate) and choline (via betaine) (see Figure 1). Although folate is not a methyl donor per se, its pivotal role in methyl transfer from serine justifies its examination with respect to DNA methylation studies.

Folate is a coenzyme of one-carbon metabolism and is directly involved in biochemical pathways leading to methylation of cytosine residues of CpG dinucleotides on DNA. Deficiency in folate increases maternal plasma homocysteine and exacerbates the effect of a low methionine diet (Maloney et al., 2009). Although the effect of low dietary folate on global methylation patterns remains inconclusive, much research suggests that low folate has significant effects at the gene level (Aisling et al., 2016)

S-adenosylmethionine (SAM) is the methyl donor for numerous methylation reactions, including DNA and protein methylation. Methylation reactions involve transfer of a methyl group from SAM by a DNMT enzyme or protein methyltransferase. This process necessitates the transmethylation pathway. SAM is formed from methionine by the attachment of an ATP to the sulfur atom of methionine by methionine adenosyltransferase (MAT). Notably, when SAM is metabolized in a methylation reaction, S-adenosylhomocysteine (SAH) is produced, which can be hydrolyzed by the enzyme S-adenosylhomocysteine hydrolase (SAHH) to form homocysteine and adenosine (Van den Veyver, 2002).

Homocysteine has two potential metabolic fates. First, homocysteine can be catabolized via the transsulfuration pathway to cysteine. Here, homocysteine is condensed with serine by the enzyme CBS using vitamin B_6 as a cofactor. Next cystathionine is metabolized to cysteine and α-ketobutyrate by the enzyme cystathionine-gamma-lyase, or cystathionase. However,

homocysteine can also be remethylated to methionine in one of two ways: homocysteine can accept a methyl group from betaine to form dimethylglycine and methionine via betaine-homocysteine methyltransferase (BHMT), or a methyl group can be transferred to homocysteine from 5-methyltetrahydrofolate by MS to form methionine and tetrahydrofolate (Stipanuk, 2004).

Dietary methionine is not sufficient in providing an adequate supply of methyl groups for all methylation reactions, meaning *de novo* synthesis (i.e. via folate) and recycling (i.e. via betaine) of methyl groups is required. Methyl donors to methylation pathways include folate, betaine, or choline (through conversion to betaine) and essential cofactors include vitamin B_6 and vitamin B_{12}. Vitamin B_6 is required for transfer of methyl from serine to folate via serine hydroxymethyltransferase (SHMT), but is also required to catabolize homocysteine via CBS. Because both remethylation and transsulfuration of homocysteine depend on vitamin B_6, low levels of vitamin B_6 increase plasma homocysteine and SAH levels, although increased activity of SAH hydrolase seems to be the primary pathway (Isa et al., 2006). As SAH is inhibitory to most methyltransferases (Van den Veyver, 2002), low vitamin B_6 would also be expected to decrease global as well as gene-specific methylation.

Rationale for runt/large pig study

My objective was to examine the role of IUGR on DNA methylation and gene expression in pigs that were already shown to be at a higher risk for metabolic disease in adulthood. The reduced growth rate of IUGR pigs is a consequence of reduced blood flow (Laurin et al., 1987), which would presumably reduce the supply of all maternal nutrients, including those related to methyl metabolism. However, it is not known if changes to DNA methylation in response to a 'methyl deficient' diet will be gene-specific and, if so, which genes are affected. Moreover, it is

unknown which epigenetic changes in response to IUGR will be maintained throughout the life of the organism or how they correlate to adult disease. Here, I examine the expression of a variety of genes involved in DNA methylation as well as other genes whose methylation is expected to change in response to IUGR in adult Yucatan pigs with disease outcomes.

For this objective, I utilized tissues from large and runt pigs, which, as outlined above should have developed in a methyl donor-deprived environment. The reason for this was twofold: first, these pigs were grown to adulthood, so I was able to choose runt pigs with strong phenotypes—in other words, pigs that had developed adult metabolic diseases. Thus, it is more likely to see changes which could otherwise be masked by biological diversity. Second, by comparing gene expression changes in these pigs with gene expression changes in folate- and vitamin B_6-deprived pigs used in my second study, I will gain more insight into the similarities and differences between these two dietary insults in early development. Our **hypothesis is that runt littermates will have been deprived of methyl donors *in utero*, resulting in reduced global and gene-specific DNA methylation corresponding to altered gene expression.** The expression of selected genes was examined using real-time quantitative polymerase chain reaction (qPCR). Numerous genes involved in methylation or known to be affected by early nutritional insults were chosen, along with genes known to change methylation status throughout the life of mammals. Global DNA methylation status was also examined. Major outcomes from the runt/large pig liver samples were later examined in the folate-deprived pig liver samples to compare the effects of these two nutritional insults during early development.

Rationale for folate- and B_6-depletion pig studies

As discussed above, the comparison of runt to normal weight littermates has advantages in that the animals are grown out and those that developed biomarkers for adult onset diseases (such as impaired glucose tolerance, type 2 diabetes, hypertension, and increased adiposity) can be targeted. However, they have the disadvantage in that there is a general limitation of maternal nutrients and one cannot determine whether methyl nutrient deprivation per se affects the methylation of the DNA in a causative manner. In order to support this connection, I have to more specifically target deprivation of methyl supply. That is the purpose of the second research approach—to specifically reduce the availability of methyl groups available for DNA methylation without reducing other developmentally required dietary components.

Much research has focussed on the role of folate in fetal development and growth, largely due to its role in preventing neural tube defects (Antony, 2007;Rondo, 2000). It has been established that manipulating methyl donors (including folate) in the maternal diet can alter DNA methylation in offspring (Wu et al., 2004), and it has even been hypothesized that reducing folate intake during pregnancy may increase neural tube defects due to limited methyl supply during cranial neural tube closure (Dunlevy et al., 2006; Weingartner et al., 2007). However, the role of inadequate folate supply in early development in epigenetic changes and, subsequently, in adult disease has not been fully elucidated. As folate is a key element in methylation pathways, including DNA and protein methylation, I hypothesize that low maternal folate will perturb cellular methylation pathways, predisposing offspring to development of metabolic disease in adult life. Further, as DNA methylation is well preserved throughout the lifespan of the organism, perturbations in global methylation and gene-specific methylation may have profound effects on the longevity of the organism.

During pregnancy, folate status is positively associated with DNA methylation (Park et al., 2005). Moreover, a folate-deplete diet has been shown to decrease placental DNA methylation in a hyperhomocysteinemic rat model while folate supplementation had the opposite effect (Kim et al., 2008). In another study looking at rat pups from protein-restricted mothers, folic acid supplementation reversed DNA hypomethylation (Mathers, 2005). Further, decreased liver promoter methylation of NR3C1, DNMT1 and PPARα was also reversed by supplementation (Lillycrop et al., 2005, and Lillycrop et al., 2007). These studies clearly indicate a role for folate in establishing early epigenetic patterns and suggest a role for folate in an epigenetic mechanism for the developmental origin of adult disease.

A reduction in dietary vitamin B_6 is also predicted to have an effect on methyl supply given its key role as a cofactor for SHMT, CBS and CTH. Numerous studies indicate that pregnant and lactating women in different parts of the world consume well below the recommended dietary allowance for vitamin B_6 (dos Santos et al., 2014; Shibata et al., 2013), which may have deleterious effects on their offspring, potentially impacting brain development and cognitive function (reviewed in Guilarte, 1993). However, little is known of the impact of maternal vitamin B_6 deficiency on epigenetics or developmental origins of adult disease. Due to the role of B_6 in reducing the SAM/SAH ratio, a proxy indicator of transmethylation, I propose that low maternal B_6 may have similar effects to low folate on epigenetic outcomes and developmental programming.

My objective was to examine the role of reduced methyl supply on DNA methylation and gene expression. As discussed previously, reduced methyl supply *in utero* increases the risk of developing disease later in life. Further, reduced methyl supply should reduce overall DNA methylation by limiting available SAM. However, it is not known if changes to DNA

methylation in response to a methyl deficient diet will be gene-specific and, if so, which genes are affected. Here, I examine the expression of a variety of genes involved in DNA methylation as well as other genes whose methylation is expected to change in response to a methyl deficient diet.

For this objective, I worked with domestic pigs deprived of folate or vitamin B_6 in early development as well as their control littermates. The purpose of this study was to determine the effect of inadequate neonatal methyl supply on DNA methylation and gene expression with the **hypothesis that neonates deprived of methyl-dependent vitamins will have reduced global and gene-specific DNA methylation of genes related to one-carbon metabolism corresponding to altered gene expression**. I used liver tissue from piglets placed on a folate- or vitamin B_6-deficient diet and control diets postnatally from previous studies by Dr. House in Manitoba.

MATERIALS AND METHODS

Sample collection (Vitamin B_6)

Pig liver samples were generously donated by Dr. James D. House. Originally, liver samples were isolated from pigs deprived of vitamin B_6 as described by Zhang et al. (2009). A total of 12 pigs were used for the study. However, one sample from the control group had to be omitted from the current study due to lack of available sample. Fourteen-day old male pigs (cross-bred commercial strains) were divided into two dietary treatments (n=6 each treatment): (1) basal diet + 3.0 mg pyridoxine HCl/kg diet (Control) and (2) basal diet + 0 mg pyridoxine HCl/kg diet (Deficient). The basal diet contained 40.15% corn starch, 20.5% casein (vitamin free), 20% lactose, 5.5% corn oil, 3.5% cellulose, 0.35% cystine and 10% vitamin and mineral

premix (providing 2.4 g sodium, 3.6 g chloride, 0.6 g magnesium, 4.2 g potassium, 9 mg copper, 150 mg iron, 6 mg manganese, 150 mg zinc, 0.21 mg iodine, 0.45 mg selenium, 4400 IU vitamin A, 440 IU vitamin D3, 32 IU vitamin E, 1 mg vitamin K, 0.1 mg biotin, 1 g chlorine, 0.6 mg folacin, 30 mg niacin, 20 mg pantothenic acid, 7 mg riboflavin, 2 mg thiamin, 35 μg vitamin B12 per kg diet). The basal diet was essentially devoid of vitamin B_6 (<0.02 mg/kg). A pair-feeding design was implemented and all pigs were housed individually with water available for *ad libitum* consumption. Pigs were first adapted to the control diet for 11 days and maintained on the treatment diet for 42 days.

All pigs were housed individually in pens with plastic-covered expanded metal flooring equipped with nipple water drinkers, individual feeders and stainless steel chains for enrichment and allowed visual contact with pigs in adjacent pens. The study was approved by the Institutional Animal Care Committee of Memorial University and conducted in accordance with the Canadian Council of Animal Care guidelines.

Sample collection (Folate)

Pig liver samples were generously donated by Dr. James D. House. Liver samples from pigs subject to folate deprivation were produced in a similar fashion to the B_6-deprived pigs described above, as described by Sokoro (2008). The basal diet (48.6% corn starch, 20% lactose, 15% casein, 5% corn oil, 5% cellulose, 2.2% monocalcium phosphate, 1.5% limestone, 0.5% salt, and 0.2% cystine and a vitamin/mineral premix providing 150% of the swine's micronutrient requirements) was supplemented with either 0.6 mg/kg folic acid (control) or no folic acid (experimental) and given to piglets for four weeks. One percent succinylsulfathiazole was also added to the diets to prevent the absorption of folate produced by gut microorganisms.

Folate deficiency was confirmed with plasma and red blood cell folate level determinations (Sokoro et al., 2008).

Male piglets (~5 kg; Cotswold Canada, Ltd.; cross-bred commercial strains) weaned at 18 days of age were assigned to two treatment groups (n=6 each treatment) and those receiving folate were matched by body weight to pigs not receiving folate supplementation and fed using a pair-feeding protocol. Feed intake was measured daily. Folate status was monitored weekly for 6 weeks. Pigs were group-housed on raised, plastic-coated mesh flooring (4 pigs/pen) in an environmentally controlled room with continuous lighting. Temperature was initially set at 30°C, and gradually reduced to 25°C over the 6-week experimental period. Each pen was equipped with a self-feeder and a nipple waterer, to permit *ad libitum* intake of feed and water. Fresh feed was offered daily and body weight was measured weekly. The study was approved by the Institutional Animal Care Committee of Memorial University and conducted in accordance with the Canadian Council of Animal Care guidelines (Olfert *et al.*, 1993).

Sample collection (runt/large)

For the current study, I selected IUGR pigs displaying higher diastolic blood pressure compared to their normal weight littermates because these pigs were most affected and therefore most likely to have other aberrant metabolic outcomes as well.

Liver samples from runt and large Yucatan miniature pigs were available from a previous study in our laboratory. All animal procedures were approved by Memorial University's Institutional Animal Care Committee in accordance with the Canadian Council on Animal Care Guidelines. Yucatan miniature pigs from six sows from the University's swine herd were studied. Within each litter, the runt (i.e. IUGR) piglet (0.73 ± 0.04 kg), defined as ~65% of the

birth weight of the largest littermate, was sex-matched to a larger (i.e. normal weight (NW)) littermate (1.11 ± 0.05 kg) and removed from the sow at 3 day old. Pigs were fed rehydrated sow milk replacer (Grober Nutrition Inc., Cambridge, ON, Canada) for 4 weeks and then weaned onto standard pelleted grower pig diet (based on wheat, barley and canola; Eastern Farmers Co-op, St. John's, NL, Canada), providing 67% of energy as carbohydrate, 12% as fat and 21% as protein. Feed was provided *ad libitum* for 5 hours/day with 24-hour *ad libitum* water access and were maintained on a 12-hour day-night cycle (lights on 0700 – 1900 h). At 10 months old, pigs were assessed for biomarkers of chronic diseases (Myrie et al., 2011, and McKnight et al., 2012) and livers harvested.

DNA isolation (global methylation)

For each sample, total DNA was extracted from up to 25 mg of flash-frozen liver tissue using the DNeasy Blood and Tissue kit (Qiagen, Toronto, Canada) as per manufacturer's instructions. Each sample was chopped into small pieces and lysed in 180 µl Buffer ATL. Following RNase treatment and a 2 min incubation, 20 µl Proteinase K was added and samples were incubated at 56°C for 1 hour with occasional vortexing. DNA was eluted with 200 µl Buffer AE and a NanoDrop® ND1000 Spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, USA) was used to measure the DNA concentration and the absorbance ratio (A260/A280 and A260/A230). DNA was precipitated in 100% ethanol and 0.3 M sodium acetate at -20°C for one hour. The sample was centrifuged at 12000 g for 15 min at 4^{0}C then washed five times with 70% ethanol to remove residual salt. The pellet was air dried then dissolved in 50 µl water (Invitrogen, Burlington, Canada). To determine DNA integrity, 1 µg was examined by agarose gel electrophoresis and visualized by ethidium bromide staining. DNA was stored at -20°C until further analysis.

Cytosine extension assay

The cytosine extension assay was used to determine the global methylation status of pig liver DNA. After extraction, DNA is cleaved through the use of the methyl-sensitive restriction enzyme *HpaII*, which cuts at the sequence CCGG, leaving a G overhang. This guanine is paired with a radiolabelled cytosine using *Taq* polymerase (and the incorporation quantified with the use of a scintillation counter. The principle behind this assay is that highly methylated DNA will have fewer sites available for cytosine incorporation and therefore incorporate less radioactive label.

Ten micrograms of a genomic DNA sample was digested in 50 U (5-fold excess) *HpaII* (New England Biolabs, Pickering, Canada). Another 10 µg was digested with 5-fold excess MspI (Promega, Madison, USA) and another 10 µg was set up minus restriction enzyme. Each reaction of 150 µl reaction volume was incubated for 5 h at 37^0C. Complete digestion was verified by the addition of 0.5 µg control plasmid DNA to a 15 µl sample of the experimental digest. Digestion was considered to be complete when the internal control gave a digest pattern identical to the pattern obtained with test plasmid and the same enzyme alone. Samples were precipitated by addition of 100% ethanol and 0.3 M sodium acetate at -20^0C overnight, washed with 70% ethanol, dried and redissolved in 25 µl DNase-free water (Invitrogen, Burlington, Canada). Two micrograms of digested DNA was used for a 25 µl single nucleotide extension reaction with 0.2 µl ^{3}H-CTP (57.4 Ci/mmol), 0.2 µl Klenow fragment, and 2.5 µl 10X Klenow buffer (Invitrogen, Burlington, Canada) in 25 µl. Samples were incubated at 37^0C for 1 h and 10 µl from each sample applied to two separate DE-81 ion exchange filter papers (Whatmans, Mississauga, Canada). Samples were washed 3 times with sodium phosphate buffer (pH 7.0), dried overnight at room temperature, placed in 10 ml scintillation vials with 4 ml scintiverse

(Thermo Fisher Scientific, Waltman, MA) and counted for 5 min using a liquid scintillation counter for tritium. Duplicates with counts differing by more than 10% were discarded and the experiment performed again. The amount of methylation in a sample was determined by subtracting disintegrations per minute (DPM) of control digests from the HpaII digest and expressed per 1 μg of digested DNA. DNA methylation was compared using a paired t-test for the folate study and unpaired t-test for the B_6 study. Higher DPM counts correlate to lower DNA methylation. Data were analyzed using Prism software (GraphPad Software) and presented as mean +/- standard error (SE).

Methyl incorporation assay

This assay was used as a second method to determine global methylation status. Extracted DNA was incubated with ^{3}H-S-adenosylmethionine and SSSI, a methylase which adds methyl groups to non-methylated CpGs. The principle behind this method is that the less methylated the DNA the more radiolabelled nucleotide will be incorporated. The reaction mixture contained 2 μg DNA, S-adenosyl-L-[methyl-^{3}H] methionine (New England Biolabs, Pickering, Canada), 3 uM (containing 2 uCi); SSS1 CpG methylase (New England Biolabs, Pickering, Canada) 3 units; buffer (Tris-HCl pH 7.9, 10 mM; NaCl, 50 mM; $MgCl_2$, 10 mM) and DNase / RNase-free water (Invitrogen) up to 30 μl. This mixture was prepared in duplicate and incubated at 37^{0}C for 1 h followed by 65^{0}C for 20 min. The methylated DNA was isolated from the reaction mixture by filtering 15 μl on a Whatman DE81 ion exchange filter and washing five times with 10 ml of 0.5 M phosphate buffer (pH 7.0), followed by 2 ml of 70% ethanol and 2 ml of absolute ethanol. The dried filter was placed in a vial with 4 ml of scintiverse (Thermo Fisher Scientific, Waltman, MA) and assayed as described for the cytosine extension assay. Data are expressed as DPM per μg DNA and compared using a paired t-test for the folate study and

unpaired t-test for the B_6 study. Higher DPM counts correlate to lower DNA methylation. Data were analyzed using Prism software and presented as mean ± SE.

RNA isolation, quality assessment and cDNA synthesis

RNA was isolated in order to determine expression levels, via RT-qPCR, of various genes that I predict will have altered expression due to reduced methyl supply. RNA was extracted using RNeasy Mini kits (Qiagen Inc., Toronto, Canada) in which 10-30 mg of flash-frozen liver tissue was homogenized in 600 µl of Buffer RLT using a mortar and pestle; the remainder of the protocol was performed according to manufacturer's instructions and the extracted RNA was eluted in 30 µl of RNase-free water. A NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington,USA) was used to measure the RNA concentration and the absorbance ratio (A260/A280). Up to 100 mg of each sample was used for RNA cleanup, to desalt and concentrate RNA samples and remove residual phenol, according to manufacturer's protocol. The purity and quality of each RNA sample was determined by spectrophotometry and with the use of an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, U.S.A.). Samples with RNA Integrity numbers (RIN) less than 8 were discarded. Genomic DNA Wipeout and reverse transcription of 800 ng of each sample was performed using the QuantiTect Reverse Transcription Kit according to the protocol in the Handbook (Qiagen, Toronto, Canada). Samples in which the RT was replaced by water (RT-ve) were used as negative controls.

RT-qPCR

The expression of a variety of genes involved in, or potentially impacted by, one-carbon metabolism were examined using RT-qPCR using SYBR Green I Master kits (Roche,

Mississauga, Canada). Primers for RT-qPCR amplification of most of the analyzed genes were designed using the Primer3 program (Rozen and Skaletsky, 2000) based on pig sequences found in the Genbank online database (http://www.ncbi.nlm.nih.gov/). Primers for NR3C1 were obtained from a previous study (Mostyn et al., 2006). Primers for GAMT were designed based on conserved sequence following homology comparison of GAMT in other species (personal communication from Dr. Tamara Smith, 2010). A cDNA pool was created for the vitamin B_6 and folate study, and separately for the runt/large study, using equal aliquots of all samples for the respective studies. For each primer pair, a standard curve was generated using a range of cDNA concentrations from 80 ng to 0.08 ng to assess efficiency. A dissociation curve was also generated to ensure single product amplification. Twenty nanograms of cDNA was used for all sample reactions. Cycling temperature was optimized for each gene (Table 1). Each gene was evaluated in either triplicate or duplicate in an individual reaction. Beta-actin was used as a housekeeping gene for all genes analyzed following confirmation that levels remained consistent between groups. The qPCR reactions were carried out in duplicate in 0.1 ml real-time PCR tube strips (Eppendorf, Mississauga, Canada) and run on an Eppendorf Realplex Mastercycler (Eppendorf, Mississauga, Canada). Duplicates differing by more than 0.5 cycles were discarded and the samples repeated. For all reactions, the thermal profile was as follows: preincubation for 10 min at 95^0C (1 cycle), followed by 40 cycles of 15 s at 95^0C, gene-specific annealing and elongation temperatures for 15 sec and 20 sec respectively (see Table 1 and Table 2), and melting curve analysis as per manufacturer's protocol. Samples were then examined on 0.8% agarose gel for band size confirmation. Threshold cycle (Ct) values from technical replicates were averaged for each sample and incorporated into calculations for relative quantity (RQ) using the $2^{-\Delta\Delta CT}$ quantification method (Livak and Schmittgen, 2001) taking efficiency values

into account. For each gene of interest, the lowest normalized expression was set as the calibrator (RQ value = 1). Data were analyzed using Prism software and presented as mean ± SE using either paired or unpaired t-test as indicated. A significance level of $p<0.10$ was used. No-template controls and no-RT controls we used for all samples.

Swine hepatitis E virus (HEV) experiment

To determine whether an aberrant RNA species (ARS) identified in certain littermates in our folate and runt/large studies was hepatitis E virus, a standard PCR-identification protocol was employed (van der Poel, et al., 2001). In brief, cDNA from two ARS-positive samples were amplified using two sets of degenerate HEV primers in a nested RT-PCR assay. Following genomic DNA wipeout, cDNA was created using Quantitect Reverse Transcription kit (Qiagen, Toronto, Canada) with 800ng liver tissue using either reverse primer HEV R1 (5'-CCATCRARRCAGTAAGTGCGGTC-3') (cDNA1) or HEVR2 (5'-GGCAGWRTACCARCGCTGAACATC-3') (cDNA2) according to the manufacturer's protocol. For cDNA1, two forward primers (HEVF1- 5'-CTGGCATYACTACTGCYATTGAGC-3' and HEVF2- 5'-CTGCCYTKGCGAATGCTGTGG-3') were used in combination with both of the reverse primers and were used with HEVR2 for cDNA2. The conditions for the reactions were 35 cycles of (94^{0}C for 1 min, 50^{0}C for 45 seconds, 72^{0}C for 1 min) followed by 72^{0}C for 10 min in a Mastercycler epgradient S (Eppendorf, Mississauga, Canada). The reaction mix contained 1X Advantage 2 polymerase mix (Clontech, MountainVview, USA), 1X Advantage 2 polymerase buffer (Clontech, MountainVview, USA), 0.5 ng/µl of both forward and reverse primer, 0.5 mM dNTP mix and 80 ng cDNA. I also performed a nested reaction on cDNA1 amplified with HEVF1 and HEVR1

Table 1: Quantitative polymerase chain reaction primers and conditions

Gene	Accession number (GenBank)	Forward primer (5'-3')	Reverse primer (5'-3')	Efficiency	R^2	Expected Amplicon length (bp)
GAMT	NA	GCCTGTGGGAGGAGGTGGC	CAGGTCTCCTCAGACAGCG	0.84	0.997	85
Actin	DQ845171.1	CCCAGCACGATGAAGA	CGATCCACACGGAGTA	0.90	0.994	60
BHMT	U53421.1	GTTCGCCAGCTTCATC	CTCCAGCTTGTCCTC	0.88	0.993	87
MTR	XM_001927058.1	CTGGAAGCCTTTCTTTGACG	TTTGGCCTCTTCACCTATGG	1.19	0.992	100
PEMT	NM_214365.1	TCTGCGTTATGACCAGGTTG	GGGTCTTGTGTTCCCATCTG	0.99	0.986	126
GNMT	NM_001110419.1	GCCAGTGACAAGATGCTCAA	GGTGACTTGGGCACATCTTT	1.15	0.993	128
CTH	NM_001044585.1	CTGAGAGTTTGGGAGGATA	CTTAGGCACAGATGAATGG	0.93	0.999	71
DNMT3a2	DQ785811.1	TGCCAAAACTGCAAGAACTG	CAGCAGATGGTGCAGTAGGA	0.91	0.992	83
DNMT1	DQ060156.1	TCGAAGGATGATGGGAAGAC	AACTTGTTGTCCTCCGTTGG	0.91	0.998	104
MTHFD	AY550929.1	CTTCGTCCTGCCTATTCGAG	CATTCACCTGCTCAGCTTCA	0.93	1.00	149
FASN	NM_001099930.1	CTCCATCACCGTGTTCCAC	CAGGCTCTGGATGCTGTCC	0.91	0.999	100
NR3C1	AY779185.1	GCTGCTGGTCCTGCTGCTC	CCTTCACATTCGGCTGCTCTGG	0.87	0.996	82

Table 2: Elongation and annealing temperatures for quantitative polymerase chain reaction of various genes

Gene	**Annealing (^{0}C)**	**Elongation (^{0}C)**
GAMT	56	72
β-Actin	53	68
BHMT	56	72
MTR	55	70
PEMT	66	72
GNMT	69	76
CTH	56	72
DNMT3a2	62	72
DNMT1	67	76
MTHFD	57	72
FASN	65	72
NR3C1	60	72

using HEVF2 and HEVR2. For the nested reaction, conditions were the same except that 30 cycles were used. Nested reactions were performed on 0.5 µl, 1 µl, and 2 µl of PCR reactions.

RESULTS

Cytidine extension and methyl incorporation assays were employed to measure changes in the global methylation level of pigs deprived of either vitamin B_6 or folate (Figure 2). Neither assay indicated any detectable difference in global methylation status between folate-deprived pigs (5877 ± 400 DPM/µg DNA for cytosine extension assay and 42,201 ± 2435 DPM/µg DNA for methyl incorporation assay) and control littermates (5533 ± 621 for cytosine extension assay and 54,134 ± 6002 DPM/µg DNA for methyl incorporation assay) or between B_6-deprived pigs (5614 ± 395 DPM/µg DNA for cytosine extension assay and 38,613 ± 6552 DPM/µg DNA for methyl incorporation assay) and control littermates (5747 ± 221 DPM/µg DNA for cytosine extension and 33,272 ± 5678 DPM/µg DNA for methyl incorporation assay).

I then examined expression of a select group of genes of interest (GOI) to see if there was an effect of reduced methyl supply on transcript expression. I chose genes involved in one-carbon metabolism or genes whose methylation status and/or expression is known to change in response to altered methyl supply. Genes selected included MTR, BHMT, DNMT1, GNMT, PEMT, FASN, NR3C1, MTHFD, DNMT3a2, GAMT, and CTH.

In the folate domestic pig study (Figure 3a), transcript expression of MTR, BHMT, DNMT1, GNMT, PEMT, FASN, NR3C1, MTHFD, DNMT3a2 and GAMT did not differ between folate-deprived pigs and control littermates. Though not significant, expression of MTHFD showed a trend for increased expression in folate-deprived pigs (p=0.1291). In the vitamin B_6

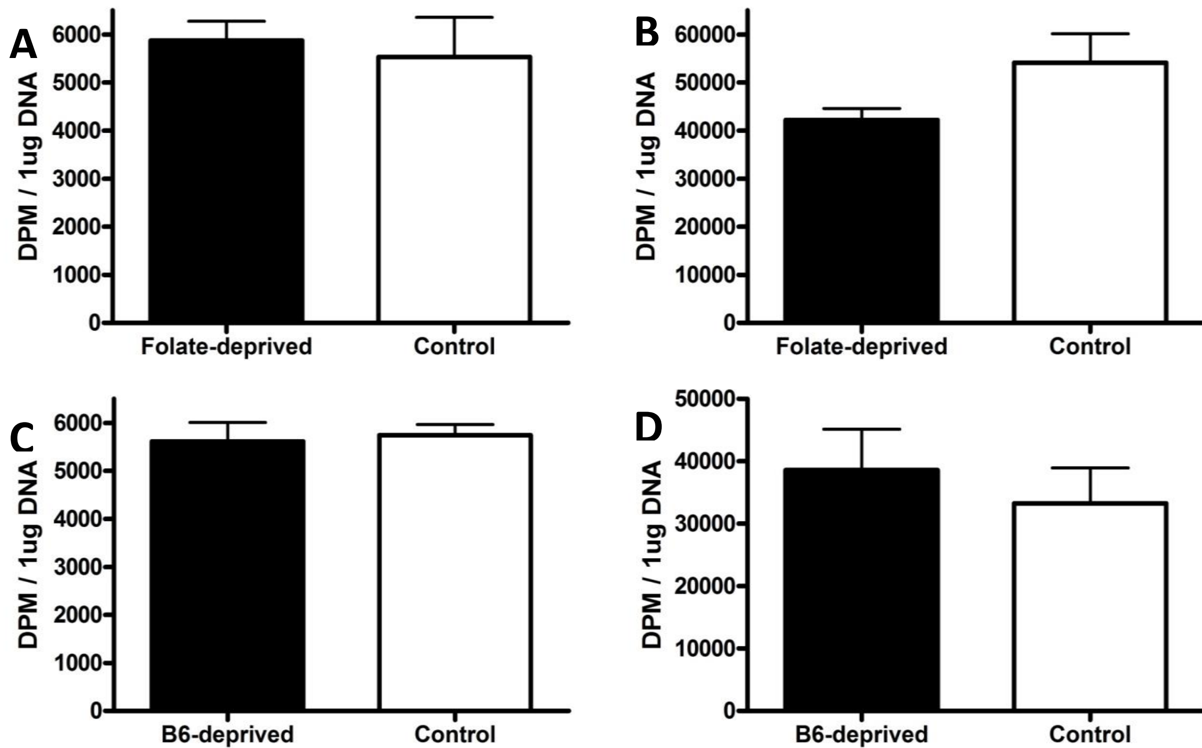

Figure 2: Global methylation analysis. Global methylation status was assessed for folate- and B_6-deprived domestic pigs. **A.** Cytosine-extension assay on folate-deprived pigs (black) (n=6) compared to control littermates (white) (n=6). **B.** Methyl-incorporation assay on folate-deprived pigs (black) (n=6) compared to control littermates (white) (n=6). **C.** Cytosine-extension assay on B_6-deprived pigs (black) (n=6) compared to control littermates (white) (n=5). **D.** Methyl-incorporation assay on B_6-deprived pigs (black) (n=5) compared to control littermates (white) (n=5).

domestic pig study (Figure 3b), transcript expression of MTR, CTH, DNMT1, GNMT, PEMT, FASN and NR3C1 expression did not differ between B_6-deprived pigs and control littermates. Expression of DNMT3a2 (p=0.0820) and BHMT (p=0.0844) were lower in B_6-deprived group than in control. In the Yucatan IUGR study (Figure 3c), expressions of GAMT, GNMT, MTHFD, FASN, NR3C1, MTR and DNMT1 did not differ between runt pigs and control littermates.

After performing RT-qPCR on the aforementioned samples, our laboratory acquired a Bioanalyzer (Agilent), and upon running these samples, I discovered an aberrant RNA species (ARS) in some of the samples that was not visible using RNA gel electrophoresis (Figure 4). The ARS is a doublet which appears between the 18S and 28S RNA bands on the Bioanalyzer electropherogram and gel image. This very distinct doublet was apparent in multiple RNA preparations from the same liver samples and, as the electrophoresis file run summary indicated, there was very little background to indicate RNA degradation. Also notable is that this band occurred exclusively in sets of littermates (n=2) in the folate study. In the IUGR study, three samples from the runt group were ARS+ while two samples from the normal weight littermate group were ARS+.

Moreover, certain samples containing this ARS were outliers with expression values more than 2 standard deviations (SD) from the mean of multiple gene expression values (data not shown). For this reason, I decided to omit samples containing this ARS from my statistical analyses. Notably, samples containing this ARS were present only in domestic pigs from the folate study and Yucatan pigs from the IUGR study. Domestic pigs from the B_6 study were unaffected.

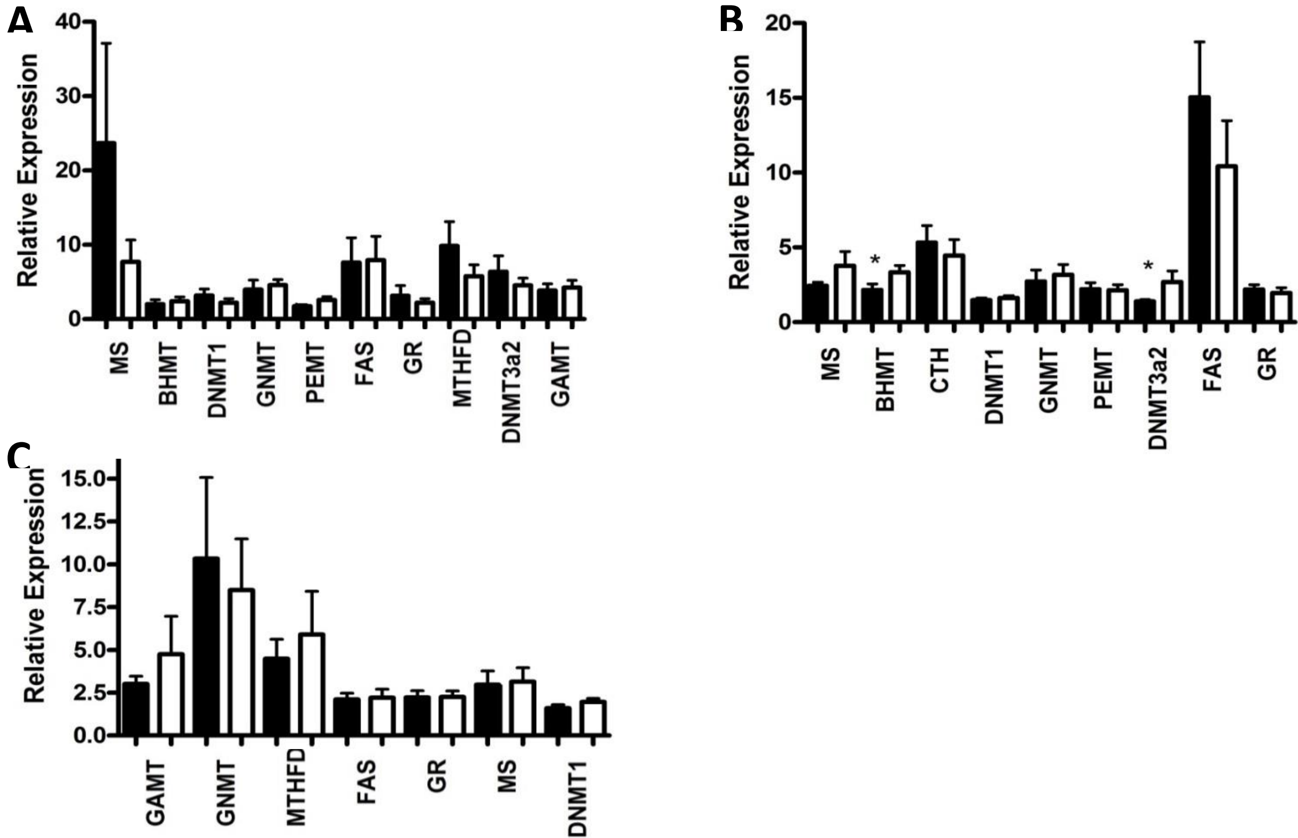

Figure 3: Relative expression of various genes of interest in pig liver RNA samples. The expression of several genes (listed in figures) associated with, or potentially affected by, methylation were normalized to β-actin. Statistical significance was determined using paired student t-test (* $p<0.1$). **A.** Folate-deprived liver samples (black) (n=6) compared to control littermates (white) (n=6) in domestic pigs. There was no significant difference identified between groups for any of the genes examined. **B.** B_6-deprived liver samples (black) (n=6) compared to control littermates (white) (n=5). The expression of DNMT3a2 and BHMT were significantly lower in B_6-deprived pigs. **C.** Gene expression in runt (black) (n=6) versus normal weight (white) Yucatan mini pigs (n=6). There was no significant difference identified between groups for any of the genes examined.

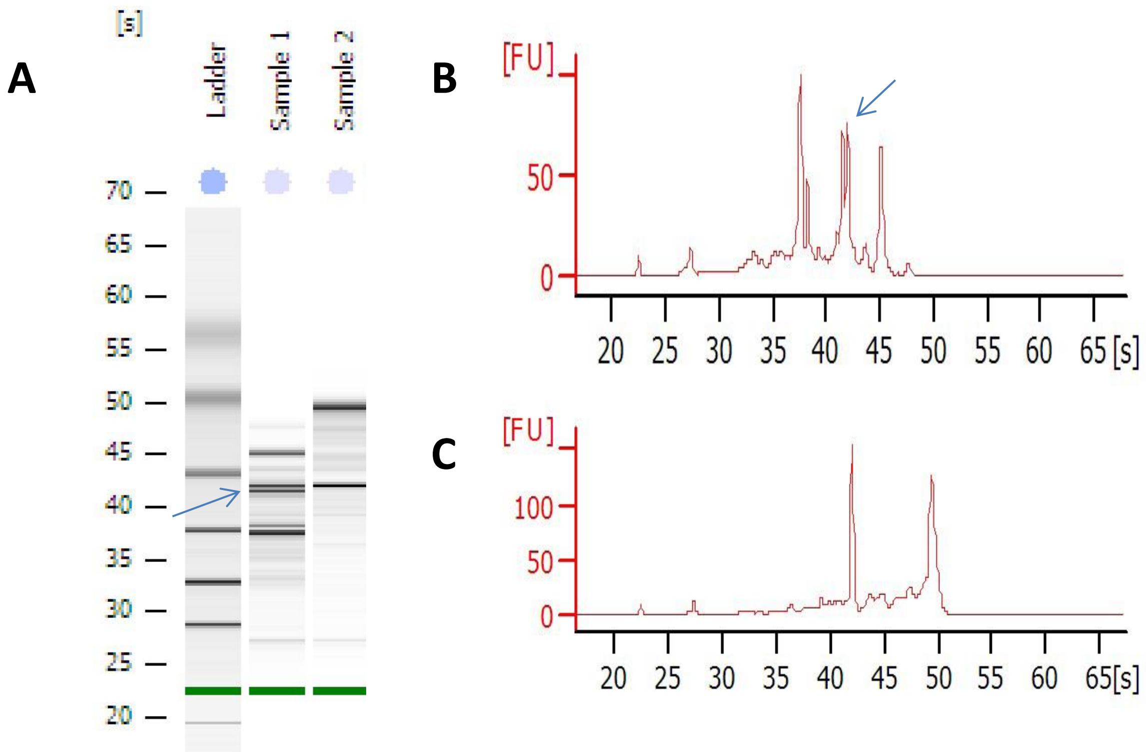

Figure 4: Bioanalytical identification of an aberrant RNA species. A. Figure representation of the gel electrophoretic analysis of pig RNA samples (Agilent Bioanalyzer). The expected 28S and 18S RNA bands are evident in sample 2 whereas an extra doublet is evident in sample 1 (arrow). **B.** Scan of sample 1. **C.** Scan of sample 2. A comparison of the peaks presented in electropherogram suggests that the Bioanalyzer algorithm is mis-identifying the 18S/28S RNA bands and that the aberrant RNA species is intermediate in size between those two RNA species.

After omitting ARS-containing samples from samples from the folate study (Figure 5a), expression levels of MTR, BHMT, DNMT1, GNMT, PEMT, FASN, NR3C1, MTHFD, DNMT3a2 and Nevertheless, when comparing samples containing ARS to samples not containing ARS in both the folate and IUGR studies, I discovered some interesting results (Figures 6 and 7). For the folate study, the ARS was apparent in liver samples from two sets of littermates. When comparing ARS+ to ARS- samples for folate-deprived samples in the folate study (Figure 6), no difference was found between groups for GNMT, PEMT, or GAMT. However, considering the very small sample size for the folate-deprived samples with ARS (n=2), a trend for decreased expression of GNMT (p=0.15) as well as increased expressions of GAMT (p=0.11) were noted. Folate-deprived samples with ARS also showed a markedly increased expression of MTR (p<0.0001), BHMT (p=0.018), DNMT1 (p=0.09), FASN (p=0.002), NR3C1 (p=0.05) and DNMT3a2 (p=0.004). When comparing ARS+ to ARS- samples in the control group from the folate study (Figure 6), no difference was found between groups for PEMT with and without ARS. A very small sample size was available for the control samples with ARS band. However, increased expression of MTR (p=0.008), BHMT (p=0.003), DNMT1(p=0.003), FASN (p=0.056), NR3C1 (p=0.003), DNMT3a2 (p=0.001), and GAMT (p=0.093) and decreased expression of GNMT (p=0.0996) and MTHFD (p=0.0003) were observed in ARS+ samples.

For the IUGR study, the ARS was apparent in liver samples from three sets of littermates. When comparing ARS+ to ARS- samples for runt samples in the IUGR study (Figure 7), no difference was found between groups for GAMT, GNMT, MTHFD, FASN or DNMT1;

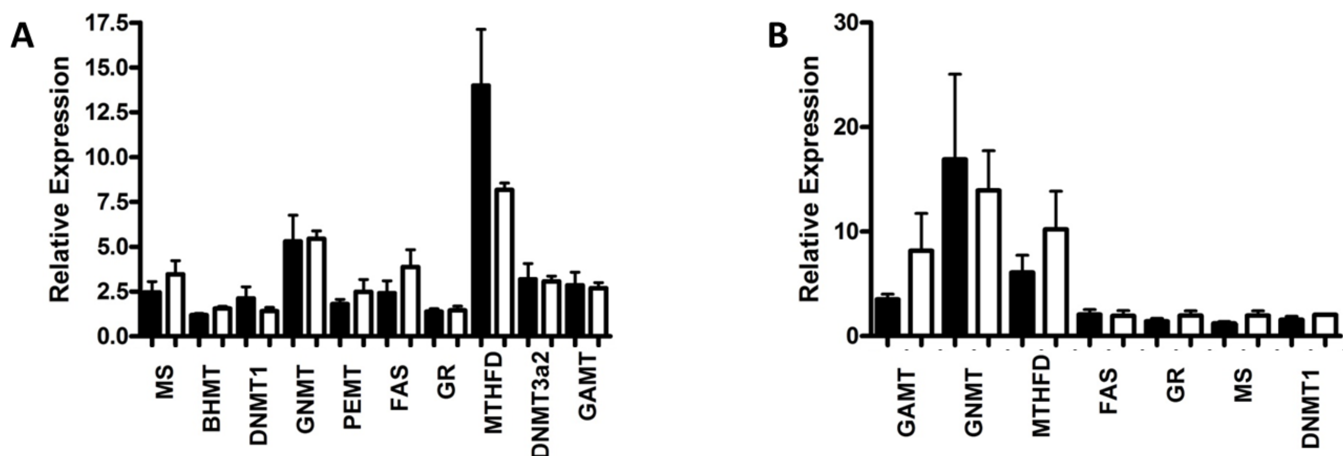

Figure 5: Relative expression of various genes of interest in control and methyl-deprived or runt pig liver RNA samples with ARS-positive samples omitted. The expression of several genes (listed in figures) associated with, or potentially affected by, methylation were normalized to β-actin. Statistical significance was determined using paired student t-test (* $p<0.1$). **A.** Folate-deprived liver samples (black) (n=4) compared to control littermates (white) (n=4) in domestic pigs. There was no significant difference identified between groups for any of the genes examined. **B.** Yucatan runt liver samples (black) (n=3) compared to normal weight littermates (white) (n=3). There was no significant difference identified between groups for any of the genes examined. GAMT still did not differ between control and folate-deprived pigs. Though not significant, expression of BHMT showed a trend for decreased expression in folate-deprived pigs (p=0.1001). For the IUGR study (Figure 5b), excluding samples containing ARS still resulted in no difference in expression for GAMT, GNMT, MTHFD, FASN, NR3C1, MTR, and DNMT1.

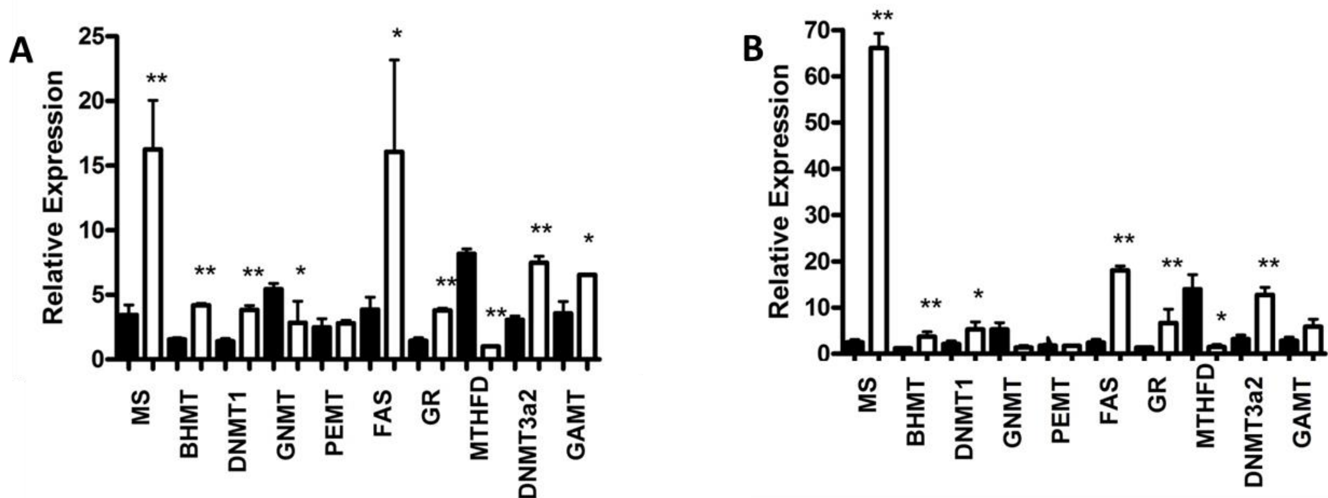

Figure 6: The effect of an ARS on the relative expression of various genes of interest in samples from folate study. The expression of several genes (listed in figures) associated with, or potentially affected by, methylation were normalized to β-actin. Statistical significance was determined using paired student t-test (* p=0.1, ** p=0.05). **A.** The expression of the genes in ARS+ (black) (n=2) and ARS- (white) (n=4) samples in the control group from the folate study. The expression was increased in the ARS+ samples for all genes with the exception of GNMT and MTHFD (expression decreased) and PEMT (expression unaffected).**B.** The expression of the genes in ARS+ (black) (n=2) and ARS- (white) (n=4) samples in the folate-deprived group from the folate study. The expression was increased in the ARS+ samples for all genes with the exception of MTHFD (expression decreased) and GNMT, PEMT, GAMT (expression unaffected).

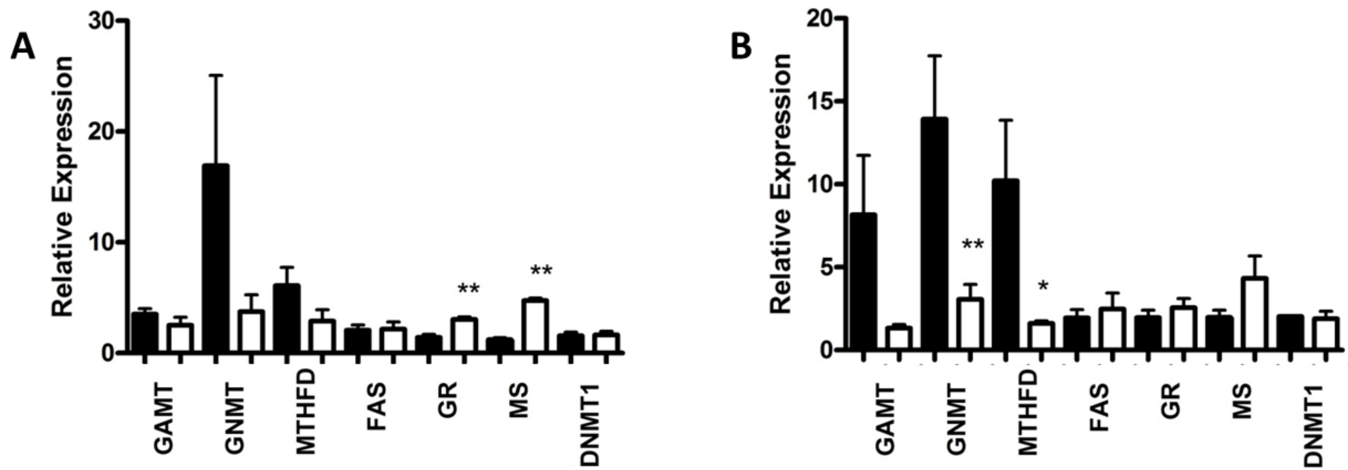

Figure 7: The effect of an ARS on the relative expression of various genes of interest (GOI) in runt and normal weight samples. The expression of several genes (listed in figures) associated with, or potentially affected by, methylation were normalized to β-actin. Statistical significance was determined using paired student t-test (* p=0.1, ** p=0.05). **A.** The expression of the genes in ARS+ (black) (n=3) and ARS- (white) (n=3) samples in the runt group from the Yucatan pig study. The expression of NR3C1 and MTR was increased in the ARS+ samples whereas the expression of all of the other genes was unaffected. **B.** The expression of the genes in ARS+ (black) (n=3) and ARS- (white) (n=3) samples in the normal weight group from the Yucatan pig study. The expression of GNMT and MTHFD was decreased in the ARS+ samples whereas the expression of all of the other genes was unaffected.

however, decreased expressions of GNMT (p=0.0499) and MTHFD (p=0.0777) were observed in ARS- samples. Expression of GAMT also showed a trend for decreased expression in ARS- samples (p=0.1297).

Next, I determined if samples containing the ARS band responded differently if the pigs were deprived of folate or if the pigs were runts. In the folate study (Figure 8a), ARS+ folate-deprived samples show a reduction in PEMT (p=0.0451) and an increase in DNMT3a2 (trend at p=0.0937) compared to ARS+ control pigs. No difference was found between groups for BHMT, DNMT1, GNMT, FASN, NR3C1, MTHFD, or GAMT. For the IUGR study (Figure 8b), runt liver samples were compared to large littermate samples to determine if the presence of ARS was indicative of a situation where the effect would be more or less pronounced in runt pigs No difference was found between groups for GAMT, GNMT, MTHFD, FASN, NR3C1, MTR or DNMT1.

Additionally, I analyzed my data to determine if sex differences existed within the IUGR Yucatan study (Figure 9). In runt samples from this study, runt ARS- females showed increased expression of GAMT (p=0.0077) while normal weight littermates showed no difference in gene expression between genders for any genes.

Based on the frequency of the infection of domestic pigs by HEV we reasoned that the ARS may represent sequences arising from this virus. However, attempts to determine if this was the case were unsuccessful (data not shown).

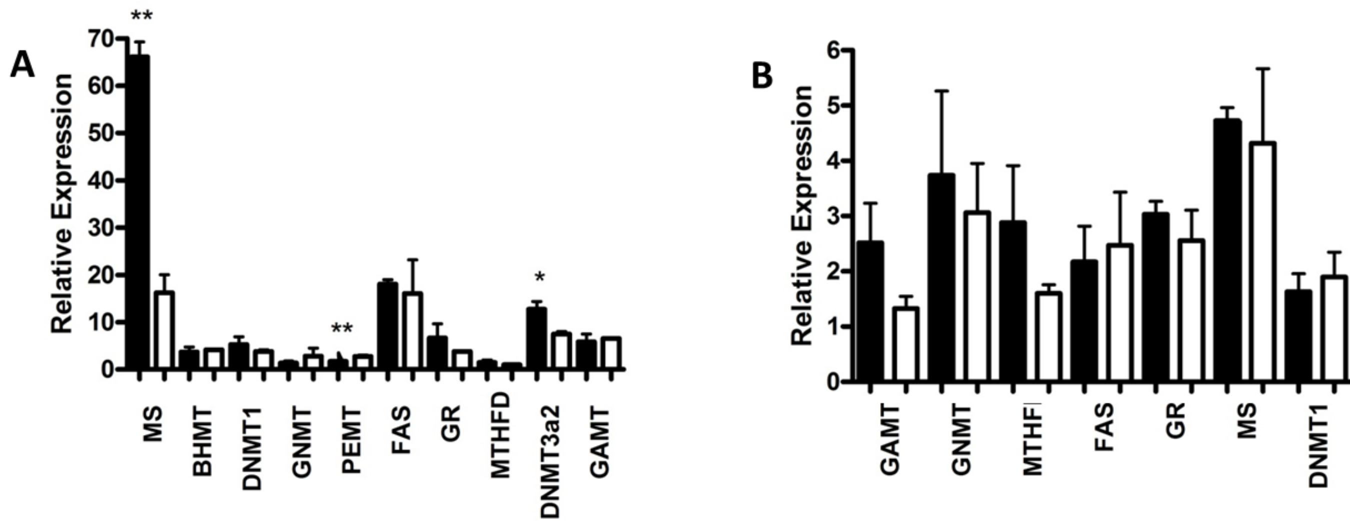

Figure 8: Relative expression of various genes of interest in ARS-positive samples from folate-deprived and IUGR study. The expression of several genes (listed in figures) associated with, or potentially affected by, methylation were normalized to β-actin. Statistical significance was determined using paired student t-test (* p=0.1). **A.** ARS+ folate-deprived liver samples (black) (n=2) compared to ARS+ control littermates (white) (n=2) in domestic pigs. The expression of MTR and DNMT3a2 were increased in the ARS+ folate samples while the expression of PEMT was decreased. The expression of all the other genes was unaffected. **B.** Yucatan ARS+ runt liver samples (black) (n=3) compared to ARS+ normal weight littermates (white) (n=3). There was no significant difference identified between groups for any of the genes examined.

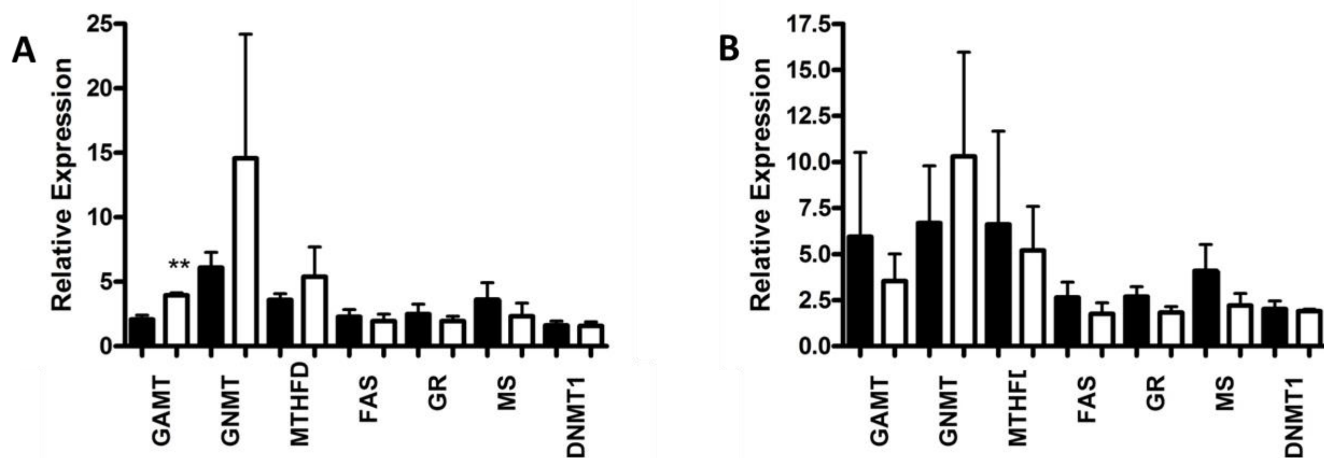

Figure 9: Relative expression of various genes of interest in male and female samples from IUGR study. The expression of several genes (listed in figures) associated with, or potentially affected by, methylation were normalized to β-actin. Statistical significance was determined using paired student t-test (* p=0.1). **A.** Runt male liver samples (black) (n=3) compared to runt female (white) (n=3). The expression of GAMT was increased in runt female samples while the expression of all other genes was unaffected. **B.** Normal-weight male liver samples (black) (n=3) compared to normal weight female (white) (n=3). There was no significant difference identified between groups for any of the genes examined.

DISCUSSION AND CONCLUSIONS

Appropriate DNA methylation is essential for embryogenesis and early fetal development (Shames et al., 2007) as inadequate establishment of DNA methylation patterns by a methyl-insufficient maternal diet, such as low folate, may induce pediatric developmental diseases and even affect health in later life (Dunlevy et al., 2006 and Waterland and Jirtle, 2004). For example, sheep fed a methyl-deficient diet in early development have altered promoter DNA methylation resulting in obesity, altered immune responses, insulin resistance, and elevated blood pressure (Sinclair et al., 2007). Moreover, targeted deletion of de novo and maintenance methyltransferases was shown to result in various developmental defects in different species, up to and including embryonic lethality (Li and Bird, 2007). However, interactions between diet, one-carbon metabolism, DNA methylation and phenotypic effect are yet to be fully elucidated.

The purpose of this study was to use two separate pig models and approaches to investigate the effect of reduced dietary methyl supply on DNA methylation and the expression of a number of genes involved in methylation pathways or expected to be affected by alterations in DNA methylation. As discussed previously, altered DNA methylation can profoundly affect gene expression. In the first approach used in this study, I compared global DNA methylation and the expression levels of various genes in folate- and vitamin B_6-deprived pigs. Both of these compounds are involved in the methylation cycle and, consequently, deprivation of them would be expected to cause alterations in methylation pathways. My second approach involved a different set of tissues obtained from large and runt pigs, the latter of which would also be presumed to be methyl-donor deprived. The reasons for using this approach were twofold: first, these pigs were grown to adulthood with several measures of risk for adult diseases, so I was able to choose runt pigs with strong phenotypes. Thus, it is more likely to see changes which

could otherwise be masked by biological diversity. Second, by comparing these pigs with folate- or B_6-deprived pigs we will gain more insight into the similarities and differences in these two dietary insults in early development. Therefore, using these two different animal models, a specific methyl-deficient model and a model in which overall nutrition is limited, I may better elucidate the connection between diet, one-carbon metabolism, and DNA methylation.

Global methylation

I performed two separate techniques to determine global DNA methylation levels (i.e. cytosine extension assay and methyl incorporation assay). Figure 2a and b) Although a trend towards lower methyl incorporation was observed for folate-deprived pigs in the methyl incorporation assay, no observable difference was detected by the cytosine extension assay. The cytosine extension assay is considered a superior assay because, unlike the incorporation assay, it is not dependent on DNA integrity, abasic sites, or UV photoproducts, which can cause artificial results in the methyl incorporation assay (Progribny et al., 1999). However, the DNA used in methyl incorporation assays did not appear to be degraded following DNA electrophoresis (results not shown). Nevertheless, because the cytosine extension assay did not indicate differences in global methylation, I cannot conclude that a significant difference exists between the two groups of pigs, at least at the level of detection of that assay and variability within my samples. For vitamin B_6-deprived pigs, no difference was shown to exist using either the methyl incorporation assay or cytosine extension assay (Figure 2c and d). This was unexpected as Progribny et al. (2006) observed DNA hypomethylation in hepatic rat tissue following as little as nine weeks of a methyl-depleted diet. Halsted et al. (2002) also found that ~50% reduction in hepatic folate levels resulted in DNA hypomethylation.

However, my study limited only folate or B_6, and other methyl donors were still present and may have compensated to maintain remethylation. Indeed, limiting any one source of methyl supply results in multiple compensatory metabolic changes (reviewed in Niculescu et al., 2002). Further, the difference in animal model should also be noted. Given that no differences in global methylation status were observed in the domestic pig models, and previous results in our laboratory did not find changes in global methylation between runts and large littermates at 3-5 days old in Yucatan pigs (Mackay et al., 2012), global DNA methylation assays were not performed for the current IUGR study.

The bulk of methylation in the mammalian genome is in the highly repetitive, non-coding portion of the genome and so, despite not finding changes in global methylation, it is still possible that changes in gene-specific methylation exist, which would potentially accompany changes in expression of genes related to methylation pathways and/or genes whose methylation status has changed. To investigate this possibility, I continued to investigate the expression of many of these candidate genes.

Gene expression

As outlined above, I looked at the expression of number of potentially relevant genes at the level of the RNA by RT-qPCR. My qPCR analysis of the B_6-deprived pigs found lower expression of BHMT and DNMT3a2 (Figure 3b). Reduced expression of BHMT was an unexpected finding as BHMT would be expected to be upregulated in response to homocysteine accumulation (due to lower CBS and CTH activities), which was 8-fold higher in the B_6-deprived pigs from that study (Zhang 2009). Indeed, reduced BHMT function results in hyperhomocysteinemia (Teng et al., 2012). Moreover, BHMT should have been upregulated

also due to lower methylneogenesis via folate, due to the lower SHMT activity that was also observed in the original study (Zhang 2009). Reduced expression of DNMT3a2 might well be expected because, as homocysteine levels increase, the SAM/SAH ratio lowers, effectively reducing the available SAM for methylation reactions; although this reduced expression was obviously not low enough to affect global methylation.

No differences were observed in folate-deprived pigs in comparison to control littermates (Figure 3a) for any of the genes analyzed. However, I found significant outliers in both control and experimental animals in both treatment groups. In folate-deprived pigs, two samples from each group were outliers in the expression of multiple genes with expression values two times the standard deviation from the mean. In runt pigs and control littermates, no difference was observed between groups (Figure 3c), but there was a large discrepancy within groups as well. A more detailed analysis of the original RNA with the use of a Bioanalyzer identified an aberrant RNA band in many of the samples (Figure 4). There were two samples in each group for the folate study (both littermates) and three samples in each group for the runt/large study (also littermates) with this ARS. Interestingly, the samples containing the aberrant band were also the samples with outlying expression in the folate study and may well have been responsible for discrepancies in gene expression for the runt/large study. Because of this, I discarded these samples from my initial analysis as the cause of this ARS was also affecting the expression of certain genes I was investigating.

After omitting the aforementioned samples from my analysis, I was still unable to detect any differences in gene expression for the remaining folate-deprived pigs compared to control littermates or for runt pigs compared to control littermates (Figure 5a). However, this may be due in part to reduced power in the statistical analyses. Trends could be seen for reduced

expression of MTR and FASN in folate-deprived pigs, as well as trends for increased expression of MTHFD and DNMT1. Compared to this, trends could be seen in runt pigs for reduced MTHFD, while GAMT and MTR also showed a trend for decreased expression (Figure 5b).

Interestingly, both folate-deprived and runt pigs showed a trend for reduced MTR expression in liver. Both pig models are presumed to be methyl donor deficient, so the underlying cause may be reduced methyl supply via lower MS in the folate cycle. Moreover, this trend was not observed in B_6-deprived pigs (Figure 3b), indicating that changes are independent of the SAM/SAH ratio. Decreased FASN expression in folate-deprived pigs is consistent with other research showing that fatty acid synthase expression in rats decreases following a high fat diet (Lomba et al., 2010). Moreover, their research also discovered that changes in expression accompanied changes in promoter methylation of the fatty acid synthase gene, although no correlation between expression and methylation status was established. As such, it is possible that reduced FASN expression in folate-deprived pigs results from altered gene methylation but this hypothesis requires further study.

Sex differences were also observed in the present runt/large study (Figure 9), with GAMT showing reduced expression in runt males in comparison to runt females. This indicates that males may be more susceptible to reduced nutrient intake with respect to one-carbon metabolism. Interestingly, in the brains of IUGR rats, histone acetylation is also sex-dependent with more pronounced acetylation at K9 and K14 of H3 (Ke et al., 2006). Moreover, the effects of methyl-deficient diets in sheep shown to alter promoter DNA methylation of a number of genes were also more pronounced in males (Sinclair et al., 2007).

Aberrant RNA Species

Upon discovering the ARS in liver samples of the present study, I tested other pig tissues in our laboratory to see how widespread it was (results not shown). ARS was present in other hepatic tissue from previous studies but not in intestinal tissue. This led us to hypothesize that the ARS may result from a liver-specific pathology. Perhaps the most common hepatic illness in pigs is swine hepatitis E virus (HEV), with one study finding 35% of pigs, and 54% of commercial herds, to be infected (Huang et al., 2002); prevalence rates in 2001 were 38% in the Prairie provinces and up to 80-90% in Ontario and Quebec (Dongwan et al. 2001). As such, I attempted to determine if the ARS resulted from HEV infection. This preliminary analysis did not show this to be the case. Nevertheless, disturbances in expression of genes involved in methylation pathways or known to be affected by changes in methylation were observed.

In the runt/large study, normal weight littermate samples containing ARS showed increased expression of GNMT and MTHFD compared to ARS- littermates, while ARS+ runt samples showed increased expression of NR3C1 and MTR compared to ARS- runts (Figure 7). However, GNMT and MTHFD also showed a trend towards increased expression in ARS+ runt samples. Overall, these data suggest that the effect of ARS on gene expression is largely independent of IUGR, but that the presence of ARS could lead to false results in some studies.

In my folate study, both control and folate-deprived samples with ARS displayed marked differences in expression of examined enzymes compared to samples without ARS (Figure 6). In control animals from the folate study, increased expression for MTR, BHMT, DNMT1, FASN, NR3C1, DNMT3a2, and GAMT were observed in ARS samples while MTHFD and GNMT showed reduced expression. For folate-deprived samples for the folate study, MTR,

BHMT, DNMT1, FASN, NR3C1 and DNMT3a2 displayed increased expression in ARS samples while MTHFD displayed reduced expression. Notably, the expression profile for both groups is similar, indicating that results are largely due to ARS, independent of folate supply.

BHMT lacks a promoter element (TATA or CAAT box), however a CpG island surrounds the promoter and transcriptional start site, indicating that its expression may be regulated by methylation (Ganu et al., 2011). FASN expression is also known to be regulated by DNA methylation (Lomba et al., 2010) and NR3C1 expression and promoter methylation have been shown to respond to a variety of dietary insults with an inverse relationship between methylation and expression (Lillycrop et al., 2005, and Lillycrop et al., 2007). In the folate study, BHMT expression was increased in folate-deprived ARS+ pigs and corresponding ARS+ control pigs (Figure 6), FASN expression was increased in ARS+ folate-deprived pigs, and NR3C1 expression was increased in ARS+ samples in the folate-deprived, folate control, and runt groups (Figures 6 and 7). Lillycrop et al. (2007) found increased NR3C1 expression in offspring of rats fed reduced protein during lactation, which they attributed to inability to maintain DNA methylation. However, in their study, DNMT1 expression had decreased, while in the present study DNMT1 and DNMT3a2 expression increased in folate-deprived ARS+ and folate control ARS+ groups (Figure 6). Thus, I suggest that the increase in NR3C1, FASN and BHMT in the present study is not simply due to decreased DNMT activity as suggested by Lillycrop et al. (2007). Rather, it is possible that the ARS is preventing the action of DNMTs or that DNMTs are interacting with the ARS, thereby limiting their ability to interact with the pig genome.

Notably, the effect of ARS on gene expression appears to be affected by methyl status (Figure 8a). MTR and DNMT3a2 expression are significantly higher in ARS+ folate-deprived

samples than in control ARS+ samples, while PEMT expression is lower. These differences were not observed when ARS- folate samples were compared to controls (Figure 5a). This suggests that the effect of ARS is exacerbated by methyl status in pigs. No synergistic effect was observed between runt animals and ARS (figure 8b), but the number of samples was limited (n=3) and group variability was high.

Future studies

The most pressing issue stemming from these results is to discover what this ARS represents and how it is impacting the expression of genes involved in one-carbon metabolism and methylation reactions. As this ARS was found in different strains of pigs from herds in two separate provinces, it is likely that infected animals are being used in many studies Canada-wide, and may be unknowingly affecting study results. Further, if pigs containing this ARS are being utilized for other animal husbandry practices, it is important to determine the potential impact of this ARS on the health of the pigs and safety for humans. Isolating the band corresponding to ARS after electrophoresis followed by cloning and sequencing would be the most direct method to achieve this goal. Pathogen-specific qPCR could also be employed. Further, primary culture of hepatic tissue from pigs known to be infected followed by electron microscopy could also identify the unknown RNA species if it is indeed viral.

Future studies with B_6-deprived pigs should focus on determining the cause of reduced expression of BHMT and DNMT3a2. I hypothesize that these changes may be regulated by epigenetic changes within the promoter regions of these genes, and have begun investigating this using bisulfite sequencing technology. Further, microarray or whole transcriptome shotgun

sequencing (WTSS) experiments need to be conducted to determine what, if any, other genes show altered expression in B_6-deprived pigs.

Studies regarding one-carbon metabolism on runt pigs and folate-deprived pigs need to be conducted with higher sample number. The unexpected finding of an ARS in many of the animals reduced the power of my statistical analysis significantly and, although there appears to be a trend for altered expression of numerous genes, this needs to be confirmed. Microarray or WTSS analysis for identification of other genes showing altered expression and bisulfite sequencing of promoter regions to determine if their altered expression is epigenetically regulated would be optimal. There is also strong supporting evidence that histone modifications are heritable in multicellular organisms (Schuettengrubbet et al., 2007), so this opens another avenue for future research. Chromatin immunoprecipitation sequencing (ChIP-Seq) might be of particular use here. Further, many of the genes studied may be regulated at the translational or post-translational level, so future studies should also measure enzyme activity. Finally, as sex differences were found in runt pigs, I suggest the use of only one gender for future studies.

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