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Association of maternal and nutrient supply line factors with DNA methylation at the imprinted *IGF2/H19* locus in multiple tissues of newborn twins

Yuk Jing Loke[1,2,†], John C Galati[3,4,†], Ruth Morley[2], Eric Ji-Hoon Joo[1,5], Boris Novakovic[1,5], Xin Li[2], Blaise Weinrich[2], Nicole Carson[2], Miina Ollikainen[6], Hong-Kiat Ng[5], Roberta Andronikos[1,2], Nur Khairunnisa Abdul Aziz[1,2], Richard Saffery[1,5], and Jeffrey M Craig[1,2,*]

[1]Department of Paediatrics; University of Melbourne; Parkville, VIC Australia; [2]Early Life Epigenetics Group; Murdoch Childrens Research Institute (MCRI); Royal Children's Hospital; Parkville, VIC Australia; [3]Clinical Epidemiology and Biostatistics Unit; Murdoch Childrens Research Institute (MCRI); Royal Children's Hospital; Parkville, VIC Australia; [4]Department of Mathematics and Statistics; La Trobe University; Melbourne, VIC Australia; [5]Cancer, Disease and Developmental Epigenetics Group; Murdoch Childrens Research Institute (MCRI); Royal Children's Hospital; Parkville, VIC Australia; [6]Hjelt Institute; Department of Public Health; University of Helsinki; Helsinki, Finland

[†]These authors contributed equally to this work.

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Abbreviations: MZ, monozygotic; DZ, dizygotic; MC, monochorionic; DC, dichorionic; *IGF2*, insulin-like growth factor 2;, DMR, differentially methylated region; ICR, imprinting control region; CTCF, CCCTC-binding factor; HUVECs, human umbilical vein endothelial cells; CBMCs, cord blood mononuclear cells; PETS, peri/postnatal epigenetic twins study

Epigenetic events are crucial for early development, but can be influenced by environmental factors, potentially programming the genome for later adverse health outcomes. The insulin-like growth factor 2 (*IGF2*)/*H19* locus is crucial for prenatal growth and the epigenetic state at this locus is environmentally labile. Recent studies have implicated maternal factors, including folate intake and smoking, in the regulation of DNA methylation at this locus, although data are often conflicting in the direction and magnitude of effect. Most studies have focused on single tissues and on one or two differentially-methylated regions (DMRs) regulating *IGF2/H19* expression. In this study, we investigated the relationship between multiple shared and non-shared gestational/maternal factors and DNA methylation at four *IGF2/H19* DMRs in five newborn cell types from 67 pairs of monozygotic and 49 pairs of dizygotic twins. Data on maternal and non-shared supply line factors were collected during the second and third trimesters of pregnancy and DNA methylation was measured via mass spectrometry using Sequenom MassArray EpiTyper analysis. Our exploratory approach showed that the site of umbilical cord insertion into the placenta in monochorionic twins has the strongest positive association with methylation in all *IGF2/H19* DMRs ($P < 0.05$). Further, evidence for tissue- and locus-specific effects were observed, emphasizing that responsiveness to environmental exposures in utero cannot be generalized across genes and tissues, potentially accounting for the lack of consistency in previous findings. Such complexity in responsiveness to environmental exposures in utero has implications for all epigenetic studies investigating the developmental origins of health and disease.

Introduction

Cellular function in multicellular organisms is mediated by the interplay between underlying genetic profile and epigenetic mechanisms. The most widely studied epigenetic mechanism is DNA methylation that occurs predominately at the cytosine base of the CpG dinucleotide.[1,2] The epigenome is in a rapid state of flux during prenatal development[3] and evidence shows that it is particularly susceptible to environmental influence during this period.[4] Following evidence that placenta weight and/or birth weight can convey an elevated risk for cardiovascular and metabolic disease, a number of animal studies and a small number of human studies have since concluded that it is likely that environments that restrict fetal growth may "program" future chronic disease through influence on epigenetic state in utero.[5-8]

DNA methylation-mediated changes in gene expression play a pivotal role in early embryonic development, through two waves of genome-wide demethylation coupled with selective

*Correspondence to: Jeffrey M Craig; Email: jeff.craig@mcri.edu.au
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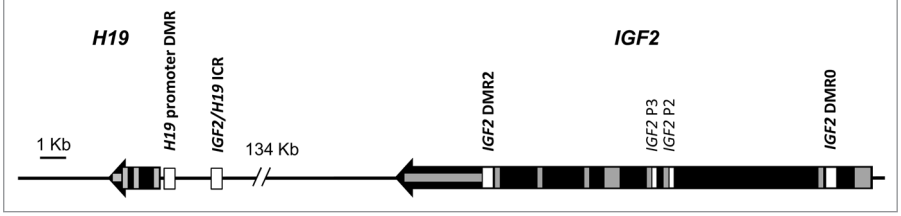

Figure 1. Structure of the *IGF2/H19* locus with DMRs. The *IGF2/H19* locus is shown with direction of transcription, exon locations (gray boxes) and DMRs (white boxes). DMRs assayed for DNA methylation in this study are indicated in bold.

re-methylation.[9-12] Expression of mammalian imprinted genes is controlled by differentially-methylated regions (DMRs), a subset of which act as master imprinting control regions (ICRs) for loci containing two or more imprinted gene.[13,14] The two most widely-studied imprinted genes are *H19* and *IGF2*, which are co-regulated within the same locus at human chromosome 11p15 (**Fig. 1**). Both are important for fetal and placental growth.[15] Within this locus, the *IGF2/H19* ICR upstream of the *H19* gene is a paternally-methylated germline DMR located within a series of binding sites for the CTCF protein necessary for long range chromatin interactions controlling gene activity.[16,17] There are at least three other DMRs within the *IGF2/H19* locus whose methylation status is somatically acquired during early embryonic development.[18,19]

In rodents, gestational environments such as maternal protein restriction,[7,20,21] low dietary folate,[22-24] low vitamin B12,[25,26] high methionine[22] and alcohol consumption[27-31] have been shown to influence DNA methylation in offspring, including at the *IGF2/H19* locus.[28,29] In humans, maternal depression/anxiety,[32,33] micronutrient supplements,[34] smoking,[35,36] famine in early gestation,[37,38] periconceptual folic acid intake,[39,40] and vitamin B12 intake[41] have also been implicated in the regulation of DNA methylation in offspring, including at the *IGF2/H19* locus.[34,36,37,39-41] However, previous studies of *IGF2/H19* DNA methylation in humans have focused on single tissues, and generally single environmental exposures (with one exception).[41]

Twins have traditionally been used to calculate the proportion of phenotypic variance explained by variation in genetic, common environmental and unique environmental factors,[42] and this approach has begun to pinpoint a role for each in establishing and maintaining levels of DNA methylation.[43,44] In our recent studies of newborn twins, we presented evidence that DNA methylation within the *IGF2/H19* locus[45] and throughout the genome[46] in humans is governed by genetic and non-shared stochastic and/or environmental factors, with latter being the largest component of variation. However, the relative contributions of specific environmental exposures to inter-individual differences in DNA methylation were not examined. Although twins share the same common (maternal) environment, they have individual supply lines, with each twin having its own umbilical cord with associated placental insertion site, placental circulation (with the exception of some monochorionic [MC] pairs) and in >99% of cases, separate amniotic sacs.[47] These factors contribute to a unique intrauterine environment for each twin within a pair. Position of cord insertion into the placenta and uneven placental allocation are significantly associated with birth weight discordance in MC twin pairs.[48-50] Velamentous insertion is also associated with increased perinatal morbidity and mortality.[51] In velamentous cord insertion, the umbilical cord inserts into the fetal membranes and the umbilical blood vessels then travel, unprotected, within the membranes to the placenta. Here, we provide a detailed analysis of the effects of ten early life factors on DNA methylation throughout the *IGF2/H19* locus, in multiple tissues at birth using data collected within the Peri/postnatal Epigenetic Twins Study (PETS).[52] Our goal was to explore the association of the shared maternal and non-shared supply line factors with *IGF2/H19* methylation at the four loci specified above. We were particularly interested in each measure's relationship with DNA methylation after taking the effects of the other measures into account, and whether the relationships held generally across the five cell types examined, or only to specific cell types. Shared factors included maternal folate and macronutrient intake, alcohol consumption, while twin-specific factors included placental size and cord insertion site. The tissues examined represent multiple lineages: mesoderm [Human Umbilical Vein Endothelial Cells (HUVECs), Cord Blood Mononuclear Cells (CBMCs) and granulocytes]; ectoderm (buccal epithelium) and extra embryonic ectoderm (placenta).

Results

Subjects and samples. Characteristics of the 67 MZ and 49 DZ twin pairs used in this study are listed in **Table 1**. In total we analyzed DNA methylation in DNA from buccal cells from 95 pairs (55 MZ and 40 DZ), CBMCs from 70 twin pairs (36 MZ and 34 DZ), granulocytes from 51 pairs (27 MZ and 24 DZ), HUVECs from 73 pairs (38 MZ and 35 DZ) and placenta from 67 pairs (34 MZ and 33 DZ).

Association of nutrient intake and lifestyle measures with DNA methylation. When all factors were taken into account and combining methylation data from all four DMRs (assays) and all five cell types, no significant associations ($P < 0.05$) were found for nutrient intake and lifestyle measures (All assays combined, **Table 2**). However, when DMRs were analyzed separately (with cell types still combined), significant associations were observed predominately at the *H19* promoter DMR (**Table 2**; **Figs. 2 and 3**; **Table S2**). Newborn offspring of mothers who had supplemental folate in the first 12 weeks of pregnancy exhibited lower mean methylation level compared with newborn offspring of mothers who did not (difference = -1.7%; $P = 0.024$; 95% CI: -3.2% to -0.2%), and similarly, a 1 standard deviation increase in serum vitamin B12 level at 28 weeks was associated with a -1.0% difference in mean methylation ($P = 0.002$; 95% CI: -1.6% to -0.4%) at this DMR. On the other hand, a 1 standard deviation increase in macronutrient score was associated with a 0.8% increase in mean methylation ($P = 0.049$; 95% CI: 0.0%

to 1.6%) over all five cell types combined. Newborn offspring of mothers who smoked before they knew they were pregnant had 2.1% higher methylation (P = 0.005; 95% CI: 0.6% to 3.6%) at this locus with all tissues combined. Interestingly, newborn offspring of mothers who smoked also had higher methylation at the *IGF2*/*H19* ICR (difference = 1.2%; P = 0.043; 95% CI: 0.0% to 2.4%) and newborns of mothers who took folate supplementation had higher methylation at the *IGF2* DMR2 (difference = 2.9%; P = 0.035; 95% CI: 0.2% to 5.6%; **Table 2**; **Figs. 2 and 3**; **Table S2**). Formal testing of differences in associations between loci suggested there may be a difference in the effect of folate supplementation between *IGF2* DMR0 and *H19* promoter DMR (difference in coefficients = 2.6%; P = 0.034; **Table S3**) and between *IGF2* DMR2 and *H19* promoter DMR (difference in coefficients = 4.7%; P = 0.005; **Table S3**), and a difference in the coefficients of vitamin B12 between *IGF2* DMR0 and *IGF2*/*H19* ICR (difference in coefficients = 1.2%; P = 0.023; **Table S3**). Interestingly, of the observed differences in associations between cell types (**Table S4**), all except one observed difference was between HUVECs and buccals (folate supplementation; difference in coefficients = -4.5%; P = 0.026; alcohol; 3.0%; P = 0.013; stress score; 1.2%; P = 0.009; **Table S4**), with the remaining difference being between Granulocytes and Buccals (difference in coefficients = 2.1%; P = 0.004; **Table S4**).

Association of gestational diabetes with DNA methylation. Overall, the presence of gestational diabetes was associated with decreasing DNA methylation when the data were combined (all assays combined, **Table 2**) and averaged over the four DMRs (assays) and five cell types examined (**Table 2**; **Fig. 3**; **Table S2**). A difference of -1.3% in mean methylation (P = 0.012; 95% CI: -2.4% to -0.3%) was observed between newborn offspring of mothers with and without gestational diabetes after controlling for the remaining measures (**Table 2**). This negative difference remained consistent across individual loci: -1.5% (*H19* promoter DMR), -0.8% (*IGF2*/*H19* ICR), -1.5% (*IGF2* DMR0) and -1.6% (*IGF2* DMR2). There was no evidence that the association of gestational diabetes with *H19*/*IGF2* methylation differed between loci (**Table S3**) or between cell types (**Table S4**).

Association of umbilical cord placement with DNA methylation. Central location of cord insertion into the placenta for newborns within pairs of MC twins exhibited a positive association with *IGF2*/*H19* methylation when considered over the four loci and five cell types examined. A difference of 3.0% (P < 0.001; 95% CI: 1.6% to 4.4%) in mean methylation between newborns with central cord insertion compared with those with non-central cord insertion was observed after controlling for all other measures examined (**Table 2**; **Fig. 4**; **Table S2**) in the combined analysis of all assays and cell types (All assays combined, **Table 2**). The observed difference was consistent at each locus (**Table 2**), namely 3.2% at the *H19* promoter DMR (P = 0.046; 95% CI: 0.1% to 6.3%), 2.4% within the *IGF2*/*H19* ICR (P = 0.005; 95% CI: 0.7% to 4.1%), 2.7% at *IGF2* DMR0 (P = 0.001; 95% CI: 1.1% to 4.2%) and 4.3% at *IGF2* DMR2 (P = 0.002; 95% CI: 1.6% to 7.0%), respectively. Moreover, the observed positive difference remained evident within cell types,

Table 1. Subject characteristics of twin pairs

Variable	
Pair-specific data	**Mean (SD) or %**
Gestational age at birth, weeks (median, SD)	37.0 (1.94)
Birth weight, g (median, SD)	2433.9 (479.7)
Sex: both male	40.5%
Sex: both female	50%
Sex: one male, one female	9.5%
Supply line factors	
Placental weight, mean (g)	419.5 (85.4)
Central cord insertion	38.8%
Non-central (velamentous or peripheral) cord insertion	**53%**
Maternal factors	
Periconceptional[a] folate supplements, >400 mg/day	90.5%
Third trimester serum vitamin B12 (pmol/L)	174.5 (69.2)
Third trimester serum homocysteine (μ-mol/L)	4.55 (2.13)
Carbohydrate intake in the first 2 trimesters (grams/day)	219.9 (82.2)
Protein intake in the first 2 trimesters (grams/day)	93.8 (43.0)
Energy intake in the first 2 trimesters (kilojoules/day)	8373.9 (3207.7)
Periconceptional* alcohol consumption[†]	50%
Periconceptional* smoking[‡]	24.1%
Maternal stress score, mean (range of possible scores 0–46)	21.3 (7.23)
Gestational diabetes	7.7%

*Defined as "before you knew you were pregnant (from conception to week 12 of your pregnancy"). [†]Women who consumed any quantity of alcohol on a weekly basis before knowing they were pregnant. [‡]Women who consumed any quantity of cigarettes on a weekly basis before knowing they were pregnant.

with 18/20 tissue-locus pairs exhibiting higher mean methylation in MC newborns with central cord insertion (**Fig. 3**), the two exceptions being a small negative difference at the *H19* promoter DMR in buccals and at the *IGF2*/*H19* ICR in placenta. In contrast, differences in mean methylation between dichorionic (DC) twins with and without central placement of the umbilical cord insertion into the placenta were more varied (**Table 2**; **Fig. 4**; **Table S2**), with only the *IGF2*/*H19* ICR region exhibiting evidence of a positive difference (of 1.8%; P = 0.001; 95% CI: 0.8% to 2.8%) and the *IGF2* DMR0 region exhibiting some evidence of a negative difference (of -1.8%; P = 0.045; 95% CI: -3.6% to 0.0%). The observed difference in association between MC and DC twins may be explainable by a difference in the coefficients of central cord insertion between placenta and non-placenta cell types, with a difference of -4.6% in coefficients between placenta and buccal tissue types being observed (P = 0.003; **Table S4**).

Association of placenta weight with DNA methylation. Similarly to cord insertion in MC twins, placenta weight exhibited evidence of a positive association with *IGF2*/*H19* methylation when averaged over all four loci and five cell types. An

Table 2. Linear regression of *IGF2/H19* methylation jointly on maternal and supply line factors over all five cell types combined

Factor	All Assays Combined		*H19* promoter DMR		*IGF2/H19* ICR		*IGF2* DMR0		*IGF2* DMR2	
	Coeff	*P* value	Coeff	*P* value	Coeff	*P* value	Coeff	*P* value	Coeff	*P* value
Had folate	0.50%	0.44	-1.70%*	0.024*	0.40%	0.69	0.90%	0.46	2.90%*	0.035*
Vitamin B12 (z-score)	-0.23%	0.24	-0.97%*	0.002*	-0.23%	0.54	0.23%	0.55	0.27%	0.63
Homocysteine (z-score)	0.27%	0.29	0.10%	0.75	0.40%	0.29	0.37%	0.30	0.17%	0.72
Macronutrients (z-score)	0.37%	0.17	0.80%*	0.049*	0.20%	0.50	0.43%	0.27	0.10%	0.77
Had alcohol	0.50%	0.27	0.00%	0.98	-0.70%	0.18	1.30%	0.14	1.90%	0.062
Smoked	0.90%	0.059	2.10%*	0.005*	1.20%*	0.043*	0.30%	0.74	-0.10%	0.90
Stress (z-score)	-0.10%	0.68	0.00%	0.98	-0.27%	0.42	0.30%	0.58	0.17%	0.65
Gestational diabetes	-1.30%*	0.012*	-1.50%	0.14	-0.80%	0.44	-1.50%	0.14	-1.60%	0.17
Central cord insertion (MC)	3.00%*	<0.001*	3.20%*	0.046*	2.40%*	0.005*	2.70%*	0.001*	4.30%*	0.002*
Central cord insertion (DC)	-0.30%	0.47	0.80%	0.28	1.80%*	0.001*	-1.80%*	0.045*	0.00%	0.98
Placenta Weight	0.53%*	0.044*	0.53%	0.18	0.87%*	0.010*	0.30%	0.57	0.27%	0.60

Coefficients measure difference in absolute percentage methylation. The "All Assays Combined" columns display the data from all assays and tissues are combined, the rest of the table displays the data resulting from associations analyzed separately for each assay. Coefficient for placenta weight, vitamin B12, homocysteine, macronutrients and stress score represents difference in mean methylation for a 1 standard deviation increase in the corresponding factor. Central cord insertion indicates central vs. non-central (peripheral and velamentous) insertion. *Coefficients with *P* values less than 0.05. Confidence intervals are shown in **Table S3**.

increase of 0.5% in mean methylation per 1 standard deviation increase in placenta weight was observed (All assays combined, **Table 2**) ($P = 0.044$; 95% CI: 0.0% to 1.1%), with the positive difference remaining consistent across loci (**Table 2**): 0.5% in the *H19* promoter DMR, 0.9% within the *IGF2/H19* ICR, 0.3% at *IGF2* DMR0 and 0.3% at *IGF2* DMR2, respectively (**Table 2**; **Fig. 4**; **Table S2**). A striking difference between non-placenta and placenta tissues was observed, with 14/16 locus-tissue pairs in non-placenta tissues exhibiting a positive association of placenta weight with DNA methylation (**Fig. 4**), compared with a negative association of placenta weight with DNA methylation being observed at the *H19* promoter region in placenta tissue, and essentially no association being observed at each of the other three loci (**Fig. 4**; **Table S2**). Formal testing of differences in coefficients of placenta weight on *H19/IGF2* methylation between cell types showed a -1.7% difference ($P = 0.032$; **Table S4**) in placenta weight coefficients between placenta and buccals tissue.

Expected antagonistic relationship of IGF2 and H19. *IGF2* and *H19* work antagonistically to promote and inhibit fetal/placental growth respectively.[15,53] As DNA methylation at *IGF2/H19* DMRs correlates positively with *IGF2* expression, negatively with *H19* expression, and is positively correlated with growth, we determined whether the associations observed between maternal and supply line factors (assumed to be also positively associated with growth) and *IGF2/H19* DNA methylation were in agreement with these relationships. Eleven of 12 relationships between supply line factors and *IGF2/H19* DNA methylation with all cell types combined (**Table 2**) had positive regression coefficients as expected; the single exception being central cord insertion in DC twins and DNA methylation at *IGF2* DMR0. These positive relationships also held true for 14 out of 18 significant relationships at the DMR and tissue level (**Fig. 4**, 95% CIs not crossing zero).

Discussion

We have undertaken a comprehensive multivariable study of the association of a range of maternal environmental factors with DNA methylation within *IGF2/H19*. This is the first study of its kind, encompassing the combined relationship of maternal micronutrient (folate, vitamin B12 and homocysteine), macronutrient, nutrient supply line factors (umbilical cord insertion point and placenta weight), lifestyle factors (alcohol consumption, smoking and perceived stress) and gestational diabetes with DNA methylation levels at multiple loci and across multiple tissues in newborn twins.

The importance of unique environment on the neonatal epigenome. The most striking finding of our study is the observed consistent positive association of central cord insertion of the umbilical cord into the placenta with *IGF2/H19* DNA methylation levels in MC twins. This finding suggests the existence of a robust positive association between DNA methylation in the *IGF2/H19* region and the quality of the insertion point of the umbilical cord into the placenta for newborns from a pair of MC twins. We also observed a positive association of placenta weight with *IGF2/H19* DNA methylation in other tissues. Together, these findings suggest that DNA methylation in this region is susceptible to the quality of the nutrient supply line, but that this is a complex relationship subject to many different influences. In particular, these data suggest that the unique environment experienced individually by each twin of a twin-pair,

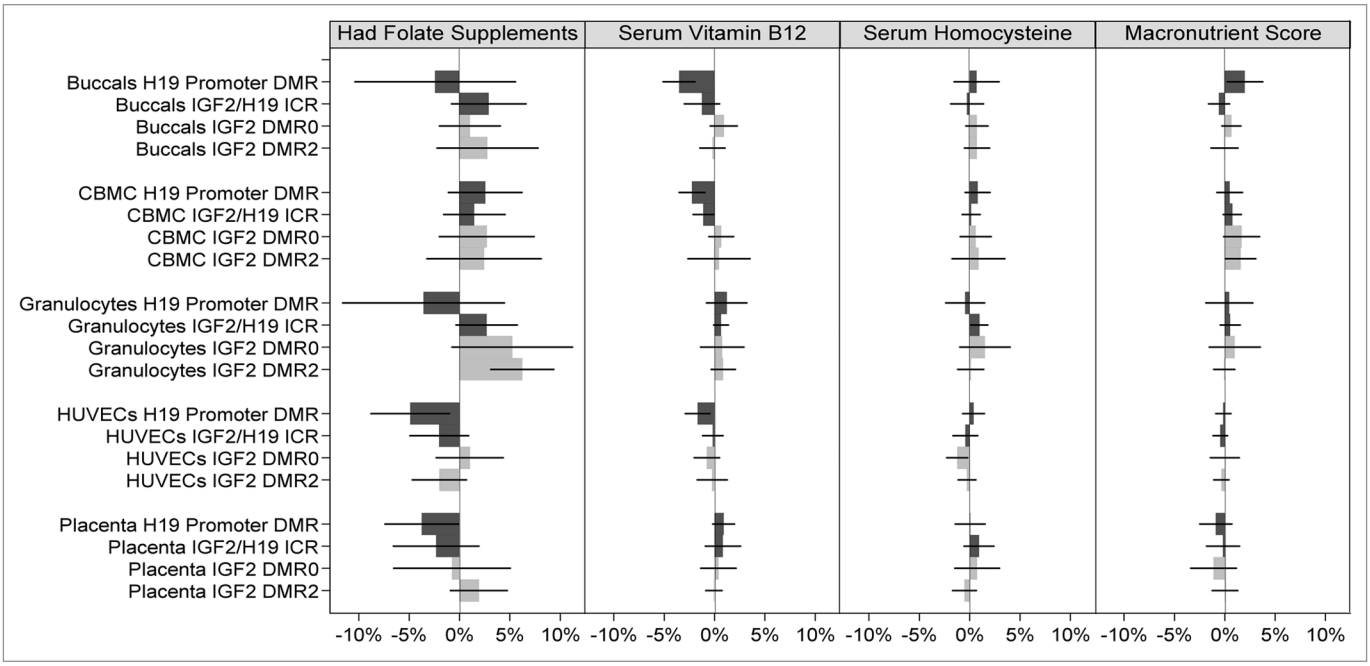

Figure 2. Association of *IGF2/H19* methylation and nutrition factors across all cell types and DMRs. Light gray bars represent methylation changes in the *IGF2* DMRs, and the dark gray bars represent changes in *H19* DMRs. 95% confidence intervals of the coefficient change are represented by a horizontal black line through each bar. The mean methylation differences corresponding to serum vitamin B12, serum homocysteine, and macronutrient are for a 1 standard deviation increase in the respective factor's z-score. Macronutrient score is obtained as the average of protein, carbohydrate and energy z-scores.

that includes the differential position of cord insertion and associated placental vascularity, can influence DNA methylation in utero. The reason why an association with cord insertion was observed for only MC twins could reflect the competition for resources that can occur within twin pairs shat share the same placenta.[48] As DNA methylation within the *IGF2/H19* locus can influence fetal growth through its effects on gene expression,[15-17] we suggest that the impeded blood flow that results from a smaller placenta size[54] or a non-central cord insertion point[55] influences fetal growth at least in part due to an increase in DNA methylation within *IGF2/H19*. Indeed, levels of *IGF2 DMR0* methylation are associated with increased levels of *IGF2* protein and increased birth weight.[56] This relationship may help to explain the previously described links between non-central cord insertion, low placenta and birth weight and increased risk of neonatal morbidity and mortality.[48-51] We suggest that future studies of twins and singletons should consider cord placement and placenta size as potential confounders. Furthermore, because of the link between low birth weight and risk for complex disease,[57] it will be important to look at the relationship between unique environmental factors and future health in studies of twins and singletons, and not just on factors common to both twins.

Gestational diabetes influences neonatal DNA methylation. A second striking finding of our study is the observed consistent negative association between maternal gestational diabetes and DNA methylation levels at *IGF2/H19* in non-placental tissues (**Table 2**; **Fig. 3**; **Table S2**). Studies of rodents have investigated such a link and both found a positive association between induced gestational diabetes and DNA methylation at the *IGF2/H19 ICR*[58,59] and *IGF2 DMR2*.[59] The discrepancy between these findings and our study could be due to the differences in the control of imprinting between species or to the multivariable nature of our study, which takes account of the potential effects of a range of factors acting simultaneously.

The complexity of IGF2/H19 methylation differences across tissue and loci associated with different maternal factors. We have reported associations between early life environment and DNA methylation in four DMRs of the *IGF2/H19* locus in multiple tissues at birth. This is the first study that has investigated multiple early environmental factors across multiple tissues and loci. Overall, we observed a complex relationship with different magnitudes and directions of effect on DNA methylation variation in association with varying gestational factors across five tissue types (**Table 2**; **Figs. 2–4**; **Table S2**). The exception is the position of umbilical cord insertion, specifically in MC twins, that showed a consistent direction of effect in all tissues. Although it has been shown that mammalian tissues can differ in their epigenetic status (e.g. refs. 46 and 60), there have been few studies of the effects of early environment on DNA methylation in different tissues. However, our findings agreed with earlier data from studies of mature mice and humans (reviewed in ref. 61) and with recent genome-scale studies: in mice of early alcohol exposure[62] and in humans of assisted reproductive technologies ART,[63] smoking,[35] maternal homocysteine levels,[64] and childhood stress.[65] Taken together, these data showed that the effects of early environment on DNA methylation in offspring are often locus-specific and therefore

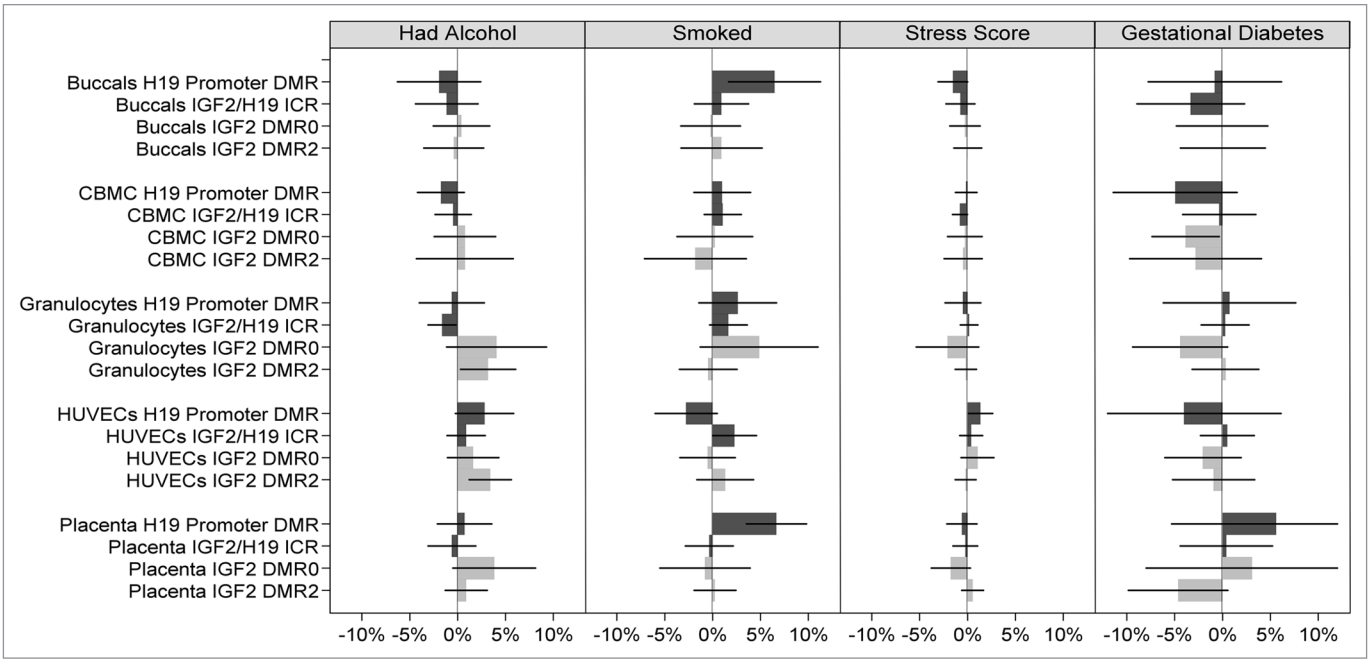

Figure 3. Association of *IGF2/H19* methylation and lifestyle factors across all cell types and DMRs. Legend as for **Figure 2**. The mean methylation difference corresponding to stress is for a 1 standard deviation increase in the stress z-scores.

effects from other environmental factors (or even the same factors) cannot be assumed to be the same for other genes or tissues. Our study also provides the first insights into the potential processes underlying the variable tissue-specific effects on genome-scale DNA methylation that we have recently described at birth.[45]

Comparison of effect direction with previously published data. Our DNA methylation data from multiple maternal factors, cell types and *IGF2/H19* DMRs allow comparisons with previous studies in rodents and humans (**Table 3**). Although the effect sizes might be small, most of the top hits in epigenome-wide epigenetic studies are only around 5% and, when occurring in gene networks, may be additive[66] and should be considered as relative rather than absolute differences.[67] A recent study showed that an *IGF2/H19* methylation difference of 5% was associated with a 2 mm difference in abdominal skin fold thickness in children age 1–10 y and that a 3.4% *IGF2/H19* methylation difference was associated with a 18 mm difference in their head circumferences.[68] This shows that small differences in methylation can be associated with sizable phenotypic effects. Furthermore, relationships between DNA methylation and gene expression have been demonstrated to be nonlinear, with small changes in DNA methylation being associated with large differences in gene expression.[20,69-71]

With reference to **Table 3**, previous data showing a positive correlation between maternal periconceptional folic acid supplementation and DNA methylation at the *IGF2 DMR0* in whole blood from 17 mo-old offspring in humans[40] agree with our positive, although non-significant, association between the same two variables in granulocyte and CBMC blood fractions taken at birth. Conversely, previous data[39] showed a negative correlation between maternal folic acid supplementation during pregnancy and DNA methylation at the first CTCF binding site within the *IGF2/H19* ICR in whole blood from newborn mouse pups, which disagrees with our positive, although non-significant, association between the same exposure-DMR combination in blood fractions taken at birth. Differences between the locations of the assayed regions within the *IGF2/H19* ICR could explain the discrepancy. Few studies have looked at the effects of prenatal alcohol exposure on *IGF2/H19* DNA methylation. Animal studies have shown a trend of decreased *IGF2/H19* ICR methylation in placenta,[28] which was comparable to our study, as reduction of placental *IGF2/H19* ICR methylation was observed in mothers who consumed alcohol during the periconceptional period.

DNA methylation at the *IGF2* DMR0 was reduced by 5.2%[37] and 2.1%[72] and reduced by 0.5% in the *H19* promoter DMR[72] in whole blood of 60-y-olds whose mothers were exposed to famine during early gestation compared with their unexposed siblings. This data can be compared with our variable of maternal macronutrient intake, as this would be mainly compromised in famine conditions. Indeed, the Dutch famine data agree with our findings of a 1.0 to 1.7% (non-significant) increase in DNA methylation at the *IGF2* DMR0 and a 0.5% (non-significant) increase in DNA methylation at the *H19* promoter DMR in CBMC and granulocyte blood fractions for every 1 standard deviation increase in macronutrient score (**Table 3 and Fig. 2**). Finally, a positive association between maternal periconceptional smoking and DNA methylation at the *IGF2* DMR0 in whole blood at birth (significant in males only[36]) agrees with our data of a positive, but non-significant association between DNA methylation at the *IGF2* DMR0 in neonatal blood fractions (**Table 3**; **Fig. 3**).

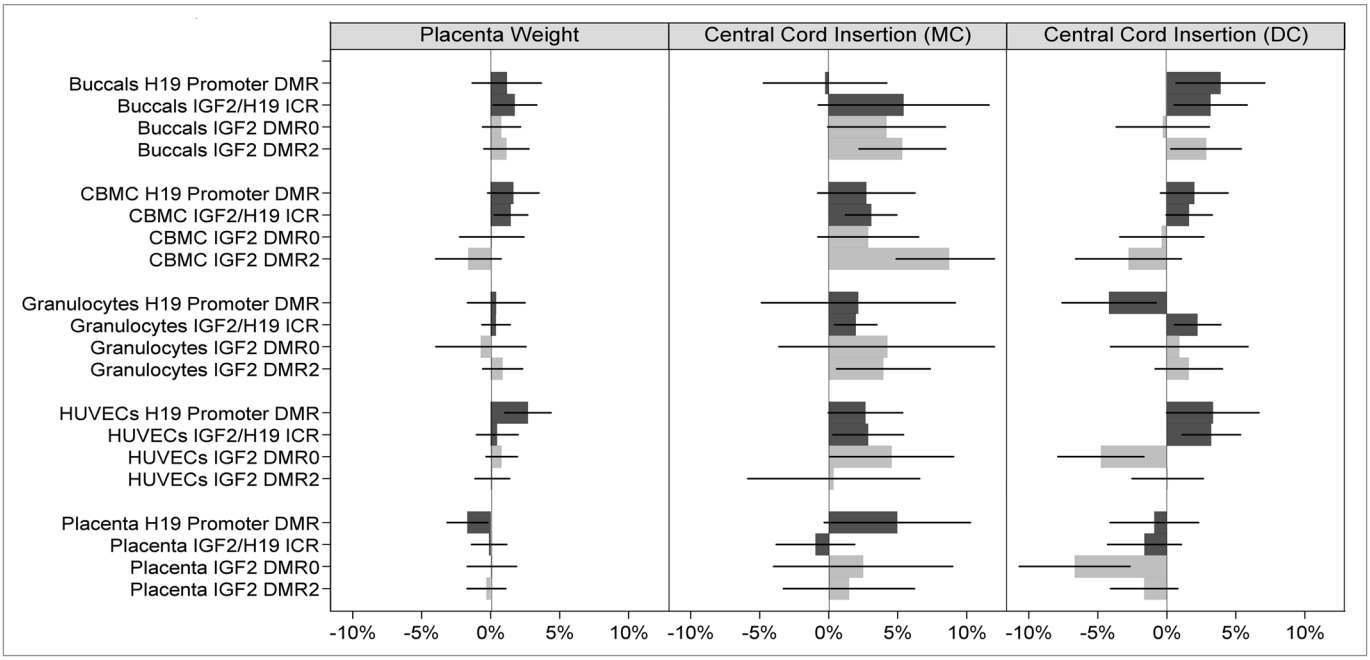

Figure 4. Association of *IGF2/H19* methylation and supply line factors across all cell types and DMRs. Legend as for **Figure 2**. Mean methylation differences are regression coefficients obtained from multivariable regression analysis, whereby regression coefficients are converted to percentage difference by multiplying each regression coefficient with average standard deviation. The mean methylation corresponding to placenta weight is for a 1 standard deviation increase in placenta weight z-score.

We focused on the *IGF2* locus because *IGF2* and *H19* the genes within are highly expressed in utero where they contribute to fetal and placental growth.[15] However, other genes involved in fetal growth, imprinted or otherwise, will need to be examined in future studies to determine how widespread these effects are.

Conclusions

For the first time we present evidence for the effect of specific non-shared environments and for locus-specific effects of a number of shared environments on the neonatal epigenome. Our study has a number of strengths including the use of multiple cell types, multiple DMRs within the *IGF2/H19* locus and the rich data on maternal and supply line factors collected. In addition, our data underwent stringent QC analysis and excluded CpGs previously shown to be under genetic influence.[45] We also present evidence that in some cases, effects of a particular environment may be locus- or tissue-specific. Further studies will need to be conducted to determine the effect of the small effect sizes we found (1.6% to 3.0% absolute differences for significant associations in all tissues/DMRs combined [**Table 2**] and up to 8.7% for absolute difference for assay mean/tissue associations [**Figs. 2–4**]), on levels of the corresponding genes and proteins. Other studies have shown such effects to be nonlinear[20,69,70] and it has been suggested that such effects at multiple genes may be additive.[66] Further studies are also needed to determine whether such small differences are associated with disease risk and whether they are stable over time. If these and other epigenetic differences at birth are found to confer such risk, then for shared factors, this could be addressed by targeted intervention during pregnancy.[73] As intervention is usually not possible with supply line factors, they could at least be closely monitored during pregnancy.[73] In the future, risk-associated gene- or pathway-specific risks effects could be reversed by dietary, or pharmaceutical or other interventions.[74] Indeed, such broad-based interventions have already proven successful at reversing the risks associated with fetal programming in animal models.[75] Our study has shown that the epigenetic effects of such interventions will need to be studied in multiple tissues.

Materials and Methods

Twins cohort. A subgroup of 67 monozygotic (MZ) and 49 dizygotic (DZ) twin pairs from the PETS cohort[52] were included in this study. Questionnaire data were obtained for maternal nutritional intake, lifestyle and presence of gestational diabetes at different time points of pregnancy (see below for details). The majority of the twin pairs were same sex with only 9.5% being of opposite sex within a pair. An overview of the sample characteristics and maternal data used for thus study is listed in **Table 1**. The study was performed with appropriate human ethics clearances from the Royal Women's Hospital (06/21), Mercy Hospital for Women (R06/30), and Monash Medical Centre (06117C), Melbourne.

Maternal and supply line factors. Data on maternal and supply line factors were collected as previously described.[52] Location of the umbilical cord insertion into the placenta was measured at birth and defined either as "central" (located in the inner 50% of the placental radius) or "non-central" (located with peripheral or velamentous positioning.).[50] Placental weight was measured at

Table 3. Comparisons with previously published data

Published studies						Our study		
Exposure/ reference	**Model**	**Region**	**Tissue/age studied**	**Exposure timing**	**Methylation result**	**Tissue**	**Exposure timing**	**Methylation result**
Folic acid supplements[40]	Human	*IGF2* DMR0	Whole blood/ 17 mo	Periconception	+4.5%	Granulocytes CBMCs	Periconception	+5.2% ns* +2.7% ns
Folic acid supplements[39]	Human	*IGF2/H19* ICR (CTCF1)	Whole blood/ birth	Before pregnancy	+2.2% to +2.4%	Granulocytes	Periconception	+2.7% ns
				During pregnancy	-2.9% to -3.7%	CBMCs	Periconception	+1.5% ns
Alcohol[28]	Mouse	*IGF2/H19* ICR	Placenta	Pre-implantation	Average -12% in paternal clones	Placenta	Periconception	-0.6%
Famine (lack of macronutrients)[37,72]	Human	*IGF2* DMR0	Whole blood/ 60 y old	Prenatal	-5.2% -2.0%[e]	Granulocytes CBMCs	Periconception	+1.0%[†] ns +1.7%[†] ns
		H19 promoter DMR			-0.5% ns	Granulocytes CBMCs	Periconception	+0.5%[†] ns +0.5%[†] ns
Smoking[36]	Human	*IGF2* DMR0	Whole blood/ birth	Periconception	~4% [‡,§]	Granulocytes CBMCs	Periconception	+4.9% ns +0.2% ns

Previous studies that looked at association between the factors examined in this study and IGF2/H19 methylation shown on the left are compared with the data from our study (right). *ns, not significant; [†]the value indicates the mean methylation difference in every 1 standard deviation decrease in macronutrient score;[‡]results presented for males and females and average shown; [§]significant for males only; [e]corresponding to a standard effect size of -0.6 SD units.

birth, with total placenta weight being shared equally between co-twins when values for separate placentas were unavailable (e.g., in MC twin pairs). Folate intake from nutritional supplements was obtained during the first 12 weeks of pregnancy (yes/no) and serum vitamin B12 and homocysteine levels measured from a sample of peripheral blood taken from mothers at 28 weeks gestation. Protein, carbohydrate and energy intake in the first two trimesters of pregnancy were obtained through a validated food frequency questionnaire[76] at a median gestational age of 28 weeks. Alcohol consumption and smoking before the mother knew she was pregnant (yes/no) were obtained from mothers between 19 and 24 weeks of gestation. Maternal perceived stress score was obtained via questionnaire[77] at 28 weeks gestation and the presence of gestational diabetes was ascertained by questionnaires at recruitment, 24 weeks and, where appropriate, 36 weeks' gestation.

Tissue collection and cell processing. Placenta, umbilical cords, cord blood and buccals were collected at the time of delivery and processed soon after; CBMCs and granulocytes were isolated from cord blood, and HUVECs from cords as previously described.[45]

DNA extraction, bisulphite conversion, and locus-specific methylation analysis. DNA was prepared from buccal cells via salt extraction and from all other tissues by standard phenol:chloroform extraction and ethanol precipitation as described previously.[45] A total of 500 ng to 1 μg genomic DNA was bisulphite converted using the MethylEasy Exceed Rapid Bisulphite Modification Kit (Human Genetic Signatures) and *IGF2/H19* DMRs amplified as described previously, named according to location.[45] The two in the promoter regions of *IGF2* and *H19* are referred to as *IGF2* DMR0 (chr11:2,169,514-chr11:2,169,771) and *H19* promoter DMR (chr11:2,019,654-chr11:2,019,849) respectively (**Fig. 1**; **Table S1**). *IGF2/H19* ICR (chr11:2,021,010-chr11:2,021,266) is located within the 6th CTCF site of the ICR, and *IGF2* DMR2 (chr11:2,154,349-chr11:2,154,067) is located within exon 9 of *IGF2* (**Fig. 1**; **Table S1**). DNA methylation was measured using the MassARRAY EpiTYPER (SEQUENOM Inc.). At least two replicate amplifications were performed in all instances and data was subjected to stringent cleaning steps as outlined previously.[45] Study individuals were genotyped for all known SNPs in the regions analyzed and where a SNP was found to abolish a CpG site in one or both alleles, methylation values for this CpG site or CpG unit containing the SNP were set to missing.

Statistical analysis. Multiple linear regression of methylation data jointly on the environmental measures was used to assess associations, with robust standard errors used to account for correlation of methylation data within the same subject, and to allow for potential correlation between co-twin methylation values. This approach does not impose any particular covariance structure on the data. The stability of the covariance estimation was assessed by checking the results for consistency with those

obtained from a hierarchical random-effects model. In the latter model, loci were clustered within tissue, tissues were clustered within baby and twins were clustered within mother. An order one autoregressive (AR1) covariance structure for correlation of CpG units within each locus was imposed, reflecting generally positive correlations within a locus that decay with distance. We chose to report results from the model with unstructured covariance, as these are more robust to misspecification of the covariance structure. Since it is unknown whether the associations, if any, should hold generally across all four loci and five cell types at specific loci only, or only for specific tissues, in addition to reporting overall associations pooled across all loci and tissue types, interactions by locus and by tissue were fitted to explore differences in effects across loci and across tissues. Effect sizes were also explored separately for each combination of locus and tissue, but at this level of detail, formal testing of interactions was not considered feasible. DNA methylation levels were converted to z-scores prior to analysis to make them comparable across CpG units and tissues. High correlations within the set of macronutrient/energy measures were handled by using a principle components analysis to derive summary variables. The first principal component, hereafter referred to simply as macronutrient score, was chosen for use in analyses as it captured 93% of the variation between protein, carbohydrate and energy and represented an approximate equal weighting of these three measures. To facilitate comparison of effect sizes across all environmental measures, continuous measures were converted to z-scores prior to analysis. For reporting, regression coefficients were converted to percentage difference in mean methylation by multiplying each regression coefficient by the average standard deviation of methylation across CpG units. The potential for false positives from multiple testing was addressed by limiting the number of regression coefficients estimated to four groups of ten (one group of ten factors per locus). Formal control of the false positive rate is not possible in this setting, however, due to the moderate number of potentially correlated comparisons being performed. All analyses were performed using Stata statistical software (StataCorp.).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/25908

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