

DNA methylation analysis of multiple tissues from newborn twins reveals both genetic and intrauterine components to variation in the human neonatal epigenome

Miina Ollikainen^{1,3}, Katherine R. Smith², Eric Ji-Hoon Joo^{1,3}, Hong Kiat Ng^{1,3},
Roberta Andronikos^{1,3}, Boris Novakovic^{1,3}, Nur Khairunnisa Abdul Aziz^{1,3}, John B. Carlin^{2,3},
Ruth Morley^{1,3}, Richard Saffery^{1,3,*} and Jeffrey M. Craig^{1,3,†}

¹Developmental Epigenetics and ²Clinical Epidemiology and Biostatistics Unit, Murdoch Childrens Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia and ³Department of Paediatrics, University of Melbourne, Parkville, Victoria 3052, Australia

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Mounting evidence from both animal and human studies suggests that the epigenome is in constant drift over the life course in response to stochastic and environmental factors. In humans, this has been highlighted by a small number of studies that have demonstrated discordant DNA methylation patterns in adolescent or adult monozygotic (MZ) twin pairs. However, to date, it remains unclear when such differences emerge, and how prevalent they are across different tissues. To address this, we examined the methylation of four differentially methylated regions associated with the *IGF2/H19* locus in multiple birth tissues derived from 91 twin pairs: 56 MZ and 35 dizygotic (DZ). Tissues included cord blood-derived mononuclear cells and granulocytes, human umbilical vein endothelial cells, buccal epithelial cells and placental tissue. Considerable variation in DNA methylation was observed between tissues and between unrelated individuals. Most interestingly, methylation discordance was also present within twin pairs, with DZ pairs showing greater discordance than MZ pairs. These data highlight the variable contribution of both intrauterine environmental exposures and underlying genetic factors to the establishment of the neonatal epigenome of different tissues and confirm the intrauterine period as a sensitive time for the establishment of epigenetic variability in humans. This has implications for the effects of maternal environment on the development of the newborn epigenome and supports an epigenetic mechanism for the previously described phenomenon of 'fetal programming' of disease risk.

INTRODUCTION

Evidence from animal models and humans implicates the intrauterine period as critical in influencing disease risk for a range of disorders that develop later in life. This 'fetal programming' appears to be largely independent of genomic DNA sequence and is likely to be mediated by epigenetic mechanisms (1,2). It is clear that epigenetic variation plays a considerable role in the modulation of disease risk in

humans. Unequivocal evidence exists for many cancers, in which the magnitude of epigenetic change is large at the level of both the gene and the genome, whereas evidence for less extreme changes is emerging for complex human disorders associated with metabolic, neural, reproduction, musculoskeletal and immune system dysfunction (reviewed in 3–8).

Epigenetic marks are extensively remodelled in early embryogenesis (9,10). Partially methylated gametic genomes undergo active (paternal genome) or passive (maternal

*To whom correspondence should be addressed at: Murdoch Childrens Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052, Australia. Tel: +61 383416341; Fax: +61 383416212; Email: richard.saffery@mcri.edu.au

†The authors wish it to be known that, in their opinion, the last two authors should be regarded as joint Senior/Corresponding Authors.

genome) demethylation post-fertilization with subsequent re-establishment of methylation profile in a tissue-specific manner (11). Lineage-specific methylation profiles are then passed on through cell division to daughter cells. However, mounting evidence suggests that DNA methylation may be dynamic in certain instances, even changing at specific loci throughout the cell cycle (12–14).

In addition to a fundamental role in cell lineage specification, epigenetic marks such as DNA methylation and covalent histone modification have also been identified as mediators of environmental and stochastic effects on gene expression (15,16), with or without associated phenotypic consequences such as the modulation of disease risk (17). Compelling data exist from several animal models (18–23) and experimental data are also emerging in human studies (16,24–26). Of particular interest in humans are data implicating prenatal exposure to adverse environments (such as exposure to famine or dietary supplementation) in determining the level of DNA methylation present in the offspring, specifically at imprinted genomic regions implicated in regulating fetal growth (27–29).

Genomic imprinting is a phenomenon whereby a gene is expressed in a parent-of-origin manner, with one active and one silent allele. This process is under epigenetic control (including via differential DNA methylation) at imprinting control regions (ICRs), which contain differentially methylated regions (DMRs). DNA methylation at DMRs is erased in primordial germ cells (30–32) and then re-established during germ cell development (33–35). However, the mechanisms behind regulation of imprinting establishment and maintenance in humans are complicated, as typified by a recent study showing heritable loss of *IGF2* imprinting in some individuals, independent of age, possibly predisposing to disease later in life (36).

Unravelling the determinants of epigenetic profile is problematic given the complicated interrelationship of environmental and genetic factors. Twin pairs have proven invaluable in this context by allowing the relative contributions of each of these factors to be studied independently. Monozygotic (MZ) twin pairs are widely regarded as genetically identical, and as such, phenotypic variation is presumed to be almost exclusively environmental or stochastic in origin. A comparison between MZ and dizygotic (DZ) twin pairs, which share on average half of their segregating polymorphisms, allows the relative contributions of both genetic and environmental/stochastic factors to be estimated (37).

Since the epigenome is likely to be in constant drift, due to both stochastic and environmental factors, there is a great potential for epigenetic variation within MZ twin pairs (38,39). Differences in the epigenome may explain the incomplete penetrance and variation in the age at onset and severity of diseases in such pairs (40,41). Several studies have described MZ twin pairs showing both phenotypic (disease-related) and epigenetic discordance (15,42–48). A single study has reported epigenetic drift in small numbers of MZ twin pairs of widely varying age, potentially exacerbated by differing environmental histories (39). However, this may be locus-specific, as no evidence of such drift was found following a similar examination of the *IGF2/H19* locus in a large number of adolescent and middle-aged MZ twins (49). The situation is further complicated by data from

a small number of studies that have identified differential methylation and/or imprinting in MZ twin pairs at birth, primarily in the context of environmental perturbation (such as *in vitro* fertilization) or the presence of a pathological condition (50–52). In summary, the relative contribution of genetic, environmental and stochastic factors to epigenetic profile remains unclear, particularly in relation to the establishment of the neonatal epigenome.

In this study, we investigated the level of epigenetic discordance in MZ and DZ twin pairs at four DMRs at the *IGF2/H19* locus, in multiple tissues collected at birth. Methylation differences within MZ twins were predicted to reflect a net result of environment-induced and stochastic epigenetic changes accumulated *in utero*, whereas differences within DZ pairs reflect these factors *plus* the contribution of underlying genetic variation. Comparison of variation within MZ and DZ twin pairs allows an estimate of the relative contribution of each of these factors to the overall establishment of the neonatal epigenome.

RESULTS

IGF2/H19 DMRs

We chose to study all four previously defined human *IGF2/H19* DMRs to obtain a full picture of imprinting at this locus, compared with many previous studies that have looked at only one or two in isolation. The human *H19* locus contains a DMR critical for controlling imprinting and expression of *IGF2* and *H19* (53,54). This region has seven binding sites for the insulating factor CTCF, of which the sixth is the most widely studied. Specific methylation changes in this region have been associated with altered *H19* and *IGF2* gene expression and have been described in diseased states such as cancer (54–56). Despite this, unequivocal evidence for methylation of all CTCF sites on the paternal allele in non-pathogenic states has previously been reported in humans, suggesting that the regulatory capacity of this ICR derives from the activities of multiple sites (57). In the current study, methylation at this region was measured using the *H19 CTCF6* DMR assay. Methylation levels at a second *H19*-associated DMR, located at a CpG island-associated gene promoter (*H19 promoter DMR*), were also measured. Further, we studied two well-characterized *IGF2* DMRs, DMR0 (58,59) [also known as *IGF2* DMR and located proximally in the *IGF2* gene (49)], and a DMR in exon 9 of *IGF2* (*IGF2 ex9 DMR*) (Fig. 1 and Supplementary Material, Table S1). Note that for simplicity we are using DMR and methylation assay names interchangeably.

IGF2/H19 DMRs show inter-individual and tissue-specific methylation variability in newborns

We measured DNA methylation using Sequenom MassARRAY EpiTYPER, which is a bisulphite-based technology that relies on base-specific cleavage and mass spectrometry (MS) to measure the level of methylation in DNA fragments containing one (for example, *H19 promoter DMR* CpG₃) or more (for example, *H19 promoter DMR* CpG_{7_8}, containing CpGs 7 and 8 inclusively) CpGs (60). This method provides

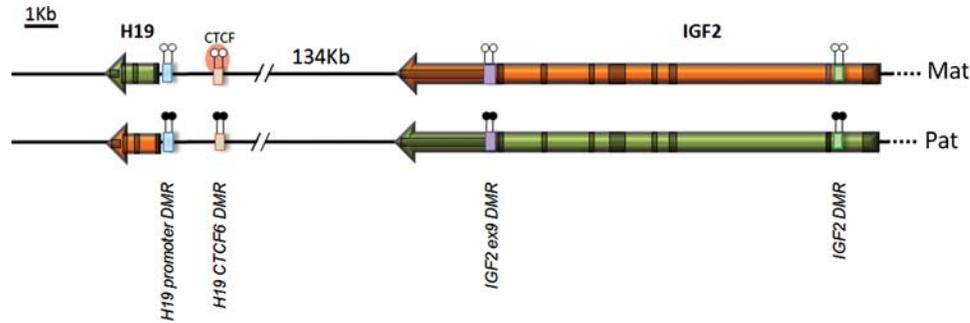


Figure 1. The *IGF2/H19* locus with locations of the studied DMRs. *H19* and *IGF2* gene expression is reciprocally controlled. In the maternal allele, CTCF binds to the major ICR (CTCF-binding site 6), which is unmethylated (white lollipop). Binding of the CTCF insulator protein prevents the expression of *IGF2* (orange arrow) and enables expression of *H19* (green arrow). In the paternal allele, CTCF-binding site 6 is methylated (black lollipop) preventing the binding of CTCF; *IGF2* is expressed and *H19* silenced. Grey boxes denote exons.

an accurate measurement of the average level of DNA methylation present within a sample of DNA (61,62). Each of the four methylation assays described above, ranging in size from 255 to 375 bp, generates DNA methylation data for multiple CpG units. Following our stringent data cleaning, the four assays produced a total of 26 analytic CpG units containing one (16 units), two (9 units) or three (1 unit) individual CpG sites. Each unit produces a single methylation value following EpiTYPER analysis regardless of the number of CpG sites contained therein. DNA methylation at each DMR was measured in up to five different cell types—cord blood mononuclear cells (CBMCs) and granulocytes, human umbilical vein endothelial cells (HUVECs), buccal epithelial cells and placenta—from each of 56 MZ and 35 DZ newborn twin pairs. Different combinations of tissues were available for each twin pair (Supplementary Material, Table S2).

Methylation values for individual CpG units and amplicon means showed differing degrees of variability across individuals and tissues, but were generally approximately normally distributed (Fig. 2). A notable exception was CpG₁₀ in the *H19 CTCF6 DMR* assay, which showed a distribution skewed towards zero in all tissues tested. The mean methylation level across all samples and tissues for each DMR generally approximated to 50% and ranged from 44 to 50% (Supplementary Material, Table S3 and Fig. 2). However, the mean DMR methylation for specific DMR/tissue combinations varied widely across individuals, ranging from 14% for the *IGF2 DMR* in a single granulocyte sample to 99% for the *IGF2 ex9 DMR* in a single HUVEC sample (Fig. 2).

We also examined the potential for preferential amplification of a single parental allele as the primary cause of a high-level of methylation variation. Genomic polymorphisms in the primer-binding site within genomic DNA are thought to be the major cause of such skewed amplification. In order to address this, we performed DNA sequencing of all four DMRs from all individuals in the study. We found no evidence of any polymorphisms of this nature likely to lead to allelic skewing. Additionally, we performed bisulphite sequencing for a proportion of randomly picked amplification products known to be heterozygous for SNPs within regions of interest. The results confirmed that in all cases, both alleles contributed to the methylation values obtained by EpiTYPER analysis (data not shown).

We next investigated the possibility of tissue-specific regulation of DNA methylation imprinting at the *IGF2/H19* locus by examining whether mean methylation levels differ markedly between tissues. To achieve this, the mean methylation value of each DMR was regressed separately upon tissue type in a linear mixed model. All four DMRs showed very strong evidence ($P < 0.001$) of tissue-specific regulation of mean DNA methylation levels (Supplementary Material, Table S4). *IGF2 ex9 DMR* showed the greatest variation in DMR mean methylation between tissues, whereas the *H19 CTCF6 DMR* showed the least variation (Fig. 2 and Supplementary Material, Table S3). The mean methylation for both *IGF2 DMRs* in HUVECs (76 and 64% for *IGF2 ex9 DMR* and *IGF2 DMR*, respectively) was substantially higher than in other tissues (31 and 44% for buccal; 50 and 46% for CBMC; 46 and 49% for granulocytes; 30 and 50% for placenta, respectively). In general, mean methylation values showed a greater degree of tissue-dependency than DMR-dependency (Fig. 2 and Supplementary Material, Table S3).

Methylation of *IGF2/H19*-associated DMRs show varying degrees of correlation at birth

We next investigated whether methylation might be coordinated within and between DMRs by examining correlations between CpG units. Figure 3 shows the correlation of mean methylation values obtained for all CpG units analysed across all tissues. Methylation levels of CpG units were generally strongly correlated within each DMR and were positively correlated between different DMRs, with some exceptions (e.g. *H19 CTCF6 DMR* CpG₁₀ and *IGF2 DMR* CpG₁). However, the strength of correlation varied markedly between tissues, with buccal cells showing the strongest correlation (median of pairwise correlations 0.42) and granulocytes the weakest correlation (median 0.11) across the four *IGF2/H19* DMRs examined (Supplementary Material, Fig. S1). The *H19 promoter DMR* showed the highest within-amplicon correlation of methylation values of all DMRs in all tissues tested (median Spearman's correlation coefficient $\rho = 0.84$). *IGF2 ex9 DMR* (median $\rho = 0.77$) also showed a higher within-assay correlation than *H19 CTCF6 DMR* and *IGF2 DMRs* (median correlations of 0.50 and 0.57 respectively).

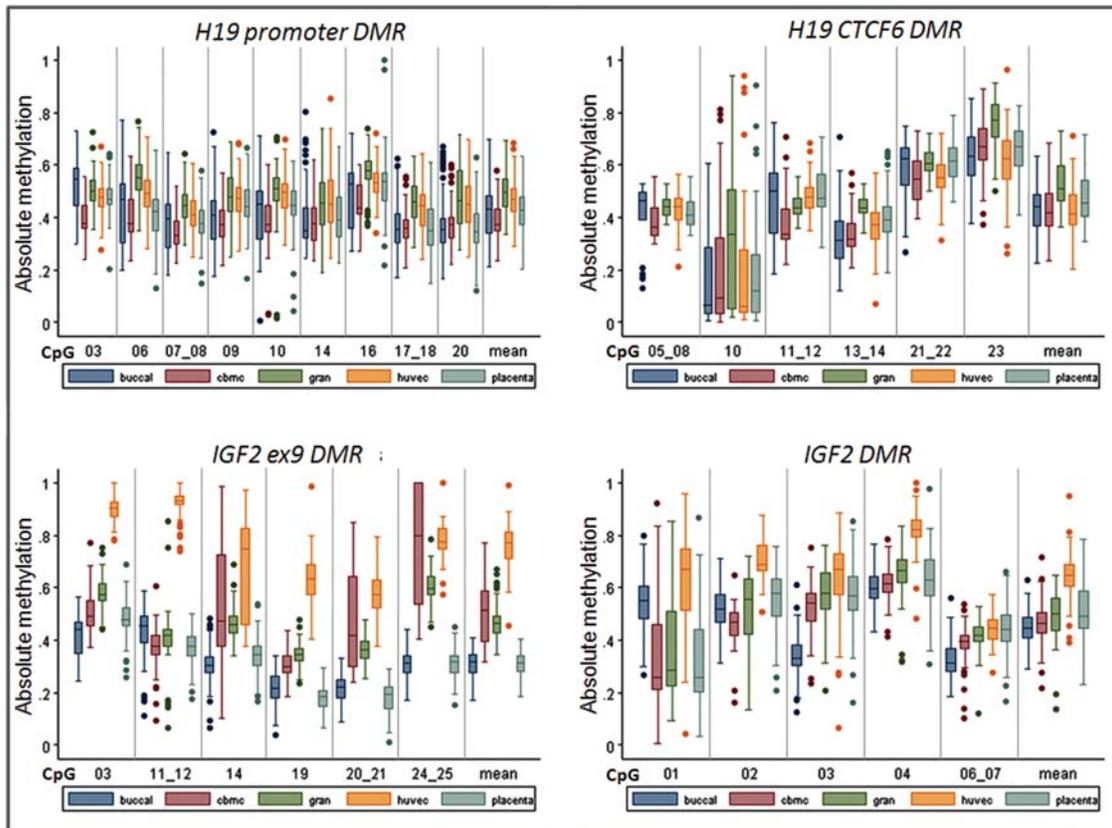


Figure 2. Methylation levels at four DMRs at the *IGF2/H19* locus. Box and whisker plots of methylation values (y -axis) of all individuals are shown for each CpG unit and DMR mean (x -axis) for each of five cell types separately. Gran, cord blood granulocytes.

In addition, CpG unit methylation correlated more within *H19*-associated DMRs and within *IGF2*-associated DMRs than between DMRs from different genes across all tissues (Fig. 3).

Newborn twin pairs show many within-pair methylation differences at specific CpG sites within DMRs

In general, the median within-pair methylation differences at individual CpG units for all DMRs were small, ranging from 3 to 4% absolute methylation difference for MZ and from 4 to 7% absolute methylation difference for DZ twin pairs (Supplementary Material, Table S5 and Fig. 4). However, the range of within-pair methylation differences varied considerably between specific CpG units. In MZ twin pairs, maximum within-pair differences in DNA methylation were 28% (*H19 promoter DMR CpG₁₄*), 52% (*H19 CTCF6 DMR CpG₁₀*), 49% (*IGF2 ex9 DMR CpG₁₄*) and 54% (*IGF2 DMR CpG₃*). In DZ twins, the maximum within-pair differences were 35% (*H19 promoter DMR CpG₆*), 82% (*H19 CTCF6 DMR CpG₁₀*), 26% (*IGF2 ex9 DMR CpG₁₄*) and 41% (*IGF2 DMR CpG₁*) (Supplementary Material, Table S5 and Fig. 4). Within-twin pair methylation differences in MZ pairs also varied between tissues, being higher in HUVECs and placenta (medians ranging from 4 to 8% and 3–9%, respectively), than in the other tissues (medians ranging from 2 to 5%).

None of the MZ or DZ twin pairs showed consistent high within-pair methylation discordances across all DMRs or tissues examined. However, for each DMR, a number of twin pairs had consistently high mean within-pair methylation differences (greater than 20%), but generally in only a single tissue. This included six twin pairs (one MZ, five DZ) for the *H19 promoter DMR*, 11 pairs (five MZ, six DZ) for the *H19 CTCF6 DMR*, four pairs (three MZ, one DZ) for the *IGF2 ex9 DMR* and three pairs (two MZ, one DZ) for the *IGF2 DMR*.

IGF2/H19-associated DMR methylation levels are genetically influenced in most (but not all) newborn tissues

We examined the evidence for a genetic contribution to methylation levels by comparing the distribution of intraclass correlation coefficient (ICC) values within DZ and MZ [regardless of chorionicity, i.e. whether they had separate placentas (dichorionic, DC) or a shared placenta (monochorionic, MC)] twin pairs. The highest median ICC values were seen for MZ twin pairs in buccal cells (0.76) and CBMCs (0.80) (Fig. 5A). Median ICC values were typically higher for MZ twin pairs relative to DZ twin pairs in buccal cells (matched-pairs signed-ranks $P < 0.001$), CBMCs ($P < 0.001$), HUVECs ($P = 0.02$) and placental cells ($P = 0.02$), whereas there was little evidence of an effect in granulocytes ($P = 0.22$) (Fig. 5A). The difference between MZ and DZ twins

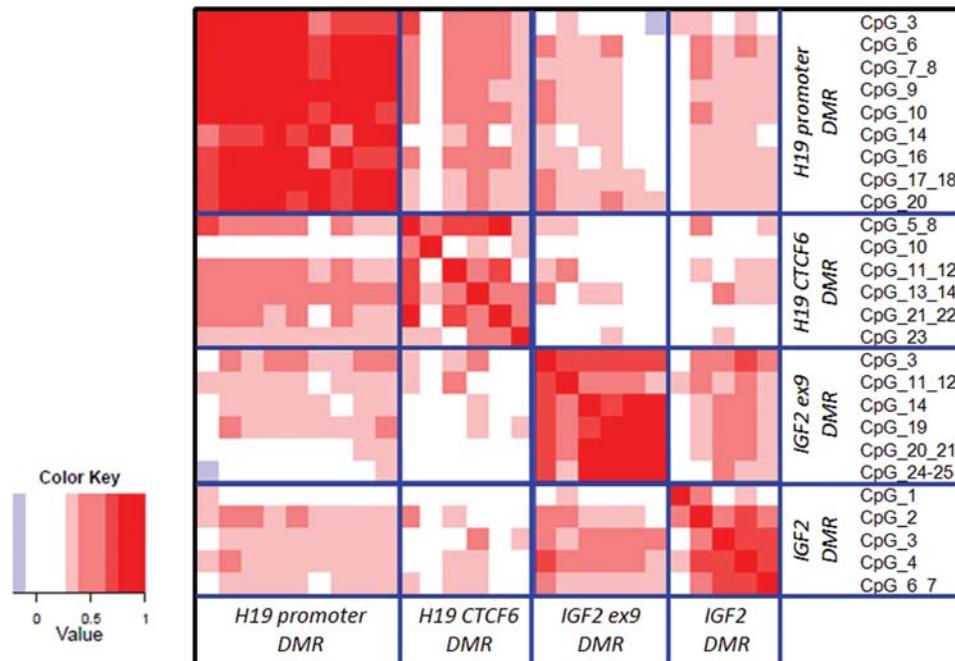


Figure 3. Correlation of DNA methylation at individual CpG units within and between all four DMRs. Spearman's correlation coefficients were calculated using data from all tissues combined. Studied DMRs and CpG units are shown on the right. Spearman's correlation coefficients for the 325 pairwise combinations of CpG units ranged from -0.21 to 0.95 (median 0.31 , interquartile range, 0.14 – 0.50). Correlation values are colour coded as shown in the colour key.

in median ICCs was most pronounced in placenta (0.48 for MZ versus 0.04 for DZ twins) and buccal tissue (0.76 for MZ versus 0.29 for DZ twins) (Fig. 5A). To investigate the effect of chorionicity on within-pair methylation differences, we calculated ICC across all CpG units, according to tissue, zygosity and chorionicity (Fig. 5B). We found some evidence for an effect of chorionicity on within-pair differences in buccal tissue ($P = 0.04$), CBMCs ($P = 0.03$) and HUVECs ($P = 0.01$). However, there was little evidence for an effect in granulocytes ($P = 0.20$) and placenta ($P = 0.20$), and there was no consistent direction to the effect across tissues. Figure 5 shows that in general the effect of chorionicity on within-pair difference in DNA methylation is small when compared with the effect of zygosity as evidenced by MZMC and MZDC twin pairs having more similar ICC distributions to each other than to DZ twin pairs. Thus, the genetic background of twins (MZ versus DZ) is a stronger determinant of within-pair methylation similarity than the number of placentas within an MZ pair.

DNA methylation levels within *IGF2/H19* DMRs are not associated with measurable phenotypes in newborn twins

As epigenetic changes associated with gene regulation are intrinsic to development, it is possible that birth weight (BW) and/or gestational age (GA) are correlated with absolute DNA methylation levels at specific loci. In order to examine this in our twin cohort, we independently examined the association between both BW [as BW standard deviation score (BWSDS) for GA, to account for variations in BW associated with early delivery] and GA with individual CpG unit and DMR mean methylation levels. Correlations were calculated

separately for each tissue type. No strong correlations were found between BWSDS and methylation level, with the Spearman correlation coefficients (ρ) across all CpGs ranging from -0.23 to 0.33 . However, mean methylation level across all CpG sites of the *H19 promoter* DMR and the *H19 CTCF6* DMR showed weak evidence of a correlation to BWSDS in CBMCs ($\rho = 0.21$, $P = 0.03$ and $\rho = 0.28$, $P = 0.003$, respectively). No strong correlation between methylation and GA was found for any CpG unit in any tissue examined (Spearman's correlations ranged from -0.41 to 0.33). To minimize the potential genetic effects on this analysis, correlations between methylation difference and BW discordance (BWD) were investigated for MZ twins only. We did not observe any strong correlations with consistent directionality between BWD and within-twin pair methylation discordance, with the Spearman correlation coefficients ranging from -0.45 to 0.58 (from 166 total correlations examined).

DISCUSSION

In this study, we have carried out an extensive analysis of DNA methylation levels in five tissues at birth from 56 MZ and 35 DZ twin pairs representing multiple germ cell layers. The key finding is that DNA methylation levels in newborns are regulated at multiple levels in response to genetic factors in combination with environmental and/or stochastic influences. The cumulative effect on methylation status varies in a tissue-dependent manner.

The *IGF2/H19* locus is the most widely studied imprinted region in mammals and is tightly regulated by a paternally methylated germline *H19* DMR (Fig. 1). It contains multiple CTCF-binding sites that facilitate or inhibit the interaction

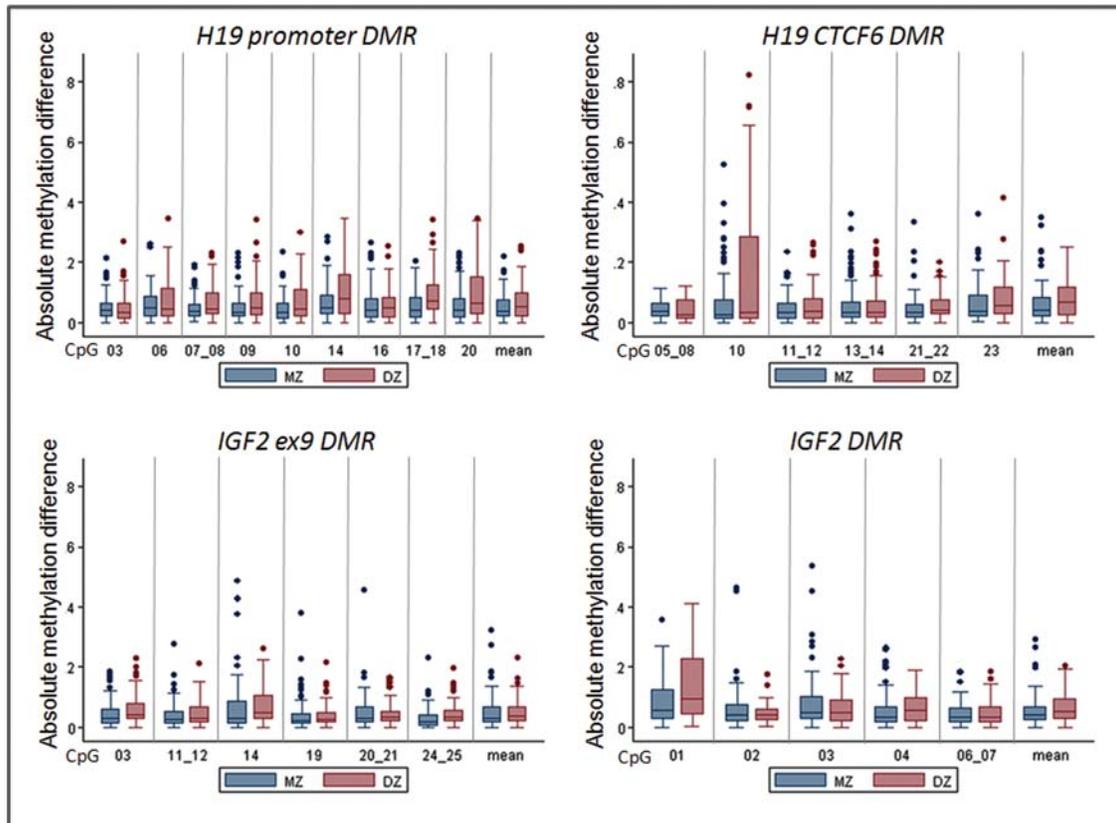


Figure 4. Within-twin pair, absolute difference in methylation values in MZ and DZ twin pairs at four DMRs at the *IGF2/H19* locus. Box and whisker plots of within-twin pair methylation discordance (y-axis) is shown in all tissues together for each CpG unit and DMR mean (x-axis). Twin pairs were divided into two groups based on zygosity (blue, MZ twin pairs; red, DZ twin pairs).

of the *IGF2* promoter with downstream enhancer elements via binding of the insulator protein CTCF to unmethylated DNA (reviewed in 63).

Methylation levels at the *IGF2* DMR have been reported to show familial clustering and to be largely stable over several decades (36,49,64,65), suggesting that any variation observed at this locus occurs earlier in development, most likely *in utero*. To date, the use of assisted reproductive technologies, alterations in one-carbon donor bioavailability and exposure *in utero* to environmental factors have all been implicated in the disruption of DNA methylation levels at the *IGF2/H19* region in humans (27–29,66). In addition, preliminary evidence for a genetic contribution to methylation at this locus has been reported (36,49,65). However, data are fragmented and often contradictory in nature, with little information available on the potential for tissue-specific sensitivities to the level of epigenetic variability established *in utero*.

Tissue-specific methylation variation at imprinted regions in newborns

As expected for an imprinted region, mean DMR methylation levels across all twin pairs and tissues varied from 44 to 50% within the *IGF2/H19* locus (Supplementary Material, Table S3). However, methylation variation fell into two broad categories. Whereas variation of mean methylation at both *H19* DMRs and at *IGF2* DMR was largely independent

of tissue of origin, there was a clear tissue-specific dependence of methylation levels at the *IGF2 ex 9* DMR (Fig. 2 and Supplementary Material, Table S3). Methylation of cord blood-derived cells at *IGF2 ex9* DMR was comparable with that previously reported for peripheral blood leucocytes (67,68) and whole cord blood (53), and lower placental methylation at this locus was also consistent with previous findings (53). Our findings therefore support previous data, indicating that the *IGF2 ex9* DMR is a somatic rather than germline DMR with methylation levels established post-zygotically (58,69). These data also support the recent findings of highly variable tissue-specific methylation levels at imprinting associated DMRs (70). In contrast to a previous report of a lack of methylation at the *H19* promoter in placenta (53), we found a pattern of methylation typical of germline imprinting. The CpG units we studied and those of Guo *et al.* (53) are different, although located at the same *H19* promoter, indicating the importance of individual CpG sites in the regulation of imprinting at this locus.

Median methylation values in the *H19 CTCF6* DMR were very similar across all tissues, supporting an important functional role of this region and suggesting a functional constraint on the ‘allowable’ level of methylation variation at this locus. Furthermore, CpG_5_8 within this region, which contains the binding site for the CTCF insulating factor, was among the least variable CpG units of all those tested, across all tissues (Fig. 2 and Supplementary Material, Table S3).

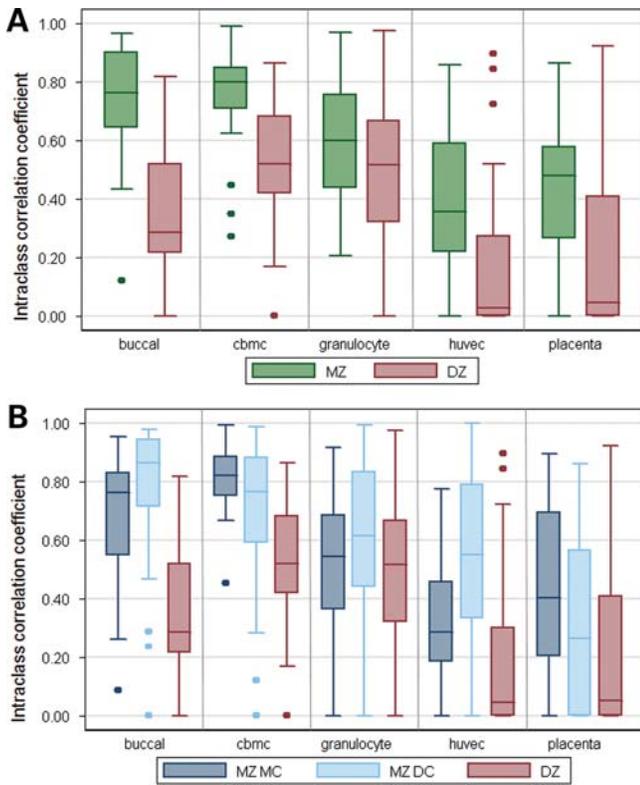


Figure 5. Effect of (A) zygosity and (B) chorionicity on DNA methylation values at the *IGF2/H19* locus. ICC was used as a measure of within-twin pair methylation and was calculated for each cell type separately for all three groups of twins. ICC values are shown (y-axis) in all DMRs together, separately for each cell type (x-axis).

Methylation values at individual CpG units within the *IGF2/H19* DMRs were positively correlated, more so within than between the two genes. However, the strength of correlation varied markedly between tissues highlighting the tissue variability in absolute methylation at each of the four DMRs and the tissue-specific roles of some of those DMRs as mentioned earlier. Correlation of methylation of CpG units between DMRs again implies coordinated regulation at the *IGF2/H19* locus and, mechanistically, could reflect higher-order chromatin folding observed within the region (59,71).

Multiple examples of within-twin pair differences in DNA methylation highlight the importance of the intrauterine milieu for epigenome establishment

Methylation differences within twin pairs were generally small, with median absolute differences for DMR means ranging from 3 to 4% in MZ and 4 to 7% in DZ twin pairs. However, the absolute mean within-twin pair discordance at *IGF2 DMR* (6%) was twice as high as what was previously reported for the same DMR when comparing periconceptual famine-exposed individuals to their unexposed same-sex siblings (2.7%) (27). In addition, when looking at individual CpG units, we found methylation discordances of up to 82% in DZ twin pairs and up to 54% in MZ twin pairs. Such extreme variation highlights the potential for differences in intrauterine environment between individuals of the same

MZ twin pair (whether sharing or having separate placentas) to dramatically alter the developing epigenetic profile of one or both twins, without any known environmental exposure, such as famine, during the prenatal development.

Tissue-specific effects were also observed for within-pair methylation discordance across all DMRs. HUVECs and placental cells showed the highest median within-pair methylation discordances across all DMRs (Supplementary Material, Table S5). One might speculate that both placenta and HUVECs are more susceptible to environmental effects than the other cell types studied here, based on their role in shielding the developing pregnancy from adverse maternal circulating factors. Despite the overall small median within-pair methylation differences at each DMR as a whole, individual CpG units showed large variation depending on the tissue type (Supplementary Material, Table S5). Taken together, these findings warrant a wider, genome-scale examination of potential changes in DNA methylation profile induced during the intrauterine development of MZ twins.

Interestingly, the *H19 CTCF6 DMR* CpG_5_8 also showed the lowest variation in within-pair methylation discordances across all tissues (ranging from 0.00 to 0.11), highlighting the crucial role of these CpGs in the control of imprinting at this locus. In combination with the data above, this suggests that the CTCF regulatory binding site at the *H19* DMR shows functionally constrained variation in methylation levels at birth.

Evidence for a genetic component to methylation level at imprinted dmrs

We found higher ICC values for MZ compared with DZ twin pairs in four out of five tissues tested. This suggests a genetic contribution to overall methylation level at *IGF2/H19* DMRs, consistent with previous data obtained in whole blood from a large number of adolescent and middle-aged twins (49,72). This genetic contribution, reflecting the heritability of methylation levels at the studied DMRs, might be due to genetic differences elsewhere in the studied locus, in a close proximity to it, or elsewhere in the genome. However, as with all twin studies of this nature, estimating heritability by fitting a standard mixed model to twin pairs, which assumes an equal level of environmental variation between MZ and DZ counterparts, may not be valid in all instances (73). Possible sources of environmental variation between classes of twins (independent of genetics) include the biological mechanism of twin splitting and nature of placentation, e.g. size, morphology and number of placentas.

Interestingly, ICCs observed in our study for different tissue types obtained at birth indicates that the impact of genetic variation may not be equal across tissues and CpG units. Also, environmental and stochastic factors are most likely to contribute to the methylation discordances observed here, but in the majority of instances this represents a smaller effect than underlying genetics.

In contrast to zygosity, the statistical evidence for an effect of chorionicity on DNA methylation variation in MZ twin was weaker and in opposite directions in different tissues. We conclude that, at least with respect to the *IGF2/H19* locus, sharing a placenta does not always result in a more similar epigenotype

and that conclusions about the effect of chorionicity on DNA methylation in general cannot be made from a single tissue.

Absolute methylation levels at the IGF2/H19 locus and newborn characteristics

Many imprinted genes are implicated in the regulation of fetal growth and development (74) and have also been postulated to be especially sensitive to environmentally mediated changes in DNA methylation (27,75,76). In mice, *Igf2* and *H19* are implicated in placental development and regulation of fetal growth (74,77,78). Low BW is commonly considered a marker of compromised prenatal development in human studies. In contrast to the previous findings obtained by the analysis of whole blood (29), we did not identify an association between the *IGF2* DMR methylation level (or any other DMR tested) with BW, in any tissue tested. This discrepancy may reflect the relatively small sample sizes of both studies or tissue-specific differences in methylation at this locus identified in the current study. Interestingly, our data do support a similar general lack of association between DMR methylation levels and BW obtained in a large study of 10 individual DMRs in chorion and amnion tissue from several hundred pregnancies (70).

Conclusions and limitations

Our study has a number of strengths. Our unique perinatal twin cohort allows us to investigate the genetic basis of DNA methylation through a comparison of MZ versus DZ twin pair methylation variation (controlled for maternal and shared obstetric factors). Within-pair comparisons of MZ twins (controlled for genetic factors) meant we could also examine the contribution of intrauterine environmental and stochastic factors to neonatal methylation. Unlike previous studies in humans, we have measured methylation in five tissue types allowing us to describe how methylation varies across tissues and unrelated individuals and within DZ and MZ twin pairs. We also studied four different DMRs within a single imprinted region, whereas others have previously looked at only one or two such DMRs (27,49). Our methylation data have undergone a stringent quality control process, including technical replication of each data point to reduce the effect of measurement error and genotyping of individuals to ensure that methylation values are not distorted by SNPs that disrupt potential sites of CpG methylation.

Given that the methylation levels at germline DMRs are by definition established during gametogenesis, any differential methylation detected during post-zygotic development either represents a loss of fidelity in transferring this methylation profile to daughter cells during cell division, or indicates that these DMRs have their methylation level controlled as part of a tissue-specific developmental programme. In contrast to germline DMRs, factors contributing to tissue-specific DMR differences at birth are less clear given the current state of knowledge regarding the regulation of this locus and its postulated role in regulating fetal growth and development (53,79–81).

Despite the obvious advantages afforded by studying twins, there are also caveats that need to be considered in data interpretation. We studied cord blood which may be partially

shared at least between MZMC twin pairs. Although none of the twin pairs in this study had twin to twin transfusion syndrome, caused by excessive blood sharing, the exact degree of sharing cannot be ascertained and therefore any confounding of within-pair comparisons cannot be measured or corrected for. However, several factors make us confident that this was not a major problem in the current study. CBMCs and granulocytes have similar ICCs to other tissues (Fig. 5) and have similar ranges of within-pair differences in methylation (Supplementary Material, Table S5).

A second potential limitation to our study is the use of tissues containing heterogeneous cell types in which each cell type could contribute to the observed levels of variation in our study population or within specific twin pairs. However, we found equal (or higher) levels of methylation variation in HUVECs, highly homogeneous CD31+ purified cells, than in other cell types. The examination of germline DMRs is also an advantage in mixed cell populations, as methylation levels are established prior to conception and show little cell-specific variability.

Our study adds to the growing body of literature supporting the emergence of epigenetic differences between genetically identical individuals early in life, possibly explaining much of the observed phenotypic variation seen in MZ twins, including the potential modulation of risk associated with development of complex human diseases (15,43,48,82–90). We have evidence that the intrauterine period is a sensitive time for the establishment of epigenetic variability in humans, with implications for the effects of maternal environment in addition to genetics on the development of the newborn epigenome and potentially for programming of later disease risk.

MATERIALS AND METHODS

Sample collection

All twin pairs included in this study were delivered at the Royal Women's Hospital, Monash Medical Centre or Mercy Hospital for Women (Melbourne, Australia). Written consent was obtained at recruitment during the second trimester of pregnancy and all procedures were approved by the appropriate ethics committees. Placentation was recorded via ultrasound at least twice during pregnancy and again by placental examination at delivery. For a small number of discrepancies between chorionicity calls, the full set of ultrasound scans was reviewed. Data on sex and BW were collected postnatally (Table 1 and Supplementary Material, Table S2). All DC same-sex twin pairs were genotyped by a panel of 12 microsatellite polymorphisms (Australian Genome Research Facility, Melbourne, VIC, Australia) to confirm zygosity. Ambiguous or partially failed zygosity test results were repeated. All MC twin pairs were assumed to be MZ. Tissues were collected at birth from a representative sample of 56 MZ and 35 same-sex DZ twin pairs selected from our larger Perinatal Epigenetic Twin Study (manuscript in preparation). DZ twin pairs were matched for GA and gender to MZ twin pairs where possible (Table 1 and Supplementary Material, Table S2). Umbilical cords, cord blood, placental tissue and cheek cells were collected using established protocols.

Table 1. Characteristics of the study population

	MZ	DZ
Chorionicity		
DC	19 (34%)	35
MC	37 (66%)	—
Gender		
Female	31 (55%)	16 (46%)
Male	25 (45%)	19 (54%)
GA (weeks)		
Median	36.29	37.29
SD	2.02	5.20
Min	28.29	29.86
Max	38.43	40.00
BWSDS		
Median	-0.82	-0.81
SD	0.74	0.82
Min	-2.54	-3.41
Max	1.03	2.04
BWD (%)		
Median	9.60	9.38
SD	9.36	8.90
Min	0.40	0.00
Max	41.60	43.31
Number of twin pairs by each cell type		
Buccal	40	25
CBMC	30	26
Gran	27	22
HUVEC	31	27
Placenta	23	25

A total of 182 newborn twins (91 pairs) were included in the study. This consisted of 56 MZ and 35 DZ twin pairs. The DZ twin pairs were selected to closely represent the MZ twin pair group. BWSDS was used as a measure of birth size to allow variation in length of gestation and the sex of the baby. BWD was calculated as (heavier twin–lighter twin)/heavier twin. DC, dichorionic; MC, monochorionic; Gran, cord blood granulocytes.

HUVECs were extracted from near full length umbilical cords as previously described (91). Briefly, type 2 collagenase (1 mg/ml, Worthington Biochemical Corporation, Lakewood, NJ, USA) was used to detach endothelial cells which were then further purified using CD31 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. CBMCs and granulocytes were isolated from cord blood by Ficoll gradient centrifugation (Ficoll-Paque Plus, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in Leucosep® tubes (Greiner Bio-One North America, Monroe, NC, USA). Collected fractions were further purified by ammonium chloride erythrocyte lysis. CBMCs, granulocytes and HUVECs were viably frozen in 10% DMSO and stored in liquid nitrogen for future use. Buccal cells were collected with Catch-all Sample Collection Swabs (EPICENTRE Biotechnologies, Madison, WI, USA) which were stored in -20°C until DNA extraction. Full thickness placental tissue was obtained from cotyledon cross-sections. This was washed twice in PBS and blotted dry prior to storage at -70°C for future use.

DNA extraction and bisulphite conversion

All tissue samples except buccal cells were incubated overnight at 50°C with shaking in DNA extraction buffer

(100 mM NaCl, 10 mM Tris–HCl pH8, 25 mM EDTA, 0.5% SDS), containing 200 $\mu\text{g/ml}$ Proteinase K. DNA was extracted in each cell type with a standard phenol:chloroform method and precipitated with absolute ethanol with 10% sodium acetate (3 M, pH 5.2). DNA pellets were washed with 70% ethanol and dissolved in $1\times$ TE buffer. Prior to DNA extraction, buccal cells were lysed by incubating the cells in SE buffer (0.75 M NaCl, 25 mM EDTA, pH 8.0) with 50 $\mu\text{g}/\mu\text{l}$ Proteinase K and 1% SDS at 55°C o/n. Proteins were precipitated with 50% 5 M sodium chloride. DNA was precipitated with two volumes of absolute ethanol, pellets washed with 70% ethanol and dissolved in $1\times$ TE buffer. A total of 500 ng to 1 μg of genomic DNA was bisulphite converted using the MethylEasy Xceed Rapid Bisulphite Modification Kit (Human Genetic Signatures, North Ryde, NSW, Australia), according to manufacturers' instructions. All samples within individual twin pairs were processed in parallel to ensure consistency of treatment.

PCR amplification

Primers for both *H19* DMRs and *IGF2 ex9* DMR were designed using SEQUENOM's EpiDesigner BETA (SEQUENOM Inc., Herston, QLD, Australia) tool (www.epidesigner.com) and the primers for *IGF2* DMR were obtained from a previous publication (49). To reduce methylation variability introduced during PCR (61), at least two replicate amplifications were performed in all instances. PCR amplification was performed in 15 μl reaction containing 10 ng bisulphite converted DNA, $1\times$ FastStart PCR Master mix (Roche Diagnostics Australia Pty Ltd, Castle Hill, NSW Australia) and 3 μM each of forward and reverse primers (see Supplementary Material, Table S1). The PCR cycling conditions were 95°C 10 min, followed by five cycles of 95°C 20 s, 56°C 30 s, 72°C 2 min and additional 40 cycles of 95°C 20 s, 60°C 30 s, 72°C 2 min and a final extension at 72°C 10 min.

DNA methylation measurement by sequenom MassARRAY epityping and associated data cleaning

DNA methylation levels were determined as previously described (92–95) using MassARRAY EpiTYPER (SEQUENOM Inc., Herston, QLD, Australia) system which is based on MALDI-TOF MS (60). In short, bisulphite-converted DNA was amplified with primers containing a T7-promoter tag and the amplification products cleaned with shrimp alkaline phosphatase (SEQUENOM). After purification *in vitro* transcription and T-specific cleavage was performed. Each reaction was then spotted onto a Maldimatrix-containing SpectroCHIPS (SEQUENOM) and subjected to MALDI-TOF MS. The mass spectra was collected by MassARRAY Spectrometer and analysed by EpiTYPER v.1.0 software (SEQUENOM).

A stringent quality control process was employed to remove potentially unreliable measurements prior to analysis. First, all data from CpG-containing fragments flagged by EpiTYPER as having low mass (outside MS analytical window), high mass (outside MS analytical window) or silent peak overlap (two overlapping peaks, one with no CpGs, following MS) were discarded. Secondly, CpG analytic units that failed to

produce data for >30% of samples were also discarded (unreliable CpG unit), and samples with more than 30% missing data points within an amplicon (unreliable sample) had all methylation values for that sample set to missing. Finally, technical replicates showing $\geq 5\%$ absolute difference from the median value of a set of technical replicates were set to missing and only samples with at least two successful technical replicates were analysed. Study individuals were genotyped for all known SNPs in the regions contained within assays. Methylation values at some CpGs with an overlapping SNP showed bimodal distribution of methylation (Supplementary Material, Fig. S2) with methylation values close to 100% (maternal allele with a non-CpG genotype) or 0% (paternal allele with a non-CpG genotype) in heterozygous individuals. Where an 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.

DNA methylation measurement by bisulphite sequencing

In order to confirm that both parental alleles are represented in the data generated by Sequenom EpiTYPER analysis, we carried out limited bisulphite DNA sequencing of a subset of amplification products used in EpiTYPER analysis as previously described (92,95). Up to 24 clones from individual samples were tested.

Statistical analysis

All analyses were performed using the mean of at least two technical replicates. Some analyses were performed using data from individual CpG analytic units, whereas other analyses were performed using the average methylation value across individual DMR assays; such averages were calculated for samples with >50% of all data points (CpG units) within the DMR after the data cleaning steps.

Association between methylation at different CpG units, and between methylation and BW or methylation and GA, was measured using Spearman's rank correlation coefficient (ρ). A Spearman correlation is calculated using the ranks of values, rather than the values themselves. Correlations between within-twin pair methylation difference and BW discordance were also summarized in this manner.

The contribution of genetic factors outside the *IGF2/H19* DMRs to methylation levels was investigated by fitting separate linear mixed models for MZ and DZ twin pairs at each CpG unit. Within-twin pair correlation was modelled by including a random intercept specific to each twin pair; the models had no other covariates and were fitted using restricted maximum likelihood. This model can be thought of as an extension to linear regression which decomposes the total variance into 'between-twin pair' and residual ('within-twin pair') components. The proportion of total variance attributable to 'between-twin pair' variation is known as the ICC. Within the different twin classes tested, the ICC estimates the proportion of total variance attributable to 'between-twin pair' variation. It estimates the degree of correlation between methylation values at a CpG unit for individuals in the same twin pair. If methylation is influenced by genetic factors, we would expect the ICC to be higher for MZ twin pairs com-

pared with DZ twin pairs; the distribution of ICC values obtained across all CpG units was compared between MZ and DZ twin pairs using the Wilcoxon signed-ranks test.

This process was extended by fitting separate linear mixed models for MZ twin pairs according to their chorionicity (MC or DC), allowing the calculation of separate ICCs for the two groups, which were then compared using the Wilcoxon signed-ranks test. To examine whether mean methylation levels differ between tissues, the mean methylation value of each DMR was regressed separately upon tissue type (modelled as indicator variables) in a linear mixed model. Correlation within twin pairs was modelled using twin model parametrization 2 (96) which accounts for the fact that MZ twin pairs share more genetic material than DZ twin pairs.

BWSDSs were calculated for each newborn using the Microsoft Excel add-in LMSgrowth (version 2.64) taking into account the GA. Data processing and analyses were performed using the statistical packages (R, <http://cran.r-project.org/>) and Stata (97).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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