Pervasive polymorphic imprinted methylation in the human placenta

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Abstract

The maternal and paternal copies of the genome are both required for mammalian development and this is primarily due to imprinted genes, those that are mono-allelically expressed based on parent-of-origin. Typically, this pattern of expression is regulated by differentially methylated regions (DMRs) that are established in the germline and maintained after fertilisation. There are a large number of germline DMRs that have not yet been associated with imprinting and their function in development is unknown. In this study, we developed a genome-wide approach to identify novel imprinted DMRs in the human placenta, and investigated the dynamics of these imprinted DMRs during development in somatic and extra-embryonic tissues. DNA methylation was evaluated using the Illumina HumanMethylation450 array in 134 human tissue samples, publically available reduced representation bisulfite sequencing in the human embryo and germ cells, and targeted bisulfite sequencing in term placentas. 43 known and 101 novel imprinted DMRs were identified in the human placenta, by comparing methylation between diandric and digynic triploid conceptions in addition to female and male gametes. 72 novel DMRs showed a pattern consistent with placental-specific imprinting and this mono-allelic methylation was entirely maternal in origin. Strikingly, these DMRs exhibited polymorphic imprinted methylation between placental samples. These data suggest that imprinting in human development is far more extensive and dynamic than previously reported and that the placenta preferentially maintains maternal germline-derived DNA methylation.

Introduction

The two haploid copies of the genome are not interchangeable, as both a maternally and paternally inherited copy is required for mammalian development. This is primarily due to imprinted genes, those that are expressed from a single allele based on parent-of-origin. This class of genes is particularly important for foetal and placental development (Frost and Moore 2010).

The mono-allelic expression of imprinted genes is controlled by differentially methylated regions (DMRs) that are established either as 'primary' (germline) imprints in the female or male gametes or as 'secondary' (somatic) imprints in embryonic development. Imprinted genes can occur in isolation, but are often found in centrally-regulated clusters. Imprinted DMRs are all CpG rich regions; however, paternal DMRs tend to be inter-genic while maternal DMRs are located at promoters or intra-genic regions (Kobayashi et al. 2006; Schulz et al. 2010).

Primary imprinted DMRs are erased and re-established during germline development of each generation dependent on the sex of the embryo (Kelsey and Feil 2013). Shortly after germ cell specification in the embryo there is a genome-wide removal of DNA methylation, thereby erasing all prior parentally-inherited imprints (Seisenberger et al. 2012). Methylation is then re-acquired in spermatocytes around the time of birth in males, while in females DNA methylation is not fully established until oocyte growth in adulthood (Henckel et al. 2012; Lucifero et al. 2004). Once established, imprints are then passed on to offspring upon formation of the zygote and are maintained through the dynamic epigenetic reprogramming in the early embryo. Our current knowledge about the mechanisms and dynamics of imprinting has been determined almost exclusively using mouse models; however, evidence from human studies suggests that these processes are largely conserved (Guo et al. 2014; Ishida and Moore 2013).

Currently, 151 imprinted genes have been identified in mouse (www.mousebook.org), while there appears to be less extensive imprinting in humans, with only 78 genes identified

(http://icg.otago.ac.nz). Despite this difference, the majority of the well-studied imprinted clusters are

conserved in their expression, methylation patterns and synteny (Onyango et al. 2000). In both mice

and humans, a large number of DMRs of unknown function have been reported between sperm and

oocyte (Guo et al. 2014; Smallwood et al. 2011), which raises the question as to whether imprinting is

more extensive in the genome than previously reported.

Over the past decade, multiple approaches to identify novel imprinted genes in the human

genome have been utilized including comparative methodologies for genomic features (Luedi et al.

2007), gene expression (Metsalu et al. 2014; Babak et al. 2015), and DNA methylation (Yuen et al.

2011a; Court et al. 2014; Choufani et al. 2011; Docherty et al. 2014; Das et al. 2013; Stelzer et al. 2013)

in various tissues. Importantly, these studies have resulted in the discovery and validation of many

imprinted genes; however, the candidate lists are often not replicated between studies. Until recently,

the genome-wide pattern of DNA methylation in human oocytes was not known (Guo et al. 2014),

preventing the definitive identification of novel primary imprints in humans. In this study, we describe

the dynamics of known imprinted DMRs throughout human development in somatic and extra-

embryonic tissues and identify novel germline imprinted DMRs utilizing gametic DNA methylation in

combination with a validated approach of comparing placental samples with genomic imbalances (Yuen

et al. 2011a).

Results

Characterisation of known imprinted DMRs in human development

To evaluate imprinting in the human placenta, we identified DMRs (≥3 CpGs within 500bp)

between 5 diandric (2 paternal copies of the genome and 1 maternal) and 5 digynic (2 maternal copies

and 1 paternal) triploid placentas, measured by the Illumina HumanMethylation450 BeadChip array

(450K array)(Figure 1A). 882 candidate DMRs, encompassing 6808 probes (CpG sites), were identified

(Supplementary table 1), including 43 that overlapped previously reported human imprinted DMRs

(Court et al. 2014) (Supplementary table 2).

DNA methylation at these 43 known DMRs was assessed using the 450K array in foetal and

extra-embryonic tissues and using publically available reduced representation bisulfite sequencing

(RRBS) in human gametes and embryos (Guo et al. 2014). 37 DMRs were informative for germline DNA

methylation and, expectedly, the majority showed >50% difference in DNA methylation between germ

cells, including 2 paternal and 32 maternal germline DMRs (Figure 1B). The remaining 3 DMRs (*MEG3*,

ZNF597, and *GNAS* DMR1) were unmethylated both in germ cells and the early embryo, suggesting

these are somatic DMRs (Figure 1B). Among the known DMRs, 15 are placental-specific in humans

(Court et al. 2014) and all showed differential methylation between oocytes and sperm, with a mean

difference of 67.5% (Figure 1B). These placental-specific DMRs are intermediately methylated in both

the inner cell mass (ICM) (mean 35.6%) and trophectoderm (TE) (34.1%) of the blastocyst, suggesting

that they are transiently imprinted in embryo, not just extra-embryonic TE (Figure 1B).

Identification of novel imprinted placental DMRs

We sought to identify novel imprinted DMRs by identifying CpG islands (CGIs) that showed a

profile consistent with imprinting in early human development: DMRs that were both differentially

methylated in the germline and between triploid placental samples. Firstly, CGIs that were differentially

methylated in the germline and intermediately methylated in blastocysts were identified (specific

criteria detailed in the methods), which included 1222 oocyte-specific and 288 sperm-specific DMRs

(Figure 2A). Of these candidate CGIs, 101 novel regions overlapped with the 882 DMRs identified in

triploid placental villous samples (Figure 2B). All 101 novel DMRs derived methylation from the oocyte

and the majority (N=72) showed a placental-specific pattern of imprinting (Figure 2C).

To confirm that the novel DMRs maintain imprinted allele-specific methylation throughout

development, allelic methylation was evaluated in oocytes, sperm, ICM and TE for known and novel

DMRs. This reaffirmed ~50% of DNA strands in the ICM and TE were partially/fully methylated and the

proportion of methylated alleles was not significantly different between known and novel DMRs (Figure

3A). It can be inferred that these methylated alleles were almost entirely derived from the oocyte

(Figure 3A), because it is assumed that there is almost no *de novo* methylation in the preimplantation

embryo (Seisenberger et al. 2013).

In placenta, allelic DNA methylation was assayed at known and novel DMRs using multiplexed

bisulfite sequencing (Supplementary table 5) in 16 term placental villi samples with matched isolated

trophoblast (N=12) and parental blood (N=20). 102 DMRs (32 known and 70 novel) were successfully

assayed, revealing a bimodal distribution of read methylation in term placenta and trophoblast at these

loci (Figure 3B), consistent with that in the ICM and TE (Supplementary figure 1). This bimodal

methylation pattern is indicative of mono-allelic methylation, as opposed to heterogeneous CpG

methylation expected at non-imprinted intermediately methylated loci. DMRs that contained

heterozygous SNPs showed that indeed this bimodal methylation pattern was attributable to mono-

allelic methylation (Figure 3C). Furthermore, DMRs with informative parental origin showed that mono-

allelic methylation was entirely maternally derived, with the exception of paternally methylated *H19*

(Figure 3C).

Polymorphic imprinted methylation in the human placenta

Strikingly, methylation across the novel DMRs was highly polymorphic, with 50% of

heterozygous samples demonstrating mono-allelic methylation and the remaining showing a complete

loss of imprinting at these loci (Figure 3C). The frequency of polymorphic imprinting was similar in

placental villi (50.0%) and trophoblast (49.4%) (Supplementary table 3) and allelic methylation was

highly correlated between matched trophoblast and whole villi from the same individual (N=34, R^2=0.73,

p<0.0001, Spearman Rank correlation). This suggests that the pattern of polymorphic imprinting is not

attributable to differences in cell composition. Polymorphic methylation was confirmed among the 63

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placental samples run on the 450K array (Figure 4A), which included 4 samples assayed by multiplexed bisulfite sequencing. Using hierarchical clustering, the samples did not cluster by gestational age, suggesting polymorphic methylation across these loci is also not gestational age dependent. Furthermore, polymorphic imprinting at these DMRs was placental-specific, as somatic tissues showed significantly less variability than placenta (p<2.2E-16) (Figure 4B). These data suggest that polymorphic imprinting is pervasive in the human placenta.

The placental-specific imprinted genes reported to date in human and mouse are not well-conserved (Frost and Moore 2010), therefore we evaluated the DNA methylation of orthologous regions to placental-specific DMRs in mouse development using publically available data (Smith et al. 2014; Hon et al. 2013; Smith et al. 2012). Only 6 DMRs showed differential germline DNA methylation in mouse gametes, but only one locus, orthologous to *NAV2*, showed a pattern consistent with placental-specific imprinting (Supplementary figure 2).

Timing of loss of imprinting in human embryonic lineages

To assess the developmental timing of loss of imprinting at placental-specific imprinted DMRs in somatic lineages, we took advantage of the methylation patterns observed in isolated extra-embryonic cell types. The various cells contributing to the human placenta vary in the timing of differentiation, and also the degree to which they comprise cells from embryonic origins. As extra-embryonic cell types progressively originate later in development, they are closer in origin to the embryo (Figure 5A). We found that the trophoblast and mesenchymal core of the placental villi showed the expected intermediate methylation at known placental-specific DMRs (51.3% and 46.5%, respectively). This supports that these DMRs are still imprinted in the TE and ICM after the blastocyst stage, while trophoblast originates entirely from the TE, mesenchyme is largely derived from the ICM-derived extra-embryonic mesoderm (Figure 5A). Contrastingly, the average methylation was lower in chorion (33.0%)

and amnion (8.3%) (Figure 5B), which both mostly originate later from the extra-embryonic mesoderm

(Figure 5A).

The exact developmental timing and origin of amnion and chorion are not firmly established. In

mouse and likely human, chorion is derived from both the TE and extra-embryonic mesoderm (Downs

2011; Pereira et al. 2011); while molecular data in human suggest that there may also be a later

contribution from epiblast, prior to primitive streak formation at day 15, to both chorion and amnion

(Penaherrera et al. 2012; Robinson et al. 2002). The relative loss of DNA methylation observed in

chorion and amnion compared to placental villi suggests that imprinting at placental-specific DMRs in

the embryonic lineages has already begun to be lost at the time of primitive streak formation (Figure

5B). Thus it appears that these loci are either passively or actively losing methylation from the maternal

allele, while much of the genome is acquiring *de novo* DNA methylation concomitant with somatic

differentiation (Smith et al. 2012).

Characteristics and function of placental-specific DMRs

The mono-allelic methylation of placental-specific DMRs (16 known and 72 novel) may be

attributable to targeted protection of these regions or that these regions are a vestige of embryonic

methylation patterns. To evaluate this, we first assessed whether there was evidence of *de novo*

methylation in the post-implantation placenta. 1352 CGIs were fully methylated in both oocyte and

sperm, and are therefore not imprinted, and retained intermediate DNA methylation in the ICM and TE.

In somatic tissues, almost all of these CGIs undergo *de novo* methylation to become fully methylated

(83.2%) and this was similarly observed in the placenta (70.1%) (Supplementary figure 3A), suggesting

the placenta exhibits a phase of post-implantation *de novo* DNA methylation. In addition, the majority

of placental-specific DMRs (80.7%) were not associated with previously described large partially

methylated domains characteristic to the placenta (Schroeder et al. 2013) (Supplementary figure 3B).

Together, these findings suggest that placental-specific DMRs may be specifically protected from loss or gain of methylation.

Zinc finger protein 57 (ZFP57) and di/tri-methylation of histone 3 lysine 9 (H3K9me2/3) have been shown to protect imprinted DMRs from demethylation during embryonic reprogramming in mouse (Li et al. 2008; Nakamura et al. 2012) and thus may be important to facilitate the protection of placental-specific imprinting in humans. We observed a slightly higher proportion of placental-specific DMRs (62.9%) with at least one ZFP57 consensus sequence (TGCCGC) (Quenneville et al. 2011) than CGIs genome-wide (51.5%) (p=0.04), which was comparable to known imprinted DMRs (70.8%) (p=0.63) (Figure 6A). Placental-specific DMRs were also enriched for H3K9me3 in placenta compared to somatic tissues (muscle, p=2.2E-16 and amnion, p=2.2E-16) (Figure 6B and Supplementary figure 4). However, this enrichment was not a unique feature, as all CGIs were enriched for H3K9me3 in placenta compared to somatic tissue (muscle, p=2.2E-16 and amnion, p=3.1E-14) (Figure 6B and Supplementary figure 4). These findings suggest that ZFP57 and H3K9me3 may, at least partially, be important for the maintenance of mono-allelic DNA methylation at placental-specific DMRs; however, this is likely not a specific mechanism.

To investigate the potential role for placental-specific imprinting, gene expression in placenta was assessed using publically available RNA-seq datasets (Metsalu et al. 2014; Yan et al. 2013; Bernstein et al. 2010). 50.0% of the placental-specific DMRs had an associated gene that was expressed in at least one developmental stage (RPKM>0.1) (Figure 6C, Supplementary table 6). However, gene ontology analysis revealed no enrichment for pathways typically associated with imprinted genes, such as foetal growth or placentation. The top gene ontology terms included alternative splicing, expression in the brain, splice variants, and the extracellular region (Supplementary Table 7).

Discussion

In this study, DNA methylation in human gametes and triploid placental villi was used to identify germline imprinted DMRs. We found 43 imprinted DMRs in placenta that overlapped with previously reported imprinted DMRs initially identified in somatic tissues (Court et al. 2014) and 34 of these were confirmed to be germline imprints. Fifteen DMRs reported by Court et al were not identified in this study; notably, 10 of these did not show germline differential methylation and therefore may be secondary DMRs that are not fully established in placenta. 15 known placental-specific DMRs did show differential germline methylation. This is supported by a similar finding in an independent study of oocyte DNA methylation (Okae et al. 2014); however, these findings contradict a previous report suggesting that these regions are unmethylated in human gametes (Court et al. 2014). This discrepancy may be explained by the use of parthenogenetic embryonic stem cells as a proxy for the oocyte methylome in that study (Court et al. 2014).

Recently, it was shown that many regions of the genome are differentially methylated between oocyte and sperm beyond the widely-studied imprinted DMRs (Smallwood et al. 2011; Guo et al. 2014; Kobayashi et al. 2012) and the importance of this DNA methylation during development has been unclear to date. We have determined that some of the additional differential methylation in the human germline results in more widespread imprinting, specifically in the placenta. We identified 101 novel maternal germline imprinted DMRs in human placenta and found that 72 were placenta-specific. The mono-allelic DNA methylation at these DMRs was validated by targeted multiplexed bisulfite sequencing in placenta and trophoblast, confirming maternally-derived methylation in all informative cases.

While the importance of imprinting in placental development has been well-established (Varmuza and Miri 2015), the role of placental-specific imprinting has not been fully explored. Consistent with previously reported placental-specific imprinted genes (Frost and Moore 2010), the human placental-specific DMRs we identified were not conserved in mouse. This is somewhat

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unsurprising, because although imprinting is quite robustly conserved across eutherian mammals

(Renfree et al. 2013), many of the additional germline DMRs identified thus far have not been conserved

in methylation state across species (Smith et al. 2014). There has not been a genome-wide investigation

of placental-specific imprinting in many other species and therefore the extent and role of placental-

specific imprinting in eutherian mammals is unclear.

All 101 novel DMRs we identified had maternally inherited DNA methylation, raising the

number of maternal germline imprinted DMRs to at least 150 loci in placenta. With only 2 paternally-

derived germline imprinted DMRs identified, it suggests that the maternal genome is almost solely

responsible for inter-generational epigenetic inheritance, in the form of germline imprinted DMRs. In

the assessment of methylation at all CGIs in germ cells and embryos, we observed that oocyte-derived

DNA methylation is more likely to be retained in the blastocyst; this is consistent with the majority of

imprinted domains being maternal in origin, and with previous observations made in the mouse

(Smallwood et al. 2011). The maternally-inherited DNA in the embryo is primarily passively

demethylated after fertilisation, as opposed to the active demethylation that occurs on paternal DNA

(Santos et al. 2002; Inoue and Zhang 2011); this passive demethylation may more readily allow oocyte-

derived methylation to be maintained. In addition, maternal DMRs are CG-rich (Kobayashi et al. 2006)

that may therefore be more likely to contain a CG-rich domain like the ZFP57 binding site and

preferentially acquire H3K9me3 in the placenta. The placenta may be able to uniquely maintain much of

this mono-allelic DNA methylation throughout development, perhaps through recruitment of protective

complexes. Intriguingly, unlike mice, ZFP57 is not expressed in human oocytes but is expressed in the

blastocyst (Yan et al. 2013), suggesting it may have differing roles in the protection of imprints in human

and mouse development.

The polymorphic imprinted methylation among the novel DMRs in the human placenta may be

explained by underlying genetic variants affecting promoter usage or genomic variation affecting the

surrounding epigenetic profile. Alternatively, it may be a reflection of epigenetic stochasticity present in early development, when only a small number of cells will contribute to the developed tissue, and that such methylation states are fixed in the placenta and not over-written by global remethylation as occurs in somatic lineages. The future study of genetic and genomic variations in conjunction with placental methylation will help elucidate this.

While many genes associated with placental-specific DMRs are expressed in placenta, the role of the mono-allelic methylation in regulating transcription remains unclear and is likely not consistent across loci.   Placental-specific DMRs have been shown to regulate mono-allelic expression, including *DNMT1* (Das et al. 2013; Yuen et al. 2011a) and the chromosome 19 mi-RNA cluster (C19MC) (Noguer-Dance et al. 2010). There are also examples of loci where bi-allelic expression has been documented despite a nearby imprinted DMR, such as at *RASGRF1* and *C3orf62* (Yuen et al. 2011a). This suggests that additional factors may be important for imprinted gene regulation, supported by the observation in mouse that mono-allelic methylation of *Rasgrf1* is widespread across tissues but only mediates mono-allelic expression in a subset (Dockery et al. 2009). Furthermore, polymorphic imprinted expression has been described for *IGF2R* and *SLC22A2*, despite no difference in DNA methylation (Monk et al. 2006). Therefore, it is likely that at least some of the placental-specific imprinted DMRs are functionally important; however, the placenta may also present a unique epigenetic context that allows for the acquisition of imprints, regardless of function. To understand this fully, both allelic expression and methylation will need to be interrogated across all loci in a large study population.

There are several hypotheses proposed to explain the evolution of imprinting. The most commonly discussed is the parental conflict hypothesis (Moore and Haig 1991; Haig and Westoby 1989), which suggests imprinted genes arose through competing interests of the maternal and paternal genetic contributions to the developing foetus. While some of the novel DMRs identified may fit this model, there are several aspects that do not appear to, such as the solely maternal origin of DNA methylation,

the relatively low expression of associated genes and the lack of enrichment for genes involved in

growth and development (Haig 1997). There are, however, several additional theories that may provide

insight, including the parthenogenetic protection (Hall 1990; Solter 1988) and coadaptation (Keverne et

al. 1996; Keverne and Curley 2008) hypotheses.

The parthenogenetic protection hypothesis suggests that maternally silenced genes important

for placentation and implantation would prevent progression of spurious parthenogenetic pregnancies.

This is consistent with an entirely maternal origin of methylation and perhaps with the placental-

specificity; however, imprinting of only a few genes may be sufficient for this to be effective (Spencer

and Clark 2014). The coadaptation hypothesis suggests that there is a need to coordinate foetal

development and maternal resource management (Keverne and Curley 2008; Keverne et al. 1996). This

supports the notion that the majority of imprinted DMRs would be maternally inherited, and that as the

site for nutrient exchange the placenta may be enriched for imprinted loci. Furthermore, imprinting of

placental expressed genes that may regulate maternal behaviour and metabolism through secreted

protein products, such as *NPY*, which regulates appetite (Stanley et al. 1986) or *RASGRF1*, which has

been implicated in pancreatic function (Manyes et al. 2014), may fit this hypothesis. Our data supports

that there may be multiple factors contributing to the evolution of imprinting, particularly in the human

placenta, as no one theory appears to explain all aspects.

This study has shown that differentially methylated CGIs in the oocyte can uniquely maintain

imprinted DNA methylation throughout pregnancy in the human placenta. The novel placental-specific

imprinted DMRs identified are not conserved in methylation status between mouse and human, and

appear to be highly polymorphic in maintenance of methylation in the human population. Further study

will be needed to understand the underlying factors contributing to this variability in human extra-

embryonic tissues and its importance in pregnancy and development.

Methods

Samples

All placenta, foetal tissue, and blood samples were collected through hospital postings with full

informed consent at the BC Children's and Women's Health Centre, Vancouver, BC or anonymously

through the Embryopathology Laboratory at British Columbia Children's and Women's Hospital,

Vancouver, BC. These samples have been previously described and utilized for studies of DNA

methylation (GSE44667, GSE69502) (Price et al. 2012; Yuen et al. 2011b; Yuen et al. 2011a). Collection of

samples was approved by the University of British Columbia Clinical Ethics Review Board and the ethics

committee of the Children's and Women's Health Centre of British Columbia (H04-70488, H06-70085).

Control placental chorionic villous samples (N=75) included 40 pregnancies delivered at term,

and 35 delivered preterm or miscarried. The range of gestational ages was 6.0-41.6 weeks and included

37 male and 38 female conceptions. The preterm births were associated with one or more of the

following: preterm labour, premature rupture of membranes, chorioamnionitis, placental abruption,

and incompetent cervix. All samples were chromosomally normal assessed by standard karyotyping or

comparative genome hybridization, as previously described (Robinson et al. 2010). Two to four

independent sites were taken from each placenta, from which DNA was pooled after extraction from

chorionic villous. Maternal contamination was excluded using microsatellite markers (Robinson et al.

2010). To identify imprinted DMRs in the placenta, chorionic villous samples from 5 diandric and 5

digynic triploids pregnancies were assayed. The average (range) gestational age for diandric and digynic

triploids were 11.2 (8-13) weeks and 9.4 (8-12) weeks (p=0.18), respectively.

Thirty-two foetal tissues, including brain (N=7), spinal cord (N=7), muscle (N=9), and kidney

(N=9) were collected from second trimester foetuses, as previously described (Price et al. 2012). Adult

female whole blood samples (N=10) were collected from control women. Extra-embryonic cell types

(N=31), including cord blood, cord, amniotic membrane, chorionic membrane, 1st, 2nd and 3rd trimester

trophoblast and mesenchyme, and decidua (maternal), were isolated from control placental samples.

Trophoblast and mesenchyme were enzymatically separated from whole villi, as previously described

(Henderson et al. 1996; Robinson et al. 2010); trophoblast included both cytotrophoblast and

syncytiotrophoblast, but are referred to as trophoblast throughout. Isolated mesenchyme often

contains a small proportion of contaminating trophoblast cells, therefore data from mesenchyme should

be interpreted with caution.

DNA methylation

Illumina HumanMethylation450 array

Using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), DNA was extracted and

purified for all samples. For each sample, 750ng of DNA was bisulfite converted using the EZ DNA

Methylation Kit (Zymo Research Corporation, Irvine, USA). DNA from 5 diandric triploids, 5 digynic

triploids, 63 control placental villi, 32 foetal tissues, 10 adult blood, and 19 cord blood and placental cell

types were run on the Infinium HumanMethylation450 BeadChip array (Illumina Inc., San Diego, USA).

The arrays were processed as per the manufacturers' guidelines and data was collected on the HiScan

System (Illumina Inc., San Diego, USA).

Using GenomeStudio software (Illumina Inc., San Diego, USA), background intensity levels were

subtracted and corrected values were exported into R version 2.14.0 (R Development Core Team 2011).

Probes that met the following criteria were omitted before analysis, using additional annotation (Price

et al. 2013): X and Y chromosome, detection p-value>0.01 in at least one sample, no average B-value in

more than one sample, XY cross-hybridizing (Chen et al. 2013), and a SNP in the target C or G. The B-

values for the remaining probes were then converted to M-values and normalized using subset-quantile

within array normalization (SWAN) (Maksimovic et al. 2012).

Reduced representation bisulfite sequencing

DNA methylation for human oocytes (N=5), sperm (N=5), zygotes (N=3), 2-cell embryos (N=3), 4-cell embryos (N=3), 8-cell embryos (N=3), morulae (N=3), ICM (N=3), and TE (N=3) from blastocysts was obtained using publically available low-input RRBS data (GSE49828) (Guo et al. 2014). All bisulfite sequencing data described in this study was processed in the same manner, unless otherwise specified: quality was evaluated using FastQC Version 0.11.2, adaptors and quality trimming using Trim Galore! Version 0.3.7, reads were aligned and methylation calls performed in Bismark Version 0.14.0 (Krueger and Andrews 2011) and data was analysed in SeqMonk Version 0.29.0. All software can be found at http://www.bioinformatics.babraham.ac.uk/projects/. Reads were combined from each biological replicate to represent a DNA methylation profile for each development stage and gamete. Probes were generated using positions from the CpG island track from UCSC (https://genome.ucsc.edu/) with ≥5 CpGs with at least one methylation call. The library quality measures and coverage are detailed in Supplementary table 4.

For comparison to mouse, DNA methylation for oocytes, sperm, ICM, TE, E6.5 epiblast, E6.5 extra-embryonic ectoderm, placenta, heart, liver and brain was obtained from publically available datasets (GSE51239, GSE42836, GSE34864) (Smith et al. 2014; Hon et al. 2013; Smith et al. 2012). For the placental data (GSE42836) published methylation calls were utilized (Hon et al. 2013). Orthologous regions for the known and novel placental-specific DMRs (N=88) were determined using the liftOver tool in UCSC (https://genome.ucsc.edu/cgi-bin/hgLiftOver) to convert coordinates from the human genome (hg19) to the mouse genome (mm10). 88.6% of placental-specific DMRs had a mapped orthologous region in the mouse genome, a total of 58 loci had informative methylation calls.

Multiplex bisulfite sequencing

Isolation of DNA and bisulfite conversion for 48 samples (16 placental villi, 12 trophoblast, 16 maternal blood and 4 paternal blood) were performed as described above. 151 PCR assays were designed for the 43 known and 101 novel DMRs (Supplementary table 5). Primers were designed to

contain no SNPs or CpGs within the primer sequences and each assay to interrogate at least one SNP.

Parallel PCR reactions with a final concentration of 1xHotStar PCR buffer, 0.2mM dNTPs (Life

Technologies, Carlsbad, USA), 1U HotStar Taq (Qiagen, Hilden, Germany) and 0.4µM each primer pair

and 2ng DNA were performed in a 15µL reaction volume in 384-well plates. Plates were prepared on

the Agilent Bravo Workstation (Agilent Technologies, Santa Clara, USA). Cycling conditions included a 15

minute 95°C denaturation, followed by 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for

90 seconds, with a 5 minute final extension at 72°C and were performed on the C1000 Touch Thermal

Cycler (Bio-Rad, Hercules, USA).

PCR products were pooled for each sample and SPRI-purified using Sera-Mag carboxylate-

modified Magnetic SpeedBeads (Fisher Scientific, Loughborough, UK) as per manufacturer's

recommendations. A-overhang addition was done using 1x NEB buffer 2, 0.2mM dATP, 12.5U Klenow

fragment (3'->5' exo-) (New England Biolabs Inc., Ipswich, USA) in a 25µl reaction incubated at 37°C for

30 minutes. Following SPRI purification, Illumina adapters were ligated using the Quick ligation kit (New

England Biolabs Inc., Ipswich, USA) in a 40µl reaction. Following another SPRI purification, libraries were

amplified (11 cycles) and barcoded using the Sanger 8-base index in a 50µl reaction containing: 1x High

Fidelity buffer, 1U Phusion High Fidelity Taq (New England Biolabs Inc., Ipswich, USA), 0.2mM dNTPs,

0.1µM each primer . Final libraries were SPRI purified and concentration and quality were assessed by

the 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, USA) and Kapa library quantification

(Kapa Biosystems, Wilmington, US). The 48 barcoded libraries were pooled and run on the Illumina

MiSeq System (Illumina Inc., San Diego, USA) using 150bp paired-end reads. Allelic methylation levels

and distributions were extracted from the Bismark BAM files for reads with a linear match to the

genome using a custom script (Supplementary file 1), which compared the bases in the reads to the

expected SNPs designed into the amplicons.

Gene expression

RNA-seq

Expression of genes associated with placental-specific DMRs in early development was assessed using three publically available RNA-seq datasets (Metsalu et al. 2014; Yan et al. 2013; Bernstein et al. 2010). Single cell RNA-seq was utilised for human oocytes, blastocyst (epiblast, primitive endoderm, and TE) (GSE36552) and mRNA-seq for term placenta (GSE56524) and total RNA-seq for mid-gestation placenta (GSE18927). The single-cell RNA-seq data was trimmed using Trim Galore! Version 0.3.7 and mapped using TopHat 2.0.12 guided by gene models from Ensembl release 61. Quality assessment was performed using the RNA-Seq QC module of SeqMonk. The mRNA-seq for term placenta was aligned using TopHat (Trapnell et al. 2009) with bowtie 1, and --color --quals options (since the data are in colour space). Aligned BAM files for the total RNA-seq in mid-gestation placenta was obtained directly from Gene Expression Omnibus (GSE18927).

Gene expression as reads per kilobase per million mapped reads (RPKM) was quantitated over exons with merged isoforms, correcting for transcript length using the RNA-seq pipeline quantitation in SeqMonk Version 0.29.0. Placental-specific DMRs were assessed for associated gene expression, based on the closest TSS, using the UCSC Genome Browser gene annotation (Supplementary table 6). Three genes were missed due to gene annotation differences between UCSC and ENSEMBL, the gene annotation track in SeqMonk Version 0.29.0, resulting in 85/88 DMRs being reported. The data is presented using the natural log transformed RPKM+1.

Histone modifications

ChIP-seq

Publically available ChIP-seq data for H3K9me3 in placental villi, amniotic membrane and foetal muscle (GSE18927) (Bernstein et al. 2010) was assessed in SeqMonk Version 0.29.0. Relative enrichment was assessed by calculating the difference (delta) between placenta and somatic tissue

(muscle or amnion) for each 1000bp probe. Delta enrichment values for placental-specific DMRs and

CGIs were compared to all other genome-wide probes using a *t*-test.

Statistical analyses

All statistical analyses were done using RStudio version 0.97.551 (RStudio Team 2015) and R version

3.0.3(R Core Team 2014), unless otherwise specified.

Identifying imprinted DMRs

Using the DMRFinder tool in the CHARM package (Aryee et al. 2011), candidate DMRs were

identified between diandric (n=5) and digynic (n=5) triploid samples (Supplementary table 1). Known

imprinted DMRs were those that overlapped a previously reported human imprinted DMR with

adequate coverage on the 450K array (N=57) (Supplementary table 2) (Court et al. 2014). Germline

novel DMRs were CGIs (defined by UCSC Genome Browser (https://genome.ucsc.edu/)) that: 1) were

>50% differentially methylated between sperm and oocytes, 2) intermediately methylated (15-60%,

based on the 90th percentile observed at known imprinted DMRs) in the blastocyst (average of ICM and

TE), 3) >5% difference in methylation between diandric and digynic triploids, and 4) matching parental

origin of DNA methylation between triploid and gametes. Placental-specific DMRs were defined as

those with >25% and <75% DNA methylation in placenta and <25% in somatic tissues.

Allelic DNA methylation

Reads from the RRBS data for oocyte, sperm, ICM and TE (Guo et al. 2014) were remapped

based on phase of DNA methylation within a given read. Reads were categorised into fully methylated,

fully unmethylated or mixed methylation and the proportion of each across a given DMR was

determined.

For the RRBS and multiplex bisulfite sequencing data, DNA methylation was averaged across

reads with ≥5 CpGs with a read depth of ≥5. Reads across DMRs were binned into 10% intervals to

generate density plots of the distribution of read methylation. For DMRs with heterozygous SNPs,

percent methylation was calculated for each allele separately for each informative individual sample.

Where possible, parental origin was assigned based on parental genotype information.

Features of placental-specific DMRs

The frequency of ZFP57 consensus motifs (TGCCGC) (Nakamura et al. 2007; Quenneville et al.

2011) among known imprinted DMRs, placental-specific DMRs and CGIs was determined using

customised perl scripts and compared using Chi-square test.

Gene ontology

The Database for Annotation, Visualization and Integrated Discovery (DAVID) gene ontology

program was used on genes associated with placental-specific DMRs (Supplementary table 6) (Huang da

et al. 2009a; Huang da et al. 2009b).

Data Access

Illumina Infinium HumanMethylation450 array data and multiplexed targeted bisulfite sequencing were

deposited in the Gene Expression Omnibus depository (GSE74738, GSE76273).

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Disclosure Declaration

The authors have no conflicts of interest to declare.

Author contributions

C.W.H. generated the research objectives, produced array and bisulfite sequencing data, performed

analyses, and prepared the figures and manuscript. M.S.P. generated array and expression data and

helped with experimental design and interpretation. H.S. performed computational analyses. S.A.

processed and analysed allelic bisulfite sequencing data. D.E.M. collected and sampled human foetal

tissues. G.K. and W.P.R. supported the study, assisted with experimental design and helped with

manuscript preparation.

Figure Legends

Figure 1. Characterisation of known imprinted DMRs in human development. A) Digynic triploid conceptions contain two maternal (white) and one paternal (grey) genome, while diandric triploid conceptions contain one maternal and two paternal genomes. By comparing digynic (N=5) and diandric (N=5) triploid placental villi, DMRs were identified, defined as ≥3CpGs within 500bp. An example plot shows a maternal DMR near *PEG3,* which is more highly methylated among digynic triploid samples (black line) compared to diandric triploid samples (red line) across 37 CpG sites over a ~4kb region. **B)** DNA methylation through early human development is shown for 43 DMRs, identified between triploid samples that overlap, previously reported imprinted DMRs (Court et al. 2014). DNA methylation for human germ cells, early embryonic stages (zygote, 2-cell, 4-cell, 8-cell, and morula stage embryos), ICM, and TE is an average of CpG sites across each DMR measured by RRBS. DNA methylation for placental villi, foetal tissues (brain, kidney, muscle, and spinal cord) and whole blood was an average of 450K array probes across each DMR. Primary (germline) imprinted DMRs (light grey), placental-specific DMRs (black) and secondary DMRs (dark grey) are denoted. Parental origin of DNA methylation is designated as maternal (red) or paternal (blue). White boxes in the heat map indicate DMRs with no data.

Figure 2. Identification of novel imprinted placental DMRs. A) DNA methylation is shown for oocyte-specific (left panel) and sperm-specific (right panel) DMRs in human gametes and early embryonic stages (zygote, 2-cell, 4-cell, 8-cell, and morula stage embryos), ICM, and TE. DMRs were defined as CGIs that showed a >50% methylation difference between gametes, with intermediate methylation (15-60%) in the ICM and TE. **B)** The number of DMRs that overlap between those identified between human gametes and those identified between triploid placental samples is shown. **C)** DNA methylation through early human development is shown for the 101 novel imprinted DMRs. DNA methylation for human gametes, early embryonic stages (zygote, 2-cell, 4-cell, 8-cell, and morula stage embryos), ICM and TE is an average of CpG sites across each DMR, measured by RRBS. DNA methylation for placental villi, foetal

tissues (brain, kidney, muscle, and spinal cord) and whole blood was an average of 450K array probes

across each DMR. 72 novel DMRs showed a placental-specific pattern of imprinting, defined as <25% or

>75% methylation in somatic tissues and intermediately (25-75%) methylated in placenta.

Figure 3. Imprinted methylation of novel placental DMRs. A) DNA methylation was averaged across

reads with ≥5 CpGs from RRBS. The proportion of reads that was completely unmethylated (black),

methylated (red), or had mixed methylation (grey) is shown for oocyte, sperm, ICM and TE for known

(N=43) and novel (N=101) DMRs. The proportion of methylated alleles did not differ between known

and novel DMRs (p=0.981, Chi-square). **B)** DNA methylation was averaged across reads with ≥5 CpGs

from multiplexed bisulfite sequencing in 16 placental villi and 12 matched trophoblast samples.

Distribution of read methylation across DMRs (binned in 10% intervals) is shown in the density plot, with

a density heat map in greyscale showing the distribution at each known (orange) and novel (blue) DMR

below. DMRs with a minimum read depth of ≥5 were reported. **C)** Average DNA methylation is shown

for the methylated and unmethylated allele at known (orange) and novel (blue) DMRs containing a

heterozygous SNP. Each row corresponds to one heterozygous SNP for one individual at one DMR; for

loci with more than one informative SNP, the SNP number is designated after the sample name.

Parental origin was assigned where informative, with maternal (red) and paternal (blue) mono-allelic

methylation, alleles with no differential methylation (grey) and uninformative alleles (white) denoted.

Figure 4. Polymorphic imprinted methylation in the human placenta. A) DNA methylation shows a

polymorphic pattern across known (orange) and novel (blue) DMRs among 63 placental samples from 1st

(N=5, black), 2nd (N=9, light grey) and 3rd (N=49, dark grey) trimester samples. **B)** Mean standard

deviation across all DMRs (N=144) is shown for placenta, foetal somatic tissues and adult whole blood

(p<2.2E-16, Kruskal-Wallis).

Figure 5. Timing of loss of imprinting in human embryonic lineages. A) Schematic representation of the

developmental origins of the extra-embryonic cell types is shown, with TE-derived cells (dark blue), ICM-

derived cells (green), and cells with mixed origin (turquoise) denoted. **B)** The distribution of DNA

methylation for known placental-specific DMRs that lose DNA methylation in somatic tissues (N=14) is

shown for extra-embryonic cell types isolated from term placenta. Methylation for trophoblast and

mesenchyme is an average from 1st, 2nd and 3rd trimester samples. TE-derived cells (dark blue), ICM-

derived cells (green), and cells with mixed origin (turquoise) are denoted. Extra-embryonic cell types are

ordered based on the approximate timing in development when each cell type is derived. The window

of epigenetic reprogramming highlights the period in which DNA methylation at placental-specific DMRs

is being erased in the ICM-derived lineage.

Figure 6. Characteristics of placental-specific DMRs. A) The proportion of known imprinted DMRs,

placental-specific DMRs, and CGIs with one or more ZFP57 binding site consensus sequence is shown. **B)**

Enrichment for H3K9me3, using 1000bp sliding windows with a 1000bp step, was compared between

human placenta and foetal muscle. Using the absolute difference for each probe, placental-specific

DMRs (p=2.2E-16, *t*-test) and CGIs (p=2.2E-16, *t*-test) showed a significant enrichment compared to all

other probes, in placenta. Placental-specific DMRs were not significantly different from CGIs (p=0.70, *t*-

test). **C)** Expression of genes associated with placental-specific DMRs based on closest transcription

start site (Supplementary table 6) is shown in TE, and 2nd and 3rd trimester placental villi.

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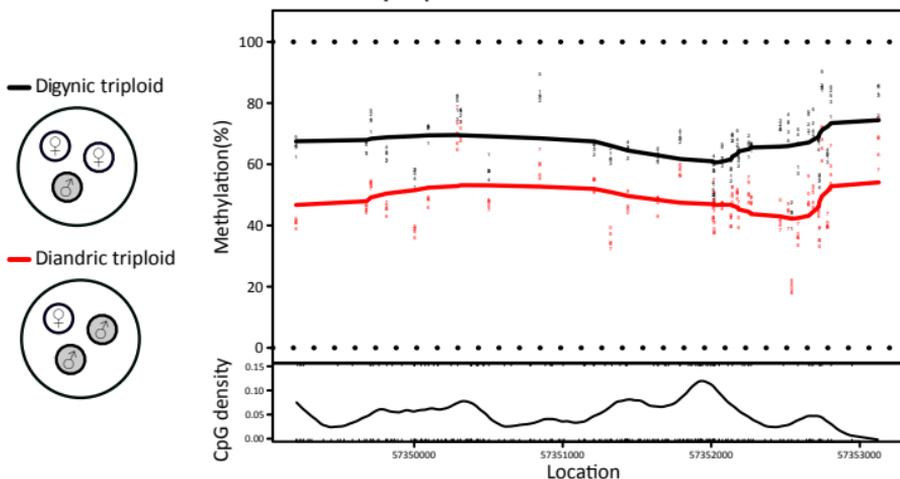Yuen RK, Jiang R, Penaherrera MS, McFadden DE, Robinson WP. 2011a. Genome-wide mapping of imprinted differentially methylated regions by DNA methylation profiling of human placentas from triploidies. *Epigenetics Chromatin* **4**: 10.
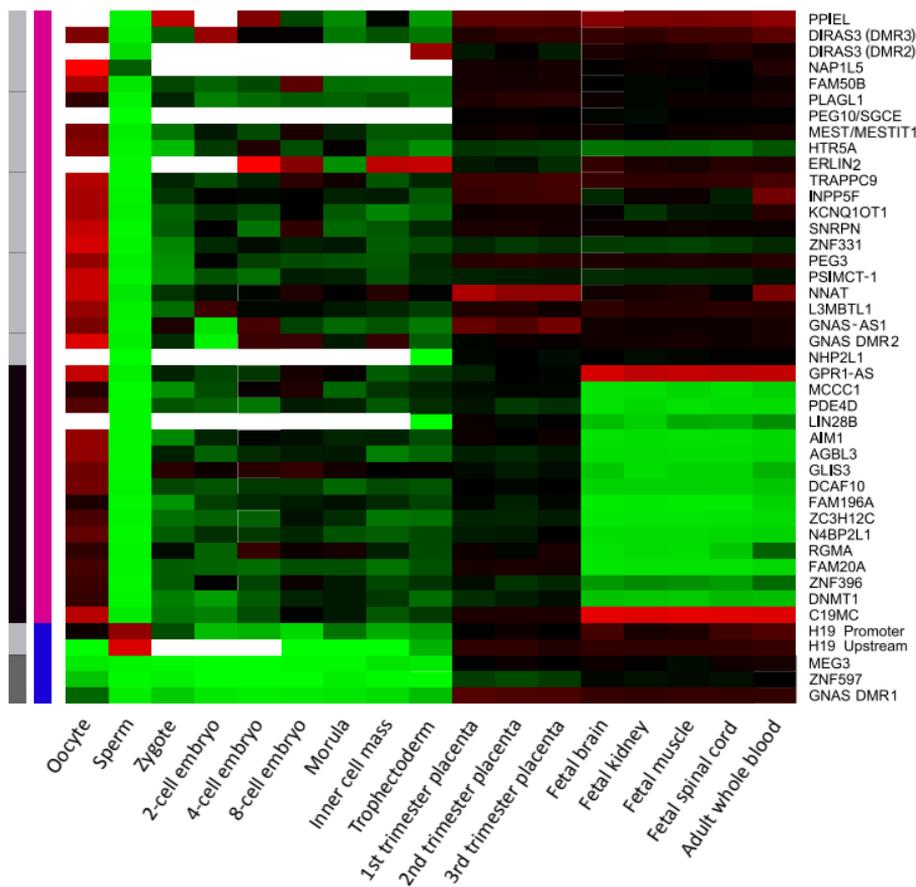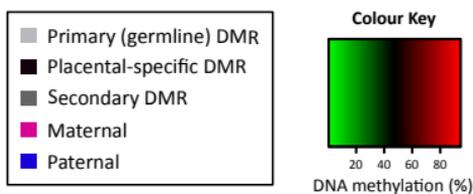
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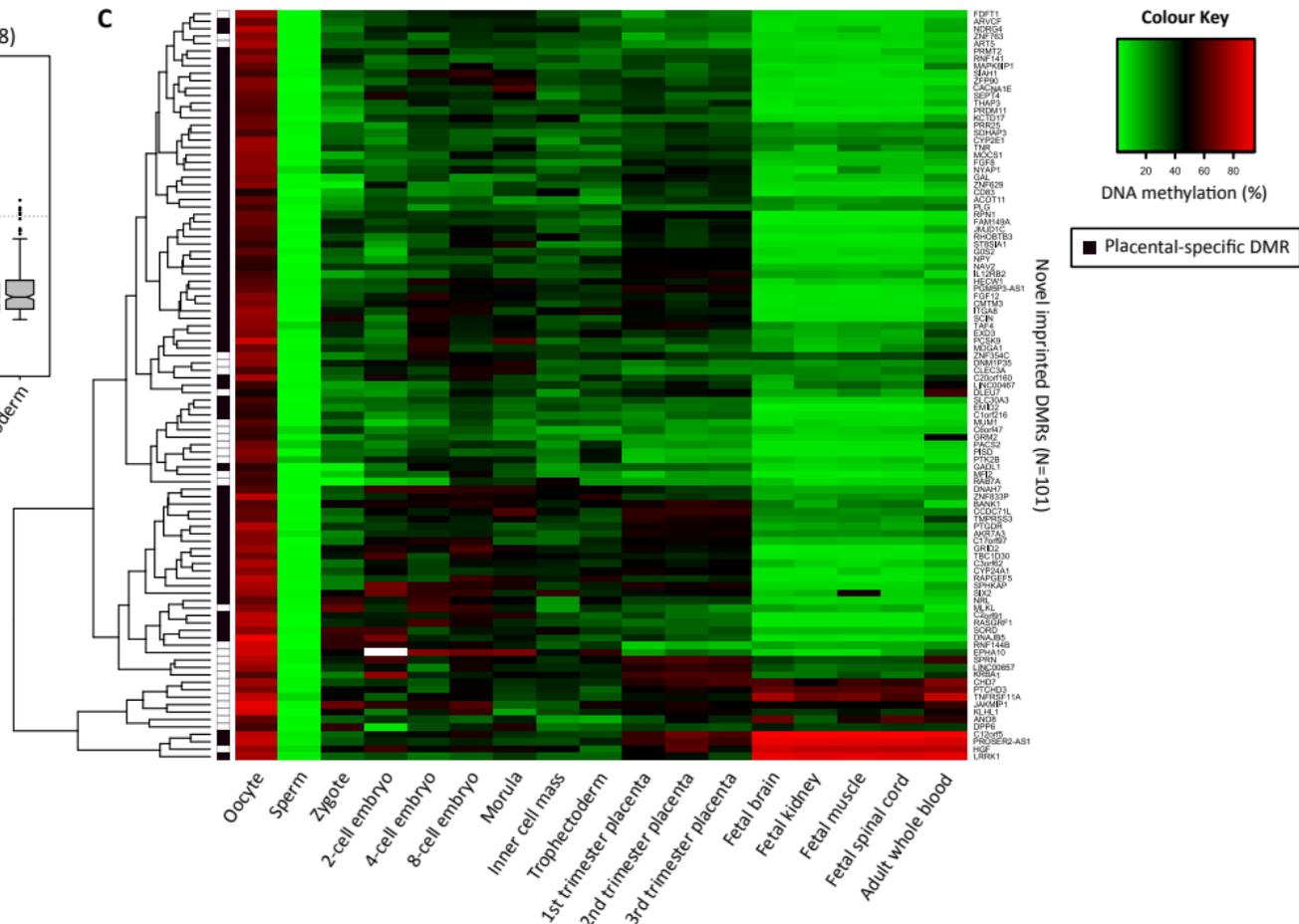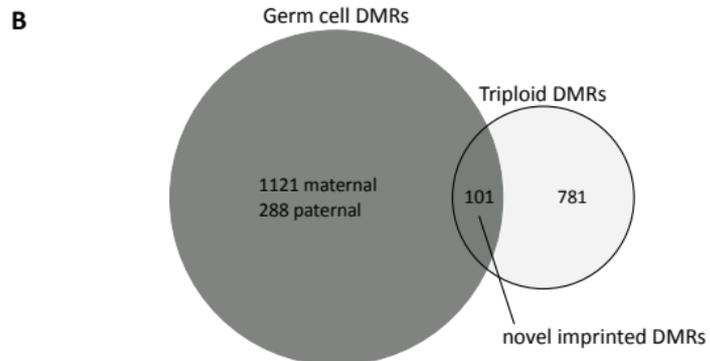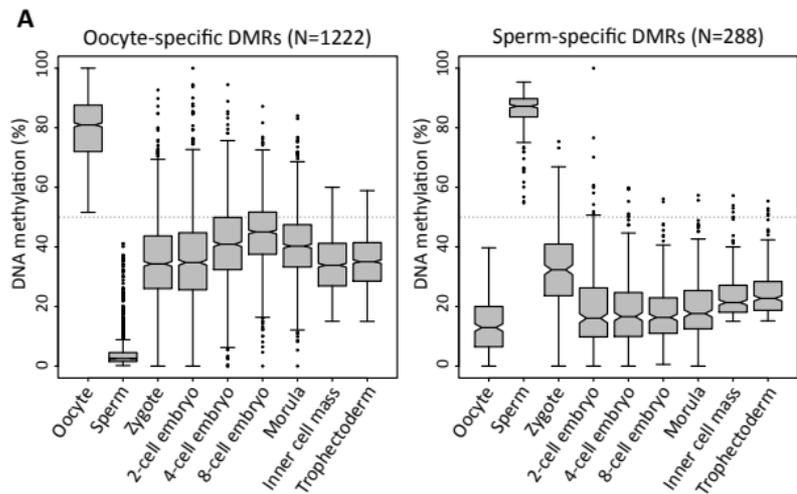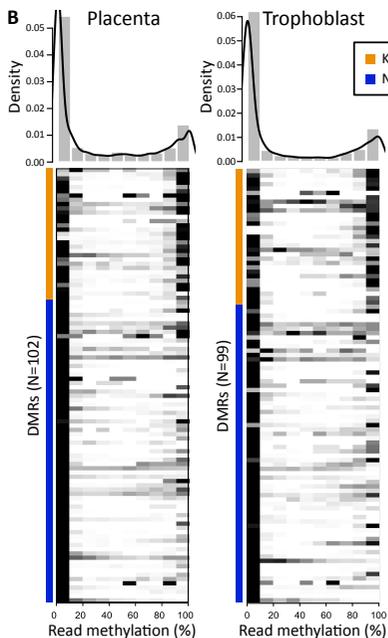
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Digynic triploid
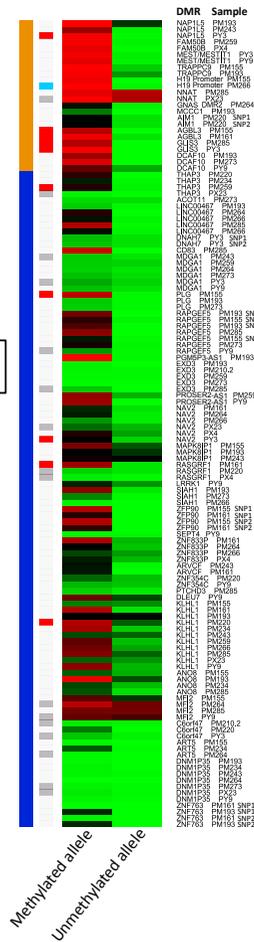
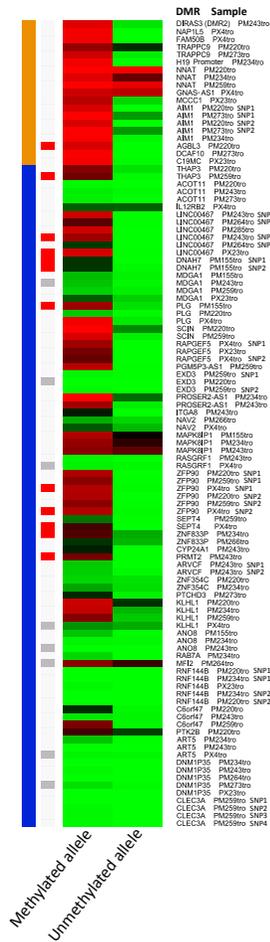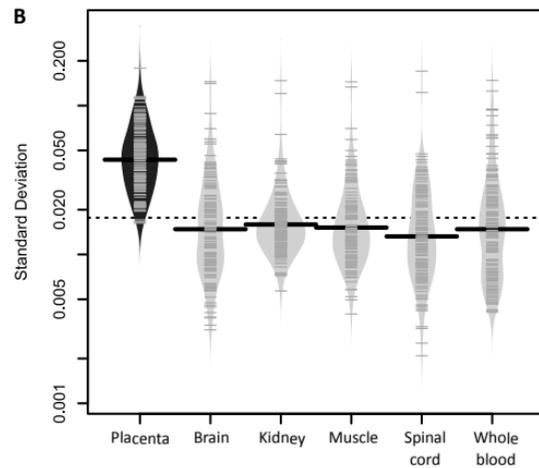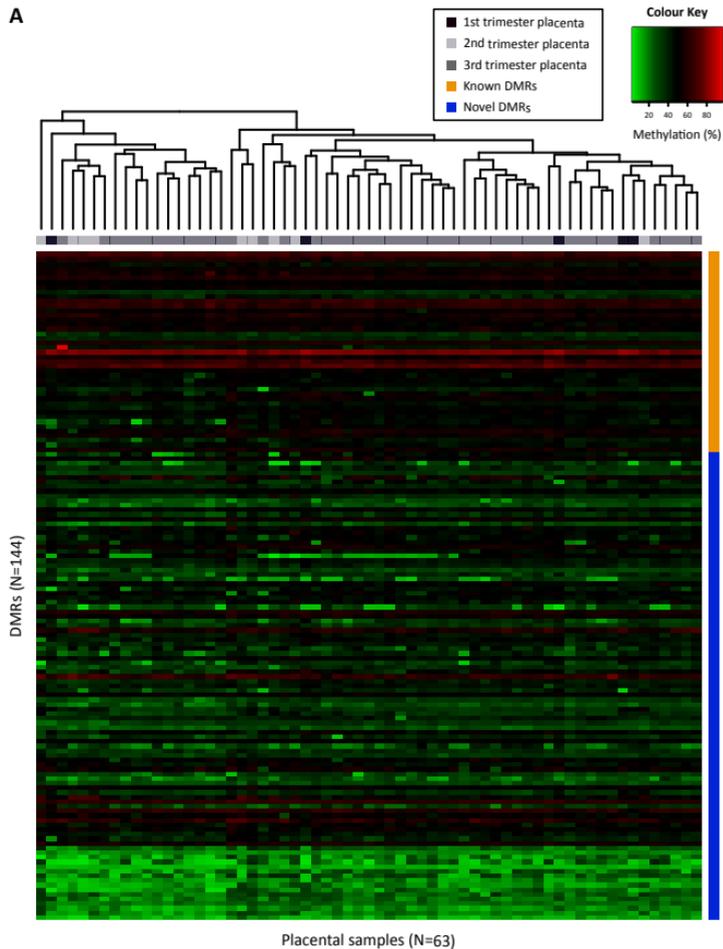Diandric triploid

Maternally imprinted DMR: PEG3 chr19:57349204-57353128

B

Colour Key

DNA methylation (%)

Primary (germline) DMR
Placental-specific DMR
Secondary DMR
Maternal
Paternal

PPIEL
DIRAS3 (DMR3)
DIRAS3 (DMR2)
NAP1L5
FAM50B
PLAGL1
PEG10/SGCE
MEST/MESTIT1
HTR5A
ERLIN2
TRAPPC9
INPP5F
KCNQ1OT1
SNRPN
ZNF331
PEG3
PSIMCT-1
NNAT
L3MBTL1
GNAS -AS1
GNAS DMR 2
NHP2L1
GPR1-AS
MCCC1
PDE4D
LIN28B
AIM1
AGBL3
GLIS3
DCAF10
FAM196A
ZC3H12C
N4BP2L1
RGMA
FAM20A
ZNF396
DNMT1
C19MC
H19 Promoter
H19 Upstream
MEG3
ZNF597
GNAS DMR1

Oocyte
Sperm
Zygote
2-cell embryo
4-cell embryo
8-cell embryo
Morula
Inner cell mass
Trophectoderm
1st trimester placenta
2nd trimester placenta
3rd trimester placenta
Fetal brain
Fetal kidney
Fetal muscle
Fetal spinal cord
Adult whole blood

A

Oocyte-specific DMRs (N=1222)

Sperm-specific DMRs (N=288)

B

Germ cell DMRs

Triploid DMRs

1121 maternal
288 paternal

101

781

novel imprinted DMRs

C

Novel imprinted DMRs (N=101)

Placental-specific DMR

Colour Key

DNA methylation (%)

A

Colour Key

1st trimester placenta
2nd trimester placenta
3rd trimester placenta
Known DMRs
Novel DMRs

Methylation (%)
20 40 60 80

DMRs (N=144)

Placental samples (N=63)

B

Standard Deviation

Placenta | Brain | Kidney | Muscle | Spinal cord | Whole blood

A Derivation of placental cell types
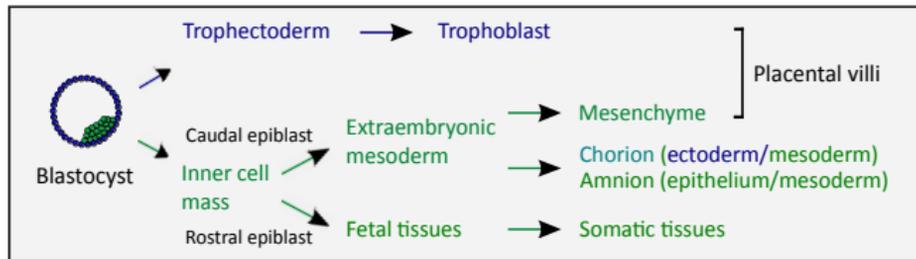
B Placental-specific DMR methylation

Developmental timing of placental-cell type derivation

A

Proportion of regions (y-axis, 0.0 to 1.0)

Legend: >2, 2, 1, 0

x-axis categories: Known germline DMRs, Placental-specific DMRs, CGIs

B

CGIs
placental-specific DMRs

y-axis: Muscle H3K9me3 enrichment (-10 to 6)
x-axis: Placenta H3K9me3 enrichment (-10 to 6)

R = 0.718

C

Colour Key
Log$_2$(RPKM+1)
0 1 2 3 4

Genes associated with placental-specific DMRs

x-axis: Trophectoderm, 2nd trimester placenta, 3rd trimester placenta

Pervasive polymorphic imprinted methylation in the human placenta

Courtney W Hanna, Maria S Peñaherrera, Heba Saadeh, et al.

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