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Pervasive polymorphic imprinted methylation in the human placenta

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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 monoallelically 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 fertilization. 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 extraembryonic tissues. DNA methylation was evaluated using the Illumina HumanMethylation450 array in 134 human tissue samples, publicly available reduced representation bisulfite sequencing in the human embryo and germ cells, and targeted bisulfite sequencing in term placentas. Forty-three 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. Seventy-two novel DMRs showed a pattern consistent with placental-specific imprinting, and this monoallelic 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.

[Supplemental material is available for this article.]

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 fetal and placental development (Frost and Moore 2010).

The monoallelic 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 intergenic while maternal DMRs are located at promoters or intragenic regions (Kobayashi et al. 2006; Schulz et al. 2010).

Primary imprinted DMRs are erased and reestablished 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 reacquired in spermatocytes around the time of birth in males, while in females, DNA methylation is not fully established until oocyte growth in

adulthood (Lucifero et al. 2004; Henckel et al. 2012). 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 (Ishida and Moore 2013; Guo et al. 2014).

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://igc.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 (Smallwood et al. 2011; Guo et al. 2014), 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 (Choufani et al. 2011; Yuen et al. 2011a;

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Das et al. 2013; Stelzer et al. 2013; Court et al. 2014; Docherty et al. 2014) 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 extraembryonic 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

Characterization of known imprinted DMRs in human development

To evaluate imprinting in the human placenta, we identified DMRs (≥ 3 CpGs within 500 bp) between five diandric (two paternal copies of the genome and one maternal) and five digynic (two maternal copies and one paternal) triploid placentas, measured by the Illumina HumanMethylation450 BeadChip array (450K array) (Fig. 1A). Eight hundred eighty-two candidate DMRs, encompassing 6807 probes (CpG sites), were identified (Supplemental Table 1), including 43 that overlapped previously reported human imprinted DMRs (Supplemental Table 2; Court et al. 2014).

DNA methylation at these 43 known DMRs was assessed using the 450K array in fetal and extraembryonic tissues and using publicly available reduced representation bisulfite sequencing (RRBS) in human gametes and embryos (Guo et al. 2014). Thirty-seven DMRs were informative for germline DNA methylation and, as expected, the majority showed $>50\%$ difference in DNA methylation between germ cells, including two paternal and 32 maternal DMRs (Fig. 1B). The remaining three DMRs (*MEG3*, *ZNF597*, and *GNAS* DMR1) were unmethylated both in germ cells and the early embryo, suggesting these are somatic DMRs (Fig. 1B). Among the known DMRs, 15 are placental-specific in humans (Court et al. 2014) and all showed differential methylation

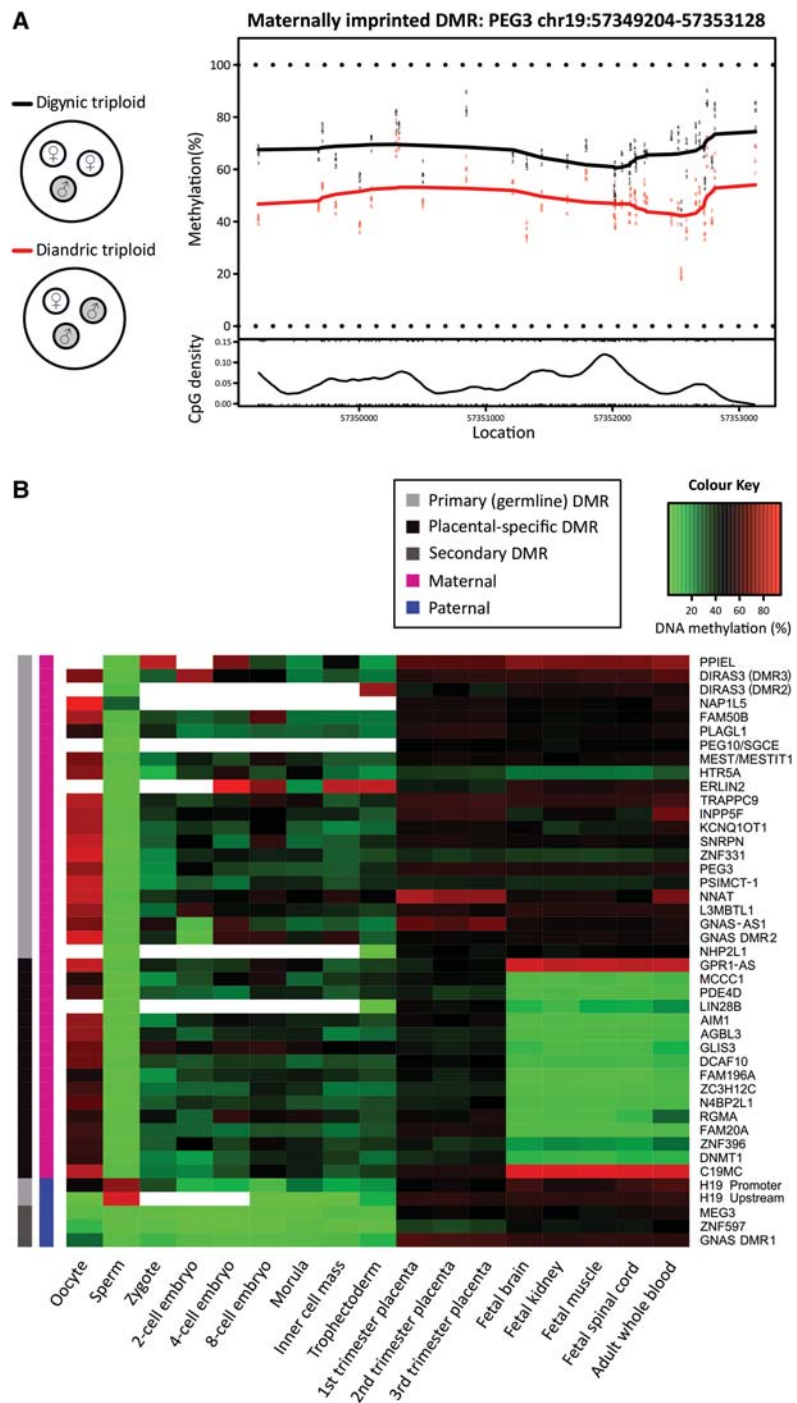


Figure 1. Characterization of known imprinted DMRs in human development. (A) Digynic triploid conceptions contain two maternal (white) and one paternal (gray) genome, while diandric triploid conceptions contain one maternal and two paternal genomes. By comparing digynic ($N = 5$) and diandric ($N = 5$) triploid placental villi, DMRs were identified, defined as ≥ 3 CpGs within 500 bp. 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 ~ 4 -kb 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, two-cell, four-cell, eight-cell, and morula stage embryos), inner cell mass, and trophoblast is an average of CpG sites across each DMR measured by RRBS. DNA methylation for placental villi, fetal 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 gray), placental-specific DMRs (black), and secondary DMRs (dark gray) are denoted. Parental origin of DNA methylation is designated as maternal (magenta) or paternal (blue). White boxes in the heat map indicate DMRs with no data.

between oocytes and sperm, with a mean difference of 67.5% (Fig. 1B). These placental-specific DMRs are intermediately methylated in both the inner cell mass (ICM) (35.6%) and trophoctoderm (TE) (34.1%) of the blastocyst, suggesting that they are transiently imprinted in embryo, not just extraembryonic TE (Fig. 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. First, 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 (Fig. 2A). Of these candidate CGIs, 101 novel regions overlapped with the 882

DMRs identified in triploid placental villous samples (Fig. 2B). All 101 novel DMRs derived methylation from the oocyte, and the majority ($N=72$) showed a placental-specific pattern of imprinting (Fig. 2C).

To confirm that the novel DMRs maintain imprinted allelic-specific methylation throughout development, allelic methylation was evaluated in oocytes, sperm, ICM, and TE for known and novel DMRs. This reaffirmed that ~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 (Fig. 3A). It can be inferred that these methylated alleles were almost entirely derived from the oocyte (Fig. 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

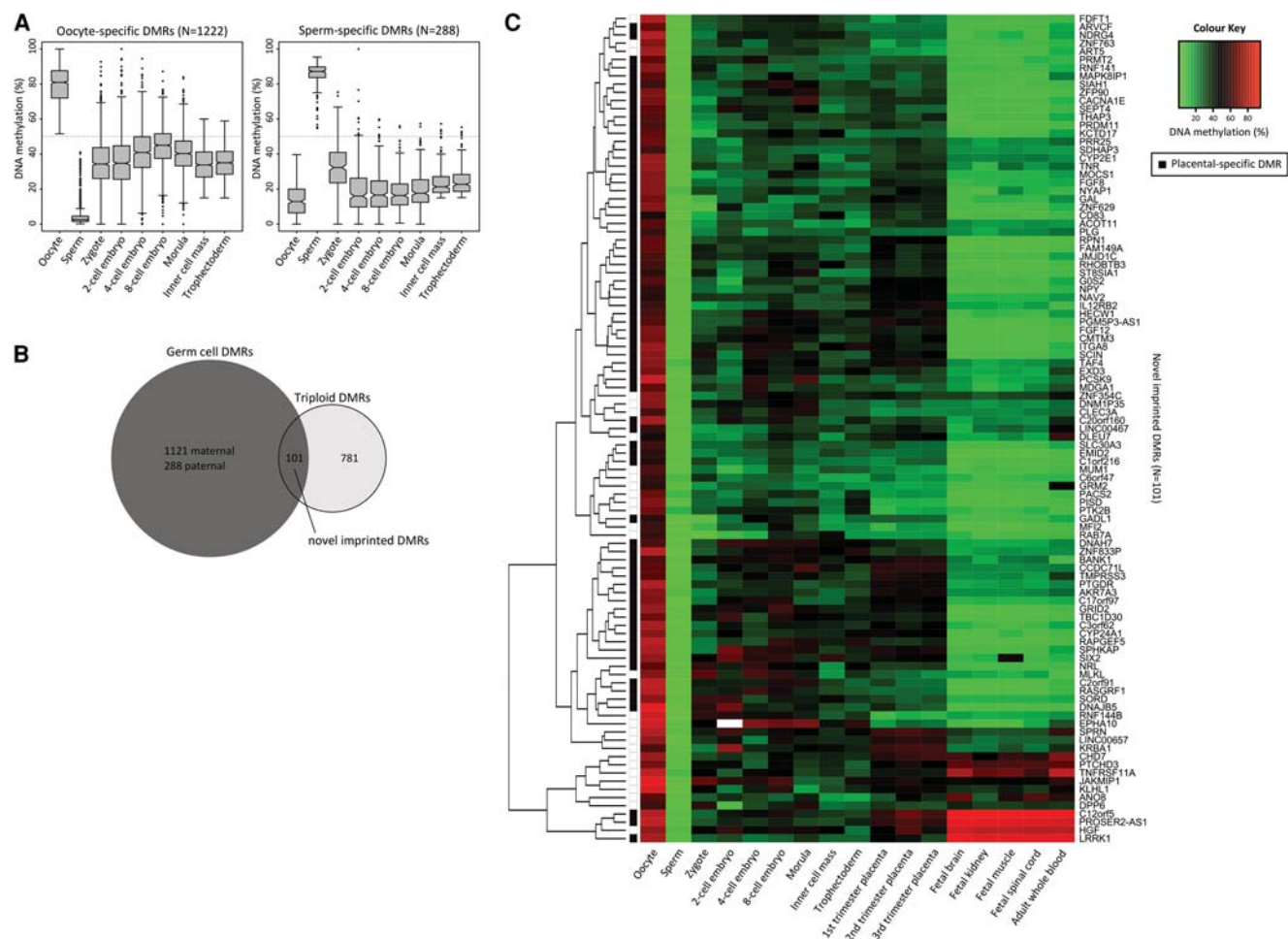


Figure 2. Identification of novel imprinted placental DMRs. (A) DNA methylation is shown for oocyte-specific (left panel) and sperm-specific (right panel) DMRs in human gametes and early embryonic stages (zygote, two-cell, four-cell, eight-cell, and morula stage embryos), ICM, and TE. DMRs were defined 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, two-cell, four-cell, eight-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, fetal tissues (brain, kidney, muscle, and spinal cord), and whole blood was an average of 450K array probes across each DMR. Seventy-two 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.

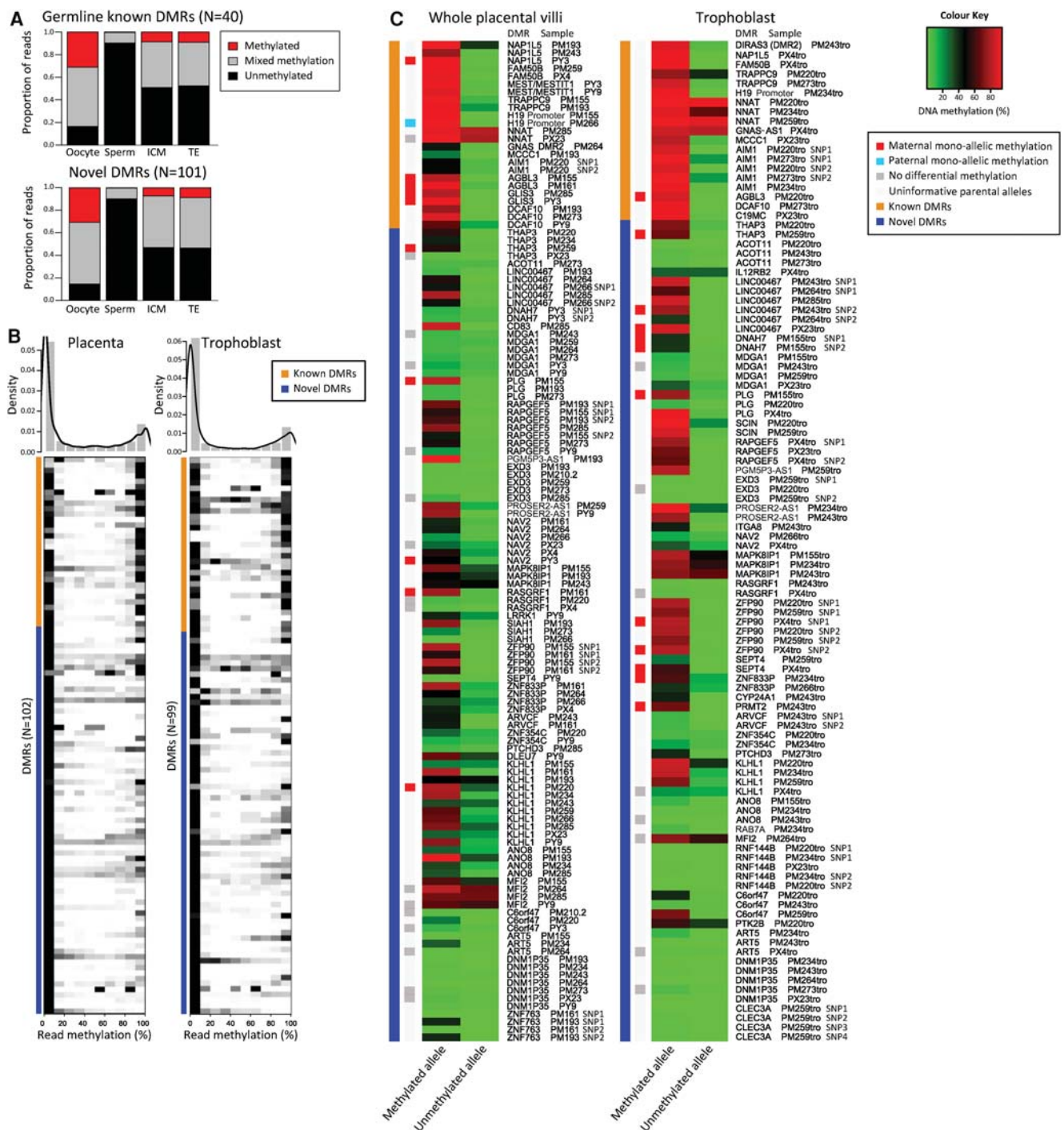


Figure 3. Imprinted methylation of novel placental DMRs. (A) DNA methylation was averaged across reads with ≥ 5 CpGs from RRBS. The proportion of reads that was completely unmethylated (black), methylated (red), or had mixed methylation (gray) is shown for oocyte, sperm, ICM, and TE for germline known ($N = 40$) and novel ($N = 101$) DMRs. The proportion of methylated alleles did not differ between known and novel DMRs ($P = 0.981$, χ^2 test). (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 heatmap in grayscale 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) monoallelic methylation, alleles with no differential methylation (gray), and uninformative alleles (white) denoted.

(Supplemental Table 5) in 16 term placental villi samples with matched isolated trophoblast ($N=12$) and parental blood ($N=20$). One hundred two DMRs (32 known and 70 novel) were successfully assayed, revealing a bimodal distribution of read methylation in term placenta and trophoblast at these loci (Fig. 3B), consistent with that in the ICM and TE (Supplemental Fig. 1). This bimodal methylation pattern is indicative of monoallelic methylation, as opposed to heterogeneous CpG methylation expected at nonimprinted, intermediately methylated loci. DMRs that contained heterozygous SNPs showed that indeed this bimodal methylation pattern was attributable to monoallelic methylation (Fig. 3C). Furthermore, DMRs with informative parental origin showed that monoallelic methylation was entirely maternally derived, with the exception of paternally methylated *H19* (Fig. 3C).

Polymorphic imprinted methylation in the human placenta

Strikingly, methylation across the novel DMRs was highly polymorphic, with 50% of heterozygous samples demonstrating monoallelic methylation and the remaining showing a complete loss of imprinting at these loci (Fig. 3C). The frequency of polymorphic imprinting was similar in placental villi (50.0%) and trophoblast (49.4%) (Supplemental 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 placental samples run on the 450K array (Fig. 4A), which included four 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 ($P<2.2\times 10^{-16}$) (Fig. 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 publicly available data (Smith et al. 2012, 2014; Hon et al. 2013). Only six DMRs showed differential germline DNA methylation in mouse gametes, but only one locus, orthologous to *NAV2*, showed a pattern consistent with placental-specific imprinting (Supplemental Fig. 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

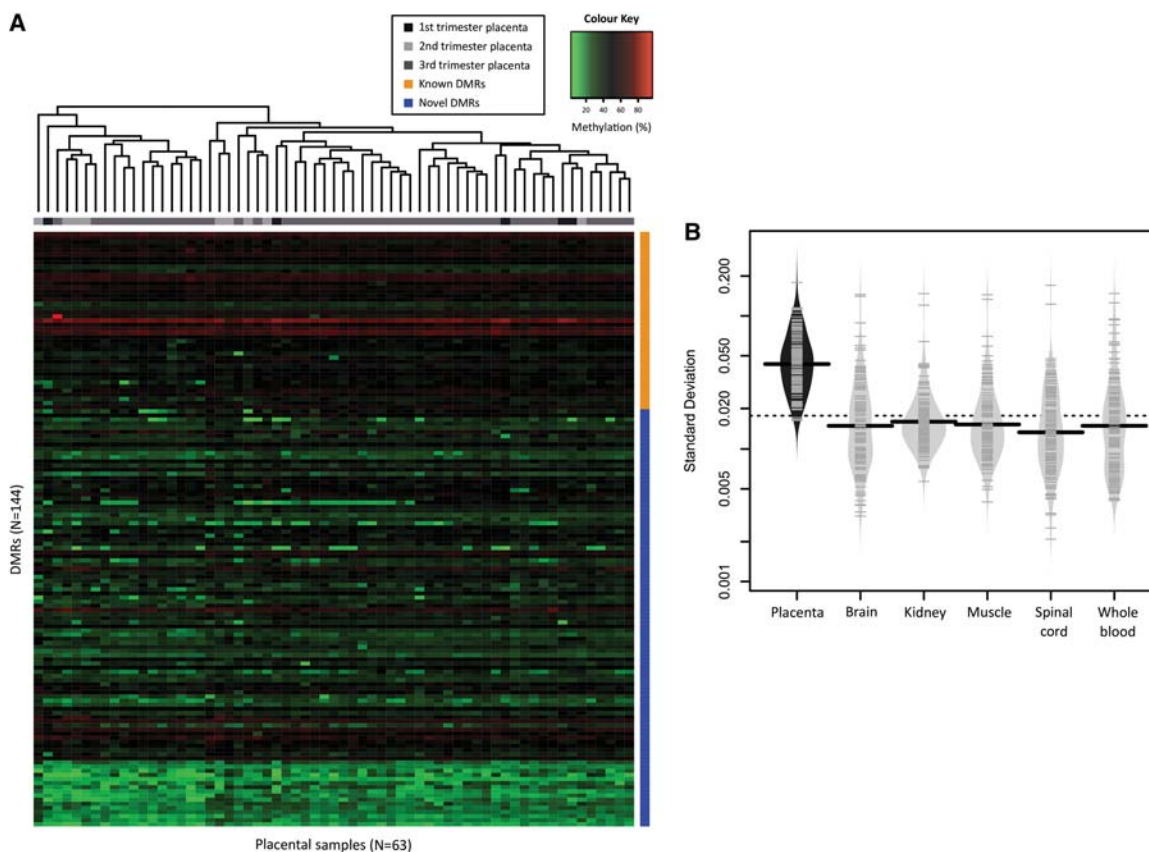


Figure 4. Polymorphic imprinted methylation in the human placenta. (A) DNA methylation shows a polymorphic pattern across known (orange) and novel (blue) DMRs among 63 placental samples from 1st ($N=5$, black), 2nd ($N=9$, light gray), and 3rd ($N=49$, dark gray) trimester samples. The color scale was set to show those samples that deviate from the intermediate range of methylation. (B) Mean standard deviation across all DMRs ($N=144$) is shown for placenta, fetal somatic tissues, and adult whole blood ($P<2.2\times 10^{-16}$, Kruskal–Wallis). Y-axis is on a logarithmic scale.

took advantage of the methylation patterns observed in isolated extraembryonic 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 extraembryonic tissues progressively originate later in development, they are more similar to the embryo (Fig. 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 a mix of cell types largely derived from the ICM-derived extraembryonic mesoderm (Fig. 5A). Contrastingly, the average methylation was lower in chorion (33.0%) and amnion (8.3%) (Fig. 5B), and both mostly originate later from the extraembryonic mesoderm (Fig. 5A).

The exact developmental timing and origin of amnion and chorion are not firmly established. In mouse and, likely, in human, chorion is derived from both the TE and extraembryonic 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 (Robinson et al. 2002;

Penaherrera et al. 2012). 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 (Fig. 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 monoallelic methylation of placental-specific DMRs (16 known and 72 novel) may be attributable to targeted protection of these regions or to the fact 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. One thousand three hundred fifty-two 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%) (Supplemental Fig. 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 of the placenta (Supplemental Fig. 3B; Schroeder et al. 2013). 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%; $P = 0.63$) (Fig. 6A). Placental-specific DMRs were also enriched for H3K9me3 in placenta compared to somatic tissues (muscle, $P = 2.2 \times 10^{-16}$ and amnion, $P = 2.2 \times 10^{-16}$) (Fig. 6B; Supplemental Fig. 4). However, this enrichment was not a unique feature, as all CGIs were enriched for H3K9me3 in placenta compared to somatic tissue (muscle, $P = 2.2 \times 10^{-16}$ and amnion, $P = 3.1 \times 10^{-14}$) (Fig. 6B; Supplemental Fig. 4). These findings suggest that ZFP57 and H3K9me3 may, at least partially, be important for the maintenance of monoallelic 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 publicly available RNA-seq data sets (Bernstein et al. 2010; Yan et al. 2013; Metsalu et al. 2014). Fifty percent of the placental-specific DMRs had an associated gene that was expressed in at least one developmental stage (RPKM > 0.1) (Fig. 6C; Supplemental Table 6). However, gene ontology analysis revealed no enrichment for pathways typically associated with imprinted genes, such as fetal growth or placentation. The top gene ontology terms included alternative splicing, expression in the brain, splice variants, and the extracellular region (Supplemental Table 7).

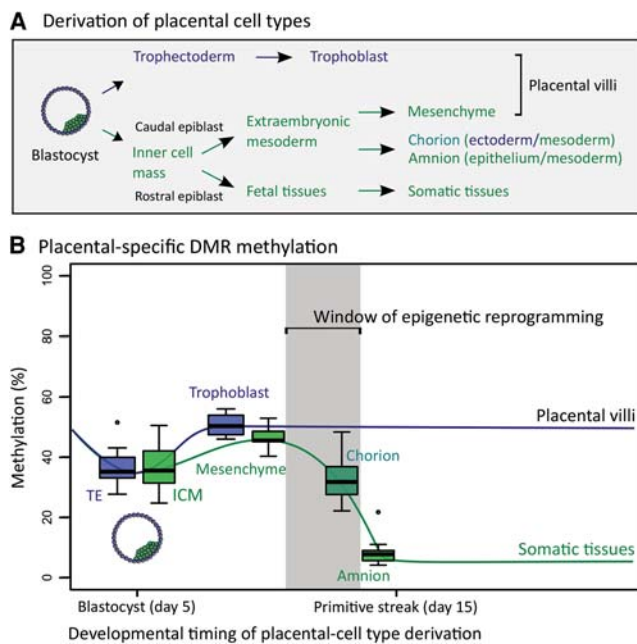


Figure 5. Timing of loss of imprinting in human embryonic lineages. (A) Schematic representation of the developmental origins of the extraembryonic cell types is shown, with TE-derived cells (dark blue), ICM-derived cells (green), and cells with mixed origin (turquoise) denoted. (B) The distribution of DNA methylation for known placental-specific DMRs that lose DNA methylation in somatic tissues ($N = 14$) is shown for extraembryonic 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. Extraembryonic 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 likely being erased in the ICM-derived lineage.

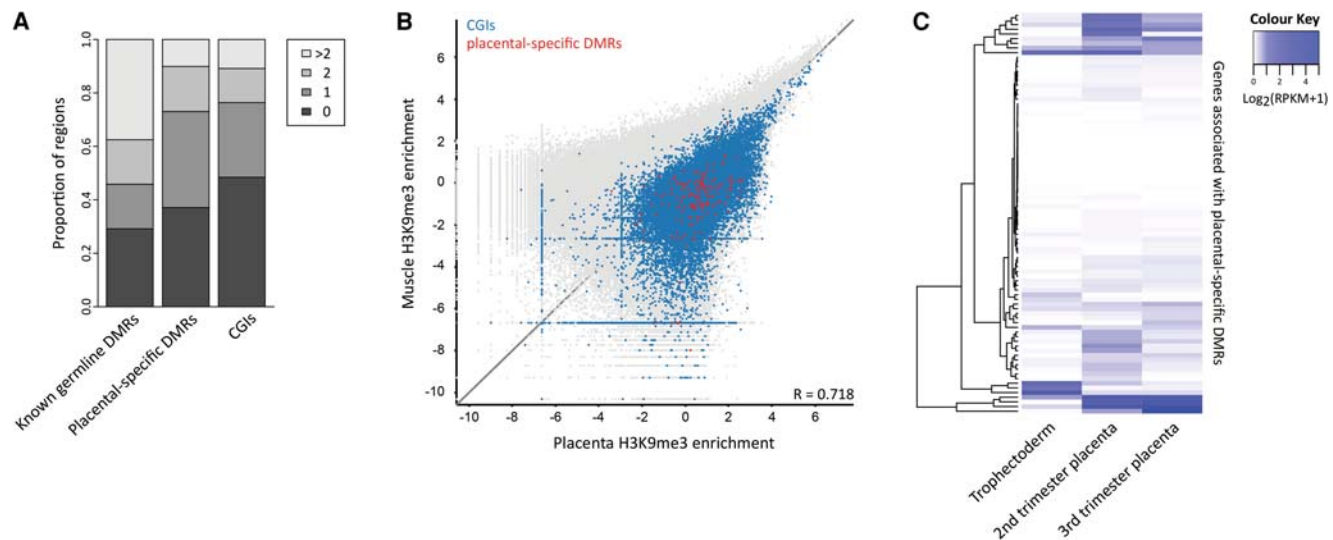


Figure 6. Characteristics of placental-specific DMRs. (A) The proportion of known imprinted germline DMRs, placental-specific DMRs, and CGIs with one or more ZFP57 binding site consensus sequences is shown. (B) Enrichment for H3K9me3, using 1000-bp sliding windows with a 1000-bp step, was compared between human placenta and fetal muscle. Using the absolute difference for each probe, placental-specific DMRs ($P = 2.2 \times 10^{-16}$, *t*-test) and CGIs ($P = 2.2 \times 10^{-16}$, *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 (Supplemental Table 6) is shown in TE, and 2nd and 3rd trimester placental villi.

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. Fifteen known placental-specific DMRs did show differential germline methylation. This is supported by a similar finding in an independent study of oocyte DNA methylation (Okoe 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; Kobayashi et al. 2012; Guo et al. 2014), 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 monoallelic 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 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 140 loci in placenta. With only two 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 fertilization, 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) and 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 monoallelic 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 that 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 overwritten 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 monoallelic methylation in regulating transcription remains unclear and is likely not consistent across loci. Placental-specific DMRs have been shown to regulate monoallelic expression, including *DNMT1* (Yuen et al. 2011a; Das et al. 2013) and the Chromosome 19 microRNA cluster (C19MC) (Noguer-Dance et al. 2010). There are also examples of loci where biallelic 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 monoallelic methylation of *Rasgrf1* is widespread across tissues but only mediates monoallelic 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 (Haig and Westoby 1989; Moore and Haig 1991), which suggests imprinted genes arose through competing interests of the maternal and paternal genetic contributions to the developing fetus. 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 (Solter 1988; Hall 1990) 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 fetal development and maternal resource management (Keverne et al. 1996; Keverne and Curley 2008). 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 behavior 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 support 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 extraembryonic tissues and its importance in pregnancy and development.

Methods

Samples

All placenta, fetal 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) (Yuen et al. 2011a,b; Price et al. 2012). 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 wk and included 37 male and 38 female conceptions. The preterm births were associated with one or more of the following: preterm labor, 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 five diandric and five digynic triploids pregnancies were assayed. The average (range) gestational ages for diandric and digynic triploids were 11.2 (8–13) wk and 9.4 (8–12) wk ($P = 0.18$), respectively.

Thirty-two fetal tissues, including brain ($N = 7$), spinal cord ($N = 7$), muscle ($N = 9$), and kidney ($N = 9$) were collected from second trimester fetuses, as previously described (Price et al. 2012). Adult female whole-blood samples ($N = 10$) were collected from control women. Extraembryonic 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 DNasey Blood and Tissue Kit (Qiagen), DNA was extracted and purified for all samples. For each sample, 750 ng of DNA was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research Corporation). DNA from five diandric triploids, five digynic triploids, 63 control placental villi, 32 fetal tissues, 10 adult blood, and 19 extraembryonic cell types were run on the Infinium HumanMethylation450 BeadChip array (Illumina). The arrays were processed as per the manufacturers' guidelines, and data were collected on the HiScan System (Illumina).

Using GenomeStudio software (Illumina), 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 ($N=4$), zygotes ($N=2$), two-cell embryos ($N=2$), four-cell embryos ($N=2$), eight-cell embryos ($N=3$), morulae ($N=3$), ICM ($N=3$), and TE ($N=3$) from blastocysts was obtained using publicly available low-input RRBS data (GSE49828) (Guo et al. 2014). All bisulfite sequencing data described in this study were processed in the same manner, unless otherwise specified: Quality was evaluated using FastQC Version 0.11.2, adapters 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 were analyzed 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 Supplemental Table 4.

For comparison to mouse, DNA methylation for oocytes, sperm, ICM, TE, E6.5 epiblast, E6.5 extraembryonic ectoderm, placenta, heart, liver, and brain was obtained from publicly available data sets (GSE51239, GSE42836, GSE34864) (Smith et al. 2012, 2014; Hon et al. 2013). 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). Of placental-specific DMRs, 88.6% had a mapped orthologous region in the mouse genome, and 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 four paternal blood) were performed as described above. One hundred fifty-

one PCR assays were designed for the 43 known and 101 novel DMRs (Supplemental 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 $1\times$ HotStar PCR buffer, 0.2 mM dNTPs (Life Technologies), 1 U HotStar Taq (Qiagen), 0.4 μ M each primer pair, and 2 ng DNA, were performed in a 15- μ L reaction volume in 384-well plates. Plates were prepared on the Agilent Bravo Workstation (Agilent Technologies). Cycling conditions included a 15-min, 95°C denaturation, followed by 30 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 90 sec, with a 5-min final extension at 72°C, and reactions were performed on the C1000 Touch Thermal Cycler (Bio-Rad).

PCR products were pooled for each sample and SPRI-purified using Sera-Mag carboxylate-modified Magnetic SpeedBeads (Fisher Scientific) as per the manufacturer's recommendations. A-overhang addition was done using $1\times$ NEB buffer 2, 0.2 mM dATP, 12.5 U Klenow fragment (3'→5' exo-) (New England Biolabs) in a 25- μ L reaction incubated at 37°C for 30 min. Following SPRI purification, Illumina adapters were ligated using the Quick ligation kit (New England Biolabs) in a 40- μ L reaction. Following another SPRI purification, libraries were amplified (11 cycles) and barcoded using the Sanger eight-base index in a 50- μ L reaction containing: $1\times$ High Fidelity buffer, 1 U Phusion High Fidelity Taq (New England Biolabs), 0.2 mM dNTPs, and 0.1 μ M each primer. Final libraries were SPRI-purified, and concentration and quality were assessed by the 2100 Bioanalyzer system (Agilent Technologies) and Kapa library quantification (Kapa Biosystems). The 48 barcoded libraries were pooled and run on the Illumina MiSeq System (Illumina) using 150-bp 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 (Supplemental 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 publicly available RNA-seq data sets (Bernstein et al. 2010; Yan et al. 2013; Metsalu et al. 2014). Single-cell RNA-seq was utilized for TE (GSE36552), mRNA-seq for term placenta (GSE56524), and total RNA-seq for mid-gestation placenta (GSE18927). The single-cell RNA-seq data were 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 color space). Aligned BAM files for the total RNA-seq in mid-gestation placenta were 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 (Supplemental Table 6). Two genes were missed due to gene annotation differences between UCSC and Ensembl, the gene annotation track in SeqMonk version 0.29.0, resulting in 86/88 DMRs being reported. The data are presented using the natural log transformed RPKM+1.

Histone modifications

ChIP-seq

Publicly available ChIP-seq data for H3K9me3 in placental villi, amniotic membrane, and fetal muscle (GSE18927) (Bernstein et al. 2010) were assessed in SeqMonk version 0.29.0. Relative enrichment was assessed by calculating the difference (Δ) between placenta and somatic tissue (muscle or amnion) for each 1000-bp probe. Values of Δ enrichment 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 (Supplemental Table 1). Known imprinted DMRs were those that overlapped a previously reported human imprinted DMR with adequate coverage on the 450K array ($N=57$) (Supplemental Table 2; Court et al. 2014). Germline novel DMRs were those that were CGIs (defined by UCSC Genome Browser [<https://genome.ucsc.edu/>]) that (1) were >50% differentially methylated between sperm and oocytes, (2) were intermediately methylated (15%–60%, based on the 90th percentile observed at known imprinted DMRs) in the blastocyst (average of ICM and TE), (3) showed a >5% difference in methylation between diandric and digynic triploids, and (4) showed matching parental origin of DNA methylation between triploid villi 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 categorized 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 customized Perl scripts and compared using a χ^2 test.

Gene ontology

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) gene ontology program was used on genes associated with placental-specific DMRs (Supplemental Table 6; Huang et al. 2009a,b).

Data access

Illumina Infinium HumanMethylation450 array data and multiplexed targeted bisulfite sequencing from this study have been submitted to the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GSE74738 and GSE76273.

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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 data and helped with experimental design and interpretation. H.S. performed computational analyses. S.A. processed and analyzed allelic bisulfite sequencing data. D.E.M. collected and sampled human fetal tissues. G.K. and W.P.R. supported the study, assisted with experimental design, and helped with manuscript preparation.

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