

# Limited evolutionary conservation of imprinting in the human placenta

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The epigenetic phenomenon of genomic imprinting provides an additional level of gene regulation that is confined to a limited number of genes, frequently, but not exclusively, important for embryonic development. The evolution and maintenance of imprinting has been linked to the balance between the allocation of maternal resources to the developing fetus and the mother's well being. Genes that are imprinted in both the embryo and extraembryonic tissues show extensive conservation between a mouse and a human. Here we examine the human orthologues of mouse genes imprinted only in the placenta, assaying allele-specific expression and epigenetic modifications. The genes from the *KCNQ1* domain and the isolated human orthologues of the imprinted genes *Gatm* and *Dcn* all are expressed biallelically in the human, from first-trimester trophoblast through to term. This lack of imprinting is independent of promoter CpG methylation and correlates with the absence of the allelic histone modifications dimethylation of lysine-9 residue of H3 (H3K9me2) and trimethylation of lysine-27 residue of H3 (H3K27me3). These specific histone modifications are thought to contribute toward regulation of imprinting in the mouse. Genes from the *IGF2R* domain show polymorphic concordant expression in the placenta, with imprinting demonstrated in only a minority of samples. Together these findings have important implications for understanding the evolution of mammalian genomic imprinting. Because most human pregnancies are singletons, this absence of competition might explain the comparatively relaxed need in the human for placental-specific imprinting.

histones | methylation | epigenetics

Genomic imprinting is an epigenetic phenomenon that results in monoallelic expression of certain genes in a parent-of-origin-dependent manner (1). Although found in angiosperm plants and marsupials, it has been suggested to be one of the most important regulatory pathways involved in the development and function of the placenta in eutherian mammals.

Imprinted genes are often found in close proximity to each other, indicating coordinate regulation by imprinting control regions (ICRs) (reviewed in ref. 1). Distal mouse chromosome 7 (mChr7) harbors the largest known cluster of imprinted genes and has conserved synteny with human chromosome 11p15.5 (hChr11). This cluster has been implicated in disorders such as Beckwith-Wiedemann and Silver-Russell syndrome, malignancies, and aberrant fetal growth (2, 3). Two ICRs controlling different sets of genes have been identified within this >1-Mb cluster, dividing it into two functionally independent groups. The centromeric domain is controlled by the paternally methylated *H19* ICR (1), whereas the telomeric potassium voltage-gated channel, subfamily Q, member 1 (*KCNQ1*)/*Kcnq1* domain is controlled by potassium voltage-gated channel differentially methylated region 1 (KvDMR1) (4), a CpG island within intron 10 of the *Kcnq1*/*KCNQ1* gene that is maternally methylated (5). In the mouse, 14 imprinted transcripts flank this ICR. The majority are expressed predominantly from the maternal allele, with imprinting of the most distal genes restricted to the placenta (6, 7). Interestingly, the placental imprinting of

genes within this domain does not depend on DNA methylation at their promoters (5, 7, 8). The KvDMR1 is also the promoter for the *KCNQ1* overlapping transcript 1 (*Kcnq1ot1*)/*KCNQ1OT1* (*LIT1*) noncoding RNA (ncRNA) (4, 9) that, because of the DNA methylation and abundance of repressive chromatin modifications on the maternal allele, is paternally expressed (6, 7). Polycomb group proteins are thought to be recruited to the paternal allele, potentially by the *Kcnq1ot1*/*KCNQ1OT1* ncRNA, contributing to the paternal repression of the imprinted genes in cis (6).

A second cluster in the mouse that contains placental-specific imprinted genes maps to mChr17. This cluster contains the maternally expressed insulin-like growth factor receptor 2 (*Igf2r*) transcript and two maternally expressed placental-specific genes, *Slc22a2* and *Slc22a3* (10). This cluster shares several similar features with the *KCNQ1* domain. These features include an intronic ICR (region 2 ICE) within the *Igf2r* gene, which is the functional promoter for the paternally expressed, unspliced ncRNA *Air* (11, 12). Also, the paternal silencing observed within the *Igf2r* domain is associated with repressive chromatin (13, 14).

In this report, we have assessed the imprinting status for the human orthologues of all reported mouse placental-specific imprinted genes. In contrast to the maternal expression observed in the mouse, the human expression is largely biallelic. We demonstrate that the lack of imprinting correlates with a lack of allelic chromatin modifications.

## Results

**Allelic Expression and Methylation Within the *KCNQ1* Domain.** To perform an investigation of imprinting across the human *KCNQ1* domain, we have analyzed allelic expression, promoter DNA methylation, and histone modifications at specific loci. All genes whose orthologues exhibit placental-specific maternal expression in the mouse, and under the control of the KvDMR1 (*NAPIL4*, *CD81*, *PHEMEX*, and *ASCL2*) (4, 6, 15, 16) were found to be expressed biallelically in human fetal tissues and in first-trimester and term placental samples (Fig. 1; see also Table 1, which is published as supporting information on the PNAS web site). In contrast, we observed conserved imprinted expression for pleckstrin homology-like domain, family A, member 2 (*PHLDA2*), *SLC22A18*, *SLC22A15*, and cyclin-dependent kinase inhibitor 1C (*CDKN1C*), with monoallelic expression being detected in all tissues at all gestational ages tested (Fig. 1 and Table 1). These genes are located immediately centromeric to the KvDMR1. The orthologues of these genes are imprinted in both the fetus and placenta in the mouse (6). This data suggests that they are most likely imprinted by a mechanism that is different to that regulating placental-specific imprinting. Im-

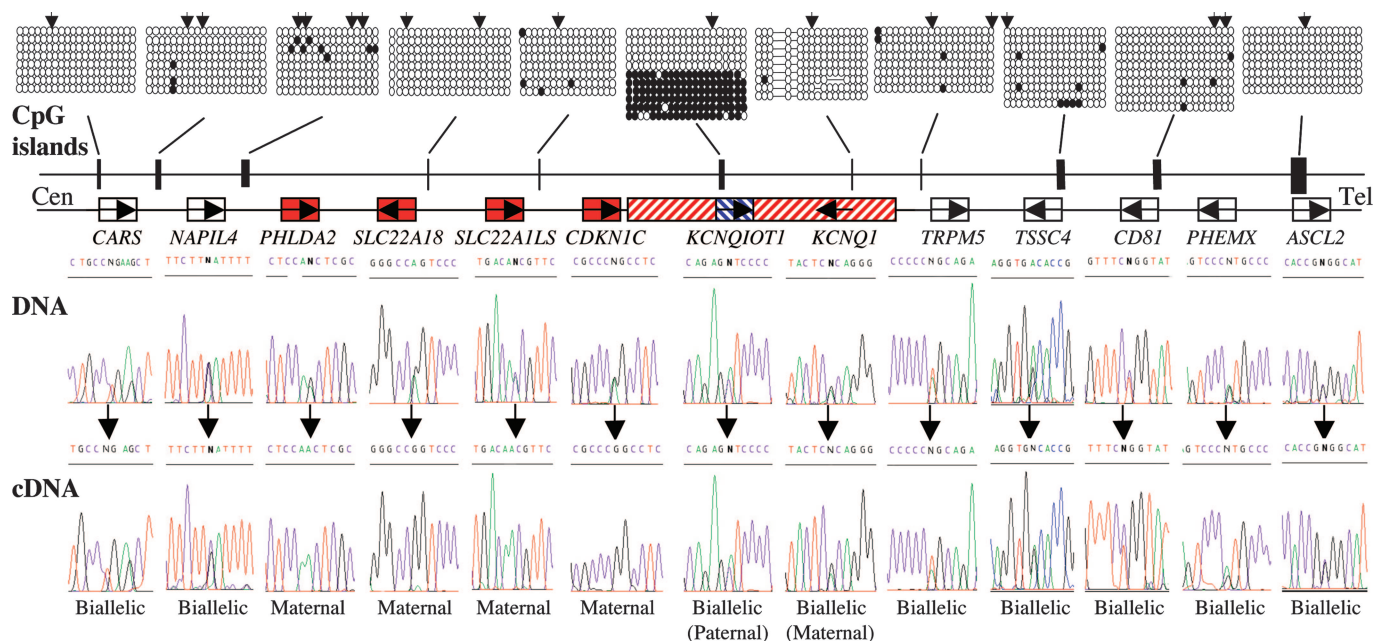
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Abbreviations: ChIP, chromatin immunoprecipitation; ICR, imprinting control region; DMR, differentially methylated region; ncRNA, noncoding RNA.

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**Fig. 1.** Schematic representation of the *KCNQ1* domain on human chromosome 11p15.5 showing the relative organization of genes and CpG islands. The methylation status of all promoter CpG islands was examined in liver-, muscle-, lymphocyte-, and placenta-derived DNA. Methylation patterns were assessed first by restriction digestion of bisulphite PCR products. The positions of the restriction sites used are shown ( $\downarrow$ ). Example of patterns obtained by sequencing bisulphite PCR product obtained from placenta-derived DNA. Similar patterns were obtained in all tissues analyzed. Each circle represents a single CpG dinucleotide on the strand, a methylated cytosine (●) or an unmethylated cytosine (○). For clarity, only the first 20 CpG dinucleotides from each CpG island are shown. In all tissues analyzed, the only evidence for differential methylation was at the human KvDMR1/*KCNQ1OT1* promoter; all other promoter CpG islands were hypomethylated. The imprinted expression for all genes in the cluster was analyzed in first-trimester fetal tissues, term placenta, and cytotrophoblast cells. DNA sequence traces for heterozygous term placenta samples are shown for all genes and the resulting RT-PCR sequences. Open boxes depict genes biallelic in all tissues throughout gestation, whereas red boxes represent ubiquitously imprinted, maternally expressed genes. The hatched red box presents the maternal-specific expression of *KCNQ1* limited to first-trimester fetal tissues only. The blue hatched box symbolizes the paternal-specific expression of the *KCNQ1OT1* transcript observed in first-trimester material only. Both of these genes are biallelically expressed in term placenta.

printing also was observed for the *KCNQ1* and *KCNQ1OT1* transcripts in early fetal tissues and first-trimester placentae, but imprinting was relaxed in term placentae (Fig. 1 and Table 1).

In the mouse, ubiquitous imprinting of *Cdkn1c* has been linked to somatic differential DNA methylation in the promoter regions of this gene, which is maintained by Dnmt1 and Lsh (7, 17). We therefore used bisulphite sequencing and Southern blotting to determine the DNA methylation pattern of all genes in the human cluster. Only one differentially methylated region (DMR) was found, which mapped to the previously described KvDMR1 (18). The promoter CpG islands of all of the other genes were hypomethylated in the placenta, liver, muscle, and lymphocyte DNA (Fig. 1 and data not shown). This result suggests that somatic allelic DNA methylation, such as at *Cdkn1c*, may be dispensable for conserved imprinting.

#### Modified Histones and Paternal Repression Within the *KCNQ1* Domain.

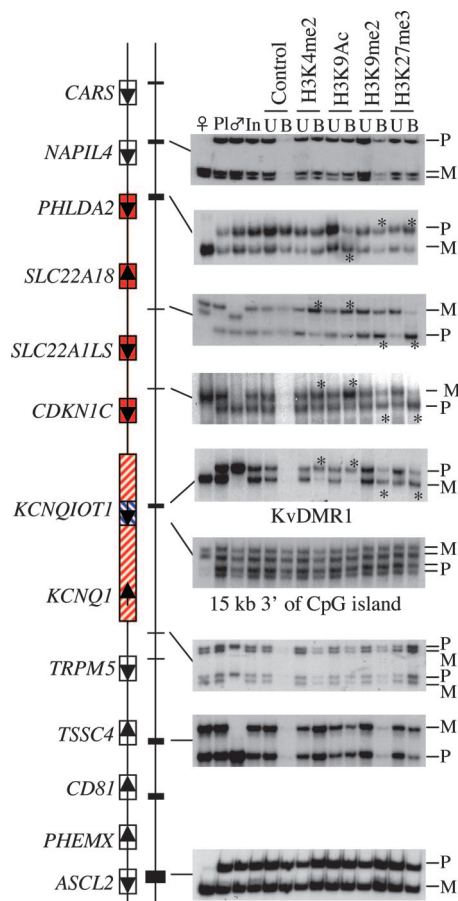
Because of the general lack of DMRs associated with the genes showing maintained ubiquitous imprinted expression, we performed qualitative chromatin studies on both sides of the KvDMR1 to determine differences between the parental chromosomes. Our analysis focused on the trimethylation of Lys-27 on histone H3 (H3K27me3) and dimethylation at Lys-9 on histone H3 (H3K9me2) as markers of repressive chromatin and on acetylation at Lys-9 on histone H3 (H3K9ac) and dimethylation of Lys-4 on histone H3 (H3K4me2) as markers of active chromatin. Qualitative chromatin immunoprecipitation (ChIP) was performed on unfixed chromatin fragments prepared from term placenta samples. In all informative placenta samples, enrichment of H3K4me2 and H3K9ac on the maternal chromosome and H3K9me2 and H3K27me3 on the paternal chromosome was limited to those genes

centromeric to the KvDMR1, which showed conserved imprinted expression. None of the other genes showed allele-specific enrichment for these chromatin markers (Fig. 2; see also Table 2, which is published as supporting information on the PNAS web site).

At the KvDMR1 region, we detected an opposite allelic pattern, with enrichment of maternal H3K9me2 and H3K27me3 and paternal H3K4me2 and H3K9ac. These data are consistent with transcription from the paternal allele at this promoter. This pattern also was present within the DMR, but not at a distance of 15 kb or 25 kb within the transcribed region of this ncRNA in a first-trimester placenta (10 and 17 weeks; data not shown) or at term (Fig. 2 and Table 2). This finding suggests that the *KCNQ1OT1* ncRNA does not cause changes to the chromatin over its entire transcribed region in placenta, at least for the modifications analyzed here. Nevertheless, *in vivo* studies suggest that this ncRNA is essential for epigenetic silencing (19).

**Polymorphic Imprinting Within the Human *IGF2R* Cluster.** To determine whether histone modifications account for placental-specific imprinting in other imprinted clusters, the imprinting status of genes within the *IGF2R* domain on hChr6q25.3 was analyzed. In the mouse, this cluster contains two genes that are paternally repressed in the placenta without the involvement of promoter DNA methylation (10, 12). Interestingly, this cluster is polymorphically imprinted in humans, with the majority of individuals showing biallelic expression (20, 21). Here we also found *IGF2R* and *SLC22A2* to be polymorphically imprinted, with monoallelic expression detected only in a minority of term placenta samples (Fig. 3 and Table 1). Four of these samples were heterozygous for both genes. Expression was concordant in each placenta, two showing maternal expression and two with





**Fig. 2.** Allele-specific analysis of histone modifications in term placenta by using antibodies against H3K4me2, H3K9ac, H3K9me2, and H3K27me3. For each ChIP, representative data for the placental (pl) and parental ( $\delta$  and  $\eta$ ) genotypes, ChIP input (In), unbound fraction (U), and antibody-bound (B) are shown. SNP accession no. and locations are given in Table 3, which is published as supporting information on the PNAS web site. The relative enrichment (\*,  $>2$ -fold) of the maternal allele in precipitated H3K4me2 (*CDKN1C* 2.1; *SLC22A18* 4.1; no enrichment at *PHLDA2* exon 2) and H3K9ac (*CDKN1C* 2.1; *SLC22A18* 2.1; *PHLDA2* 2.5), and for the paternal allele in precipitated H3K9me2 (*CDKN1C* 2.8; *SLC22A18* 2.3; *PHLDA2* 4.4) and H3K27me3 (*CDKN1C* 10.1; *SLC22A18* 6.9; *PHLDA2* 3.6), is limited to genes with ubiquitous maternal-specific expression. An opposite allelic pattern was observed at the KvDMR1 with relative enrichment of paternal H3K4me2 (3.9) and H3K9ac (2.9) and maternal H3K9me2 (2.1) and H3K27me3 (5.0) precipitation. No enrichment was observed within the transcribed *KCNQ1OT1* region.

biallelic expression for both genes. We also analyzed the imprinting of *SLC22A1* and *SLC22A3* genes in the same region. Similar to the mouse, *SLC22A1* was biallelically expressed in all tissues and at all gestational ages tested (Fig. 3 and Table 1); *SLC22A3* exhibited monoallelic expression in first-trimester placenta (maternal DNA sample was heterozygous) but biallelic expression in fetal tissues (of the only heterozygous first-trimester sample set) and term placenta (data not shown). The temporal pattern of imprinting of *SLC22A2* and *SLC22A3* is similar to that reported for mice (10).

Next we compared the DNA methylation and chromatin configuration for the four samples that showed concordant imprinting or biallelic expression for *IGF2R* and *SLC22A2*. The intronic ICR (called region 2 ICE) was the only DMR identified, and this region showed an indistinguishable DNA methylation profile between samples with imprinted or biallelic expression (21) (Fig. 3 and data not shown). We observed no allelic enrichment for active or repressive chromatin markers at gene

promoters throughout the region for all samples (Table 2; see also Fig. 5, which is published as supporting information on the PNAS web site). This lack of allelic histone modification has been reported in a human cell line, although imprinted expression was not analyzed (22). The mouse *Igf2r* gene contains two DMRs: the germ-line methylated intronic region 2 ICE and the somatic DMR at the promoter CpG island of the *Igf2r* gene (23). Both these regions are associated with modified histones (13, 14); however, in the *Eed* null mice, the imprinting of *Igf2r* is unaffected (24), suggesting that H3K27 methylation is not involved in the imprinting of this gene. This contrast with our human data suggests that a mechanism other than the analyzed histone modifications regulates this domain. The paternally expressed *Air* ncRNA might fulfill this role; however, as was reported by others (25), we were unable to determine whether the placenta samples that exhibited imprinted expression were associated with polymorphic human *AIR* expression.

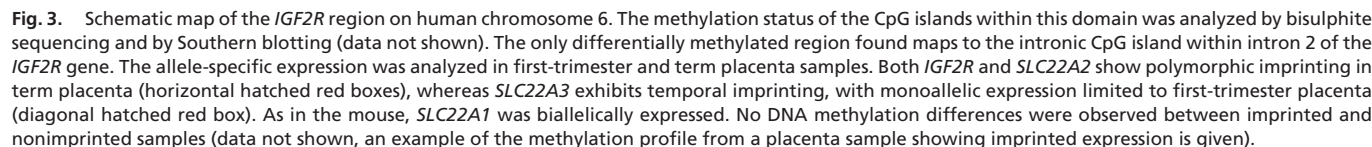
**The Biallelic Expression of *DCN* and *GATM*.** We have analyzed the human imprinted expression of genes on other chromosomes that exhibit placental-specific imprinting in the mouse, glycine amidinotransferase (*Gatm*) (mChr2) and decorin (*Dcn*) (mChr10) (26, 27). In the mouse, both are isolated imprinted genes without associated DMRs. Both human orthologues are biallelically expressed in first-trimester fetal and placenta samples, term placenta, and immunoselected trophoblast cells. The promoters of both genes are not associated with enriched chromatin modifications, suggesting that the lack of allele-specific repressive chromatin in the human placenta also accounts for the lack of imprinting (Fig. 4 and Tables 1 and 2).

## Discussion

We have analyzed the expression and epigenetic regulation of four imprinted regions in the human. This study has revealed consistent differences in imprinting patterns between mice and humans. In all regions assessed, the mouse has placenta-specific imprinted expression, regulated not by DNA methylation, but most likely by histone modifications (6, 7). Our data indicate that the discrepancy in imprinting between mouse and human correlates perfectly with the lack of allele-specific histone modifications.

Recent studies in the mouse have revealed developmental and mechanistic parallels between imprinted X inactivation and autosomal imprinting of placenta-specific genes (28–30). Both mechanisms are hypothesized to have coevolved with the development of the placenta and are thought to depend on the presence of ncRNAs and the recruitment of histone-modifying enzymes. The establishment of X inactivation requires the ncRNA, *Xist*, to be expressed from the paternal X chromosome, whereas the autosomal ncRNAs *Kcnq1ot1* and *Air* may function in a similar manner (30). However, although mouse placental *Xist* expression is imprinted, it is biallelic in the human (31, 32). Therefore, human extraembryonic random X inactivation correlates with the lack of autosomal placental imprinting, suggesting the loss of a common mechanism.

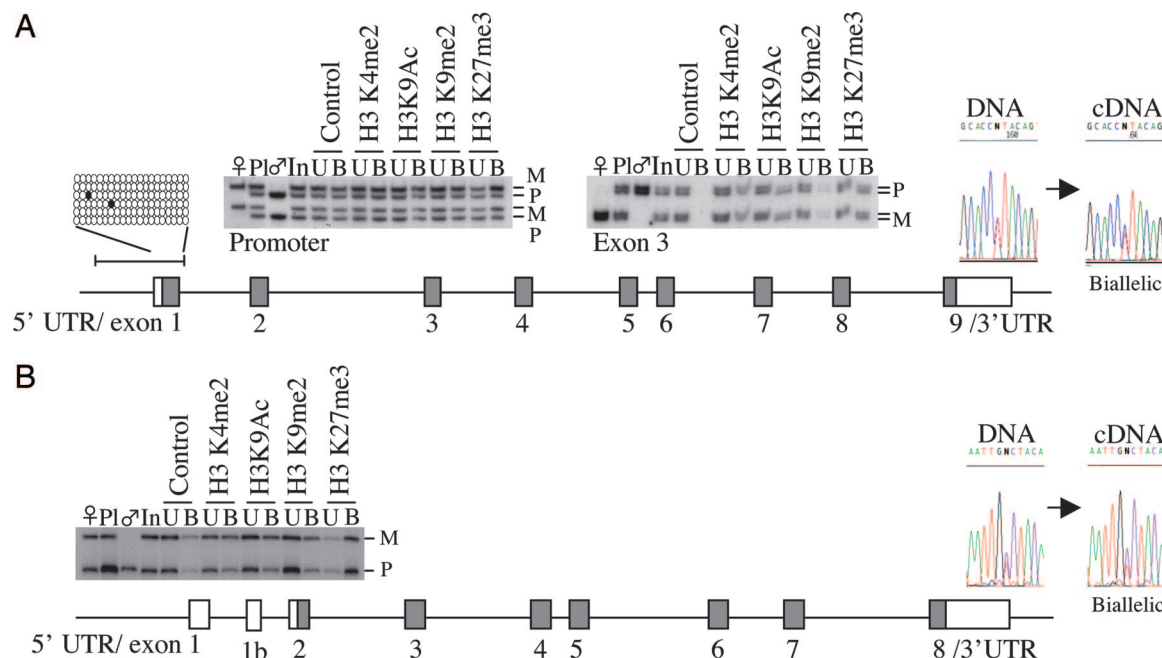
The lack of autosomal placental-specific imprinting is clearly demonstrated for the genes mapping to the *KCNQ1* domain. In the mouse orthologous region, there are 14 imprinted transcripts, of which 8 are expressed from the maternal allele in the placenta. In contrast, there are only six imprinted genes in the human, representing those that are ubiquitously imprinted. The evolutionary loss of imprinting in this cluster, therefore, is restricted to those that are placenta-specifically imprinted. It is possible that in the mouse, the placental-specific imprinting may be due to a bystander effect. To date, there is no direct evidence that the placenta-specific genes within this cluster need to be imprinted, only expressed (as in the case of *Ascl2*; ref. 16). Imprinting of these genes, therefore, may be a consequence of



We have confirmed that imprinting of the *IGF2R* gene is polymorphic between individual placentae (20, 25) and that this polymorphic trait extends to the *SLC22A2* and *SLC22A3* genes in a minority of individuals. In its imprinted configuration, the temporal expression patterns for the two placental-specific genes within the cluster mimic that of the mouse. This domain, therefore, may represent a region undergoing a transition to become biallelically expressed throughout the whole population. Interestingly, the epigenetic status at the chromatin and DNA methylation level within this domain is indistinguishable between imprinted and nonimprinted individuals. However, imprinting has been reported for *IGF2R* (opossum) and *MEST* (wallaby) genes in marsupials (35, 36), but there is no evidence for differential DNA methylation within the ICRs of both genes. These findings suggest that some imprinted genes either have developed alternative imprinting mechanisms between species or they share mechanisms that have yet to be identified.

evolution, and many explanations for the evolution of genomic imprinting implicate the placenta as a key tissue. A substantial proportion of imprinted genes are involved in the control of fetal growth and, in general, paternally expressed imprinted genes enhance fetal growth, whereas maternally expressed genes suppress it (37, 38). These parent-of-origin effects on fetal growth and development have resulted in the "parental conflict hypothesis," based on kinship theory, as the dominant theory of the origin of genomic imprinting (39). This hypothesis predicts that imprinting has evolved because of conflict of resource provisions by the mother to her offspring through the placenta and that paternally expressed genes are "selfish" and are selected to extract more resources from the mother, whereas maternally expressed genes have to balance the nutrition provision to the current fetus with that of future fetuses of the mother (but potentially different fathers). Maternally derived genes, therefore, are more conservative with regards to provision to the fetus. It is possible that mice have acquired an expansion of imprinting to enable the placenta to become more efficient over a much shorter gestational period. This expansion may have led to an accelerated requirement for resource provisioning genes and their regulators such that many more genes have become imprinted in placenta. Nevertheless, most human pregnancies are singletons in contrast to the mouse, where there is increased chance of intralitter competition. Therefore, this lack of competition relieves the pressure for maintaining placental-specific imprinting. With respect to human growth and nutrient transfer, more emphasis may lie with imprinted genes involved in postnatal adaptation and maternal behavior.

**Collection of Human Material.** A total of 45 fetal tissue sets (8–18 weeks) with corresponding maternal blood samples were obtained from termination of pregnancies at Queen Charlotte's and Chelsea Hospital, London. Local ethical approval for obtaining fetal tissues was granted by the Research Ethics Committee of Hammersmith, Queen Charlottes's and Chelsea,



**Fig. 4.** Absence of placental imprinting at the human *GATM* and *DCN* genes. (A) The human *GATM* gene maps to human chromosome 15q21.1 and its mouse orthologue exhibits maternal-specific expression limited to placenta. The human *GATM* promoter is hypomethylated in all tissues analyzed (bisulphite sequence data from placenta-derived DNA shown), and the gene is biallelically expressed in all fetal and term samples when analyzing coding SNPs in either exon 3 or the 3' UTR. No allele-specific histone modifications were detected within the promoter region or exon 3. (B) The human *DCN* gene maps to human chromosome 12q21.33, and the mouse orthologue displays maternal-specific expression limited to the placenta. Using a SNP in the 3' UTR, all isoforms of *DCN* were biallelically expressed. The figure shows the DNA sequence trace used for genotyping and a subsequent RT-PCR that amplifies all isoforms. No allele-specific histone modifications were detected within the promoter region.

and Acton Hospitals' Research Ethics Committee (2001/6028). A set of 240 term placental trio samples consisting of multiple-site placental samples with corresponding maternal and paternal blood samples were collected from consecutive consenting pregnancies at Queen Charlotte's and Chelsea Hospital (local ethics approval 2001/6029). All samples were washed in sterile PBS and snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**Enrichment for Uncultured Human Villous Trophoblasts.** A detailed protocol for the isolation of villous trophoblasts is available on request. Briefly, we used a protocol of placental tissue digestion and negative immunoselection. Dissected placental tissues from both the first trimester (8–14 weeks) and term were thoroughly washed in PBS and digested with a mixture of trypsin and DNase to release free cells. Unwanted erythrocytes were removed from the resulting cell suspension by centrifugation through a 40% Percoll solution. The resulting trophoblasts were subjected to negative immunoselection by using monoclonal anti-HLA class 1 (clone w6/32). All trophoblast cell preparations were subjected to cytokeratin 7 immunocytochemistry (>98% cytokeratin 7-positive cells; data not shown) and HLA class 1 and Vimentin to assess cell contamination (<1.3%; data not shown).

**Analysis of Allelic Expression.** We extracted total RNA from tissues by using TRIzol Reagent (Invitrogen). After digestion with RNase-free DNase 1 (Invitrogen), we generated first-strand cDNA with Moloney murine leukemia virus reverse transcriptase (Promega) by using random primers. Duplicate sets of samples were processed, with reverse transcriptase omitted to detect genomic DNA contamination of the RNA. The presence of cDNA was confirmed by using a *GAPDH* primer set.

All polymorphisms were identified by interrogating SNP

databases or genomic sequencing and confirmed by sequencing control DNAs (primer sequences are given in Table 4, which is published as supporting information on the PNAS web site). All PCR products were sequenced in both the forward and reverse orientation by using an ABI Prism 3100 DNA sequencer (Applied Biosystems).

**Methylation Analysis.** For bisulphite sequencing, DNA was converted by using EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). PCR amplification (primer sequences are given in Table 4), cloning, and sequencing were performed by using standard protocols. Combined bisulphite restriction analysis was used to assess the methylation pattern of the amplified region in the overall PCR product and to ensure that there was no cloning bias before sequencing. Southern blotting by using methylation-sensitive restriction enzymes such as *SacII* and *HpaII* was performed according to standard protocols.

**ChIP.** Twelve individual human term placenta samples were analyzed by ChIP. This method generates qualitative differences in allelic enrichment although no conclusions can be drawn on quantitative levels (40). Specificity and efficiency of the ChIP assay was determined by allele specific SSCP or HOT-STOP RFLP analysis of antibody bound (precipitated) and unbound (nonprecipitated) fractions. Appropriate allelic enrichment in the antibody bound fraction is frequently accompanied by a paralleled depletion in the unbound fraction. Mock (control) precipitations indicate specificity of the antibody, ideally, but not always, resulting in no product in the control-bound fraction. Approximately 300 mg of material was used for each ChIP assay. We carried out ChIP as described in refs. 6 and 41. Briefly, we purified nuclei on a sucrose gradient and incubated them with MNase to obtain chromatin fragments of one to six nucleosomes in length. We incubated 20  $\mu\text{g}$  of chromatin with 10  $\mu\text{g}$  of



antibody overnight at 4°C and then incubated it for 4 h with Protein-A Sepharose beads. For ChIP, we used the following antisera (Upstate Biotechnology) directed against H3K4me2 (07-030), H3K9me2 (07-212), H3K9ac (06-942), and an affinity-purified antiserum against H3K27me3 (donated by Y. Zhang, University of North Carolina, Chapel Hill). We eluted the chromatin-antibody complexes from the beads and purified the DNA by phenol-chloroform extraction and ethanol precipitation. Where possible all polymorphisms mapped within 2 kb of the transcription start site or within the first exon (primer sequences are given in Table 3). Only ChIP sample sets that showed enrichment for control regions were used in the analysis. Relative band intensities of the maternal and paternal bands

were determined by using IMAGEMASTER VDS (Amersham Pharmacia Biotech).

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