Imprinting at the PLAGL1 domain is contained within a 70-kb CTCF/cohesin-mediated non-allelic chromatin loop

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ABSTRACT

Paternal duplications of chromosome 6q24, a region that contains the imprinted PLAGL1 and HYMAI transcripts, are associated with transient neonatal diabetes mellitus. A common feature of imprinted genes is that they tend to cluster together, presumably as a result of sharing common cis-acting regulatory elements. To determine the extent of this imprinted cluster in human and mouse, we have undertaken a systematic analysis of allelic expression and DNA methylation of the genes mapping within an ∼1.4-Mb region flanking PLAGL1/Plagl1. We confirm that all nine neighbouring genes are biallelically expressed in both species. In human we identify two novel paternally expressed PLAGL1 coding transcripts that originate from unique promoter regions. Chromatin immunoprecipitation for CTCF and the cohesin subunits RAD21 and SMC3 reveals evolutionarily conserved binding sites within unmethylated regions ∼5 kb downstream of the PLAGL1 differentially methylated region and within the PLAGL1 3′ untranslated region (UTR). Higher-order chromatin looping occurs between these regions in both expressing and non-expressing tissues, forming a non-allelic chromatin loop around the PLAGL1/Plagl1 gene. In placenta and brain tissues, we identify an additional interaction between the PLAGL1 P3/P4 promoters and the unmethylated element downstream of the PLAGL1 differentially methylated region that we propose facilitates imprinted expression of these alternative isoforms.

INTRODUCTION

Genomic imprinting is defined by genotype-independent expression from either the maternal or paternal allele. This parent-of-origin expression is mediated by epigenetic modifications that differ between the two parental chromosomes (1). There are currently more than 60 imprinted genes in humans, which are largely conserved in mouse (2; http://igc.otago.ac.nz/home.html). Human diseases associated with cytogenetic abnormalities or (epi)mutations, such as transient neonatal diabetes mellitus (TNDM) and Beckwith–Wiedemann, Silver–Russell, Angelman and Prader–Willi syndromes, and targeted mouse experiments have revealed that imprinted genes are essential for foetal and postnatal growth and behaviour, with aberrant expression in adults associated with multigenic diseases and cancer (3–8).

Imprinted genes are regulated by cis-acting imprinting control regions (ICRs), which manifest as regions of allelic DNA methylation that are set during gametogenesis and are inherited throughout somatic development by the
action of the DNMT1 complex (9–11). These differentially methylated regions (DMRs) are associated with a canonical chromatin signature, which comprises H3 lysine 9 trimethylation and H4 lysine 20 trimethylation on the DNA-methylated allele, whereas H3 lysine 4 methylation is associated with the unmethylated allele and may confer protection from de novo DNA methylation (12).

Imprinted genes rarely occur in isolation, as monoallelic expression is achieved by shared cis-regulatory elements that act in distinct ways at different loci (reviewed in 1). Despite information from a few well-studied loci, still little is known how ICRs silence neighbouring genes, and there seems to be two prevailing models of regulation. In general, paternally methylated intergenic ICRs act as methylation-sensitive insulators recruiting CTCF (13), while the majority of maternally methylated ICRs act as promoters. In some cases, these differentially methylated promoters are associated with long non-coding RNAs that confer silencing of neighbouring genes in cis through recruitment of histone-remodelling complexes (14,15). However, for the majority of imprinted domains, the intricacies of ICR action are unknown, and novel mechanisms, such as allele-specific alternative polyadenylation choice, are continuously updating our insight on imprinted regulation (16). Imprinted domains are especially attractive to researchers as they permit the study of inherited epigenetic transcriptional regulation, as both the active and silent alleles are genetically identical and are present within the same nucleus, exposed to the same trans-acting chromatin regulators.

To date, the imprinted domain on human chromosome 6 associated with TNDM contains two paternally expressed transcripts, PLAGL1 (previously known as ZAC1) and the non-coding RNA HYMAI, both initiating from within the maternally methylated PLAGL1-DMR (17,18). To determine whether these transcripts are part of a larger imprinting cluster, we performed allelic expression analysis of the flanking genes in both human and mouse. This revealed that the region is a micro-imprinted domain, as all surrounding transcripts are biallelically expressed in both species, including PHACTR2, which we show exhibits allelic bias in human placenta associated with genotype and not parental origin (19). Interrogation of publicly available CTCF ChIP-seq data shows that PLAGL1 is flanked by CTCF-binding sites conserved between species in both expressing and non-expressing cells. These two CTCF/cohesin regions interact physically despite being separated by more than 70 kb, and we propose this restricts imprinting to only those transcripts contained within the chromatin loop.

MATERIALS AND METHODS

Human tissues

A cohort comprising 65 foetal tissue sets (8–18 weeks) with corresponding maternal blood samples and 96 term placental samples from the Moore Tissue bank are described elsewhere (20). An additional 96 human placenta samples were collected at Hospital St Joan De Deu (Barcelona, Spain). DNA and RNA extraction and cDNA synthesis were carried out as previously described (21). A selection of normal adult brain samples was obtained from BrainNet Europe/Barcelona Brain Bank. The human RNA panel was purchased from Clontech (Human Total RNA master Panel II). Ethical approval for adult blood and foetal tissue collection was granted by the Hammersmith, Queen Charlotte’s & Chelsea and Acton Hospital Research Ethics Committee (Project Registration 2001/6029 and 2001/6028); collection of the HSJD placental cohort was granted by the ethical committee of Hospital St Joan De Deu (Study Number 35/07) and IDIBELL (PR006/08). Written informed consent was obtained from all participants.

Mouse crosses and cell lines

To determine allelic expression in mouse, wild-type embryos and placentas were produced by crossing C57BL/6 (B) with Mus musculus castaneus (C) mice. RNA and DNA were isolated and extracted as previously described (10). Control lymphoblastoid cell lines were established by Epstein–Barr virus transformation of peripheral blood cells and propagated as previously described (22). Before chromatin immunoprecipitation (ChIP), the lymphoblastoid methylation signature throughout the PLAGL1 domain was compared with leucocytes to ensure that the transformation process had not altered the epigenetic profile. The human TCL1 placental trophoblast cell line was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics.

Allelic expression analysis

Genotypes were obtained in the foetal and placental tissues for expressed genes using PCR and direct sequencing. Sequences were interrogated using Sequencher v4.6 (Gene Codes Corporation, MI) to distinguish heterozygous and homozygous samples. Heterozygous sample sets were analysed for allelic expression using RT-PCR that incorporated the polymorphisms in the final PCR product (Supplementary Table S1). The resulting RT-PCR amplicons were sequenced in both directions. The amplification cycle number for each transcript was determined to be within the exponential phase of the PCR, which varied for each gene, but was between 32 and 40 cycles.

Pyrosequencing analysis for allelic expression quantification

Pyrosequencing was used as an accurate method of quantifying allelic expression of PHACTR2 in heterozygous placenta samples. Standard RT-PCR was used to amplify across two independent single nucleotide polymorphisms (SNPs) mapping to exon 6 (an alternative spliced exon) and the 3’ UTR, with the exception that the reverse primers were biotinylated. The entire biotinylated RT-PCR product (diluted to 40 μl) was mixed with 38 μl of binding buffer and 2 μl (10 mg/ml) of streptavidin-coated polystyrene beads. Bead–amplicon complexes were captured on a vacuum prep tool (Qiagen), and the PCR products denatured using 0.2 M NaOH. The denatured
DNA was resuspended in 40 pmol of sequencing primer dissolved in 12 μl of water, and primer annealing was achieved by heating the sample to 80°C for 2 min before cooling to room temperature. For sequencing, forward primers were designed to the complementary strand (Supplementary Table S1). The pyrosequencing reaction was carried out on a PyroMark Q96 instrument. The peak heights were determined using the pyrosequencing commercial software.

Real-time RT-PCR

All PCR amplifications were run in triplicate on either an Applied Biosystems 7500 or 7900 Fast real-time PCR machines (Applied Biosystems) following the manufacturers’ protocol. All primers were optimized using SYBR Green (Supplementary Table S1 for PLAGL1 primers, all other genes were amplified using KqCStart primers from Sigma-Aldrich), and melt curve analysis was performed to ensure that amplicons were specific and free of primer-dimer products. Thermal cycle parameters included Taq polymerase activation at 95°C for 10 min for one cycle, repetitive denaturation at 95°C for 15 s and annealing at 60°C for 1 min for 40 cycles. All resulting triplicate cycle threshold (Ct) values had to be within 1 Ct of each other. The quantitative values for each triplicate were determined as a ratio with the level of RPL19 expression, which was measured in the same sample.

Analysis of allelic DNA methylation

The DNA methylation profile throughout the PLAGL1 domain was determined using published data from our previous Illumina Infinium HumanMethylation27 BeadChIP array analysis (GEO accession number: GSE28525) (23). Promoter regions not included on this array platform were assessed by conventional bisulphite PCR. Approximately 1 μg of DNA was subjected to sodium bisulphite treatment and purified using the EZ Gold methylation kit (ZYMO, Orange, CA) and was used for all bisulphite PCR analysis. Bisulphite PCR primers for each region were used with Hotstar Taq polymerase (Qiagen, West Sussex, UK) at 45 cycles, and the resulting PCR product was cloned into pGEM-T easy vector (Promega) for subsequent sequencing (see Supplementary Table S1).

Chromatin immunoprecipitation

We used previously published CTCF ChIP-seq data sets for leucocytes and CD34 cells (GEO accession number: GSM651541) and chromatin interaction analysis with paired-end tags (ChIA-PET; GEO accession number: GSM970213 and GSM970216) (http://dir.nhlbi.nih.gov/papers/epigenomes/htgtcell.aspx) to identify potential binding sites within the 6q24-imprinted domain. For ChIP, chromatin from ~80 million cells was aliquoted into 100-μg batches and used for each immunoprecipitation reaction with Protein A Agarose/Salmon Sperm DNA (Millipore, 16-157) and specific antibody. The antibody against CTCF (07-729) was obtained from Millipore, and the antibodies against RAD21 (AB992) and SMC3 (AB9263) were obtained from ABCAM. For each ChIP, a fraction of the input chromatin (1%) was also processed for DNA purification, and a mock immunoprecipitation with a neutral unrelated IgG antibody was carried out in parallel.

Levels of immunoprecipitated chromatin at specific region were determined by qPCR using an Applied Biosystems 7900 Fast real-time PCR machine, using SYBR Green (Applied Biosystems). Each PCR was run in triplicate, and protein binding was quantified as a percentage of total input material. Normalization of ChIP levels was performed by comparing precipitation levels obtained for each specific region of interest with the level obtained for a known CTCF/cohesin-positive control region mapping to chromosome 6 (24) (see Supplementary Table S1 for primer sequences).

Chromatin conformation capture analysis

The chromatin conformation capture (3C) protocol was performed as previously described (25), with minor amendments. Briefly, HindIII was used to digest 1 × 10⁶ formaldehyde-cross-linked nuclei from leucocytes, placental cell line TCL1, mouse embryonic fibroblasts (MEFs) (for which EcoR1 was used) or 100 mg of archived tissue (overnight digestion, 1200 U, NEB). The efficiency of the restricted enzyme digestion was assessed by qPCR across each restriction site, comparing digested and undigested chromatin fractions. Only chromatin with digestion efficiency more than 70% was used. Subsequently, the DNA was ligated overnight in a 500-ul reaction volume using 1950 units of T4 ligase (Fermentas). DNA was de-cross-linked by incubating overnight at 65°C and purified using phenol/chloroform extraction. This DNA was used for both nested PCR for analysis of allelic interactions, and real-time PCR (LightCycler, Roche Applied Science) to determine the frequency of interactions, using constant primers either in the unmethylated regions ~5 kbp upstream from the DMR, within the last PLAGL1 exon, or between the P3/P4 promoters (see Supplementary Table S1). Primer efficiency and basal interaction frequencies were determined using digested and ligated bacterial artificial chromosomes (BAC) DNA (human RPII RP11 947A22; mouse RP24 399D18) as described by Braem and co-workers (26). All 3C experiments were performed in at least 3 biological samples per tissue in technical triplicates.

RESULTS

PLAGL1 and HYMAI are the only imprinted genes mapping to 6q24

To characterize the boundaries of the imprinted region on human chromosome 6, we determined the abundance and allele-specific expression of nine genes flanking PLAGL1 (DEADC1, PEX3, FUCA2, PHACTR2, LTV1, SF3B5, STX11, BC033369/LOC285740 and UTRN) using RT-PCR amplification across transcribed SNPs in first trimester foetal tissues and term placenta. No SNPs were identified within FAM164B. We confirm that human PLAGL1 transscripts from the P1 promoter and HYMAI were paternally expressed in all tissues analysed at all gestational ages. The PLAGL1 expression from the P2
promoter was biallelic when detectable. Biallelic expression of the remaining transcripts was observed in all tissues analysed, and their associated promoters were unmethylated (Figure 1A and B; Supplementary Figures S1 and S2; Supplementary Table S2).

To assess the imprinting profile within the mouse orthologous region on chromosome 10, we used various embryonic tissues and placenta (at embryonic stage E14.5) from reciprocal crosses between C57BL/6 (B) and Mus musculus castaneus (C). We confirm the ubiquitous imprinting previously described for the paternally expressed Plagl1 transcript, and observed equal expression from both parental alleles for all other genes (Supplementary Figure S3).

**Conserved paternally expressed PLAGL1 isoforms**

We recently identified and characterized several paternally expressed Plagl1 alternative transcripts in mouse (27) (Figure 1C). One of these transcripts, originating from the P3 promoter region, results in a transcript containing the protein-coding exons. As a result of expressed sequence tag (EST) and mRNA alignments, we identified an orthologous transcript originating from the equivalent P3 promoter region in the human. This transcript (reference EST AJ006354) originates from a unique promoter region upstream of the P3 promoter, and observed equal expression from both parental alleles for all other genes (Supplementary Figure S3).

**CTCF/cohesin form non-allelic boundaries flanking PLAGL1**

In an attempt to understand the mechanism restricting imprinting to HYMAI and the PLAGL1 transcripts, we interrogated genome-wide CTCF data sets to identify potential CTCF boundaries. Using two published data sets (29,30), we identified two regions of strong CTCF binding, one ~5kb downstream of the PLAGL1-DMR and between this and the non-imprinted P2 promoter, and the other within the last exon of PLAGL1. This latter region showed two independent CTCF peaks ~2kb apart (Figures 1C and 2). Using PCR on bisulphite-converted DNA, we were able to show that the CpG dinucleotides surrounding these CTCF-binding sites are unmethylated in leucocyte- and placenta-derived DNA (Figure 2B). Comparison of the sequence surrounding these binding sites identified CTCF consensus sites that contain both motifs (M1 and M2) recently described by Schmidt and co-workers (31). The M1 motifs of both regions contain CpG dinucleotides, which ensure methylation-sensitive binding. Interestingly, the second binding site within the last PLAGL1 exon (region 6, Figure 2) lacks the M2 motif and CpG dinucleotides (data not shown).

Subsequent ChIP using antisera to CTCF was performed to confirm binding in lymphoblastoid cells and the placental cell line TCL1. We observed strong CTCF enrichment within the regions downstream of the PLAGL1-DMR and in the last exon, but not within the PLAGL1-DMR itself or in the regions overlapping the P3 and P4 promoters (Figure 2C; data not shown). Recently, cohesin has been shown to play a critical role in maintaining CTCF higher-order chromatin conformation at the H19-IGF2 loci (32). To determine whether CTCF and cohesin co-localize within the PLAGL1 regions, we performed ChIP using antisera against the cohesin subunits RAD21 and SMC3 (Figure 2C). In lymphoblastoid cell lines, the cohesin subunits clearly precipitated with CTCF on one allele at the H19-ICR control region, and on both chromosomes throughout the PLAGL1 domain (Figure 2D).

**The biallelic CTCF regions adjacent to PLAGL1 physically interact to form chromatin loops**

Recent studies have revealed that CTCF is a key regulator of chromatin fibre structure and responsible for organizing chromosomal territories within the cell nucleus.
Figure 1. Schematic representation of the \textit{PLAGL1} domain on human chromosome 6p24. (A) The relative organization of the human genes and CpG islands. The orthologous mouse domain has a similar organization, except the lack of the non-coding RNA LOC285740. (B) The allelic expression for all genes in the human cluster. The sequence traces for heterozygous DNA samples from term placentas are shown for all genes, as well as the resulting RT-PCR. Black boxes depict genes that are expressed biallelically in all tissues throughout gestation, whereas blue boxes represent ubiquitously imprinted, paternally expressed transcripts. (C) A comparison of the structure and position of the imprinted transcripts within the \textit{Plagl1}/\textit{PLAGL1} transcriptional unit, including the location of the CTCF sites. (D) DNA sequences across rs2076684 SNP in the penultimate \textit{PLAGL1} exon and the RT-PCR sequence traces for each isoform in term placenta.
To determine whether the CTCF/cohesin sites physically interact, we performed 3C experiments to identify potential chromatin folding. 3C-qPCR assays were performed on adult leucocytes and brain samples, term placenta material and the TCL1 cell line, and interaction frequencies were determined between a constant HindIII site located \(500\) bp upstream to the unmethylated CTCF/cohesin binding site \(50\) to the \(PLAGL1\)-DMR and other HindIII sites throughout the locus. We observed high interaction frequencies of sites separated by \(<20\) kb from the constant fragment, which included the \(PLAGL1\)-DMR, reflecting non-specific random collision due to their physical proximity. We subsequently identified strong interactions between the CTCF/cohesin constant fragment and the CTCF/cohesin sites in the last \(PLAGL1\) exon, in different tissues irrespective of expression (Figure 3). Direct sequencing of the chimerical products from placenta and brain revealed that the interactions occurred on both chromosomes, and that the appropriate chimeric products result from the 3C ligations (Figure 3D and Supplementary Figure S6). Interrogation of publicly available ChIA-PET (35) data revealed that CTCF mediates an abundant loop identical to the one we identified (Figure 3). In addition, we also identified non-allelic interactions between the constant fragment and the HindIII sites associated with the P3/P4 promoter regions in placenta and brain (Figure 3B). We did not observe these interactions in leucocytes, which suggests this specific contact occurs only in tissues exhibiting transcription from the P3/P4 promoters and appears independent of CTCF binding. Interestingly, we observed a leucocyte-specific chimeric product between the CTCF/cohesin binding site \(50\) to the \(PLAGL1\)-DMR and a region in intron 1, which may represent a repressive interaction. These interactions were confirmed in two additional 3C experiments using constant primers in the last \(PLAGL1\) exon (Supplementary Figure S6) as well as the intervening region between the P3 and P4 promoter (Figure 3C).

CTCF sites at conservation locations physically interact in the mouse

Because the allelic expression patterns were similar between human and mouse for genes surrounding \(PLAGL1/Plagl1\), we predicted that the higher-order chromatin structure will be comparable. Interrogation of the
mouse transcription factor binding site (TFBS) data set from ENCODE (data available on the UCSC browser, NCBI37/mm9 assembly) identified two regions of strong CTCF binding at similar locations as in human (Figure 1). To confirm that these sites interact to form a chromatin loop, we performed allelic 3C on mouse embryonic fibroblast cells. This semi-quantitative analysis revealed a biallelic contact between an EcoRI constant fragment containing the CTCF site located ~4 kb upstream of the Plagl1-DMR and the binding site in the last exon ~40 kb downstream (Supplementary Figure S7).

**DISCUSSION**

By analysing the allelic expression of transcripts flanking PLAGL1/HYMAI, we show that this locus is one of only a few true conserved micro-imprinted domains. Previously reported micro-imprinted domains such as Grb10/GRB10 and imprinted transcripts that result from X-derived retrotransposition into host gene introns have subsequently been shown to influence either neighbouring or host genes (36–38).

We demonstrate that the imprinted transcriptional unit in both humans and mouse is restricted within CTCF/cohesin boundaries, suggesting that they are associated with a conserved regulatory function. Interestingly, this 70-kb loop is very similar in size to the minimal TNDM critical region identified by micro-duplications (39). By using allelic 3C, we show that the flanking CTCF/cohesin-binding sites physically interact in both species in a biallelic manner. In humans, this confirmation is maintained in both expressing and non-expression tissues, suggesting that the chromatin architecture is maintained by CTCF/cohesin and that additional transcription factors are required to promote expression. This is reminiscent of the CTCF/cohesin long-range interactions observed at the IGF2-H19 domain in lymphoblastoid cells, a cell type that does not express IGF2 despite maintained looping (22,32). In tissues that abundantly express the predominant P1-PLAGL1 transcript, we identified novel imprinted isoforms originating 45 kb downstream. The promoter regions of the P3- and P4-PLAGL1 transcripts are embedded within CpG-poor regions (the upstream 1 kb of P3 and P4 have 12 and 10 CpG dinucleotides, respectively). We hypothesize that the level of transcription from these isoforms is regulated by a combination of the endogenous promoter sequences and shared enhancers with the P1-PLAGL1 promoter, the latter conferring the allelic specificity. This is supported by the observed CTCF-independent chromatin loop in expressed tissues that juxtaposes the P3/P4 promoters and the PLAGL1-DMR so that any methylation-sensitive transcription factor binding at the PLAGL1-DMR could influence P3/P4 (Figure 4). Analysis of ChIA-PET using

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Figure 3. Chromatin interactions with the CTCF sites upstream of the PLAGL1-DMR. (A) The position of the HindIII sites used for 3C analysis, and annotated ChIA-PET data showing CTCF and POL2 interactions. (B) The looping profile in brain (black), placental TCL1 cell line (red) and normal leucocytes (blue) using a constant primer 5 kb upstream of the PLAGLI-DMR, showing strong interactions with the 3' UTR and alternative promoters of PLAGLI. The x-axis shows the position of the primers used. (C) The interaction profile in expressing tissues, brain (black) and placental TCL1 cell line (red), using a constant primer between the P3 and P4 promoters. (D) Heterozygous SNP rs2064661 in constant fragment 5kb upstream of the PLAGL1-DMR allowed for allele-specific chromatin interactions to be assessed in adult brain. Sequence traces reveal biallelic higher-order chromatin interactions between the constant fragment and various contact points throughout the PLAGL1 domain.
CTCF and POL2 revealed that this second chromatin interaction is solely present in the POL2 data set, confirming our observations (35).

A meta-analysis of microarray data recently revealed that Plagl1 is a member of a co-regulated imprinted gene network (IGN) important for embryonic growth (40). The mechanism regulating this IGN remains to be elucidated, and may be directly linked to the Plagl1 protein because it encodes a zinc finger transcription factor (41). Alternatively, it may be speculated that CTCF is a common denominator mediating expression of numerous imprinted loci via intra- and interchromosomal interactions (42). Our results suggest that the TNDM phenotype associated with paternal duplications of 6q24 likely results from the overexpression of PLAGL1, the only imprinted protein-coding gene in the region, and it remains to be determined whether PLAGL1 is central to an IGN important for non-syndromic human growth as in mouse.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Figures 1–7.

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