Human Molecular Genetics, 2014, Vol. 23, No. 23 **6275–6285**
doi:10.1093/hmg/ddu347
Advance Access published on July 3, 2014

Altered expression of the imprinted transcription factor *PLAGL1* deregulates a network of genes in the human IUGR placenta

Isabel Iglesias-Platas[1,*], Alex Martin-Trujillo[2], Paolo Petazzi[3], Amy Guillaumet-Adkins[1,2], Manel Esteller[3,4,5] and David Monk[2,*]

[1]Servicio de Neonatología, Hospital Sant Joan de Déu, Fundació Sant Joan de Déu, Barcelona 08950, Spain, [2]Imprinting and Cancer Group, [3]Cancer Epigenetics Group, Cancer Epigenetic and Biology Program, Institut D'Investigació Biomedica de Bellvitge, Hospital Duran i Reynals, Barcelona 08907, Spain, [4]Department of Physiological Sciences II, School of Medicine, University of Barcelona, Barcelona 08097, Spain and [5]Institucio Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Catalonia 08010, Spain

Received March 7, 2014; Revised and Accepted June 30, 2014

Genomic imprinting is the epigenetic process that results in monoallelic expression of genes depending on parental origin. These genes are known to be critical for placental development and fetal growth in mammals. Aberrant epigenetic profiles at imprinted loci, such as DNA methylation defects, are surprisingly rare in pregnancies with compromised fetal growth, while variations in transcriptional output from the expressed alleles of imprinted genes are more commonly reported in pregnancies complicated with intrauterine growth restriction (IUGR). To determine if *PLAGL1* and *HYMAI*, two imprinted transcripts deregulated in Transient Neonatal Diabetes Mellitus, are involved in non-syndromic IUGR we compared the expression and DNA methylation levels in a large cohort of placental biopsies from IUGR and uneventful pregnancies. This revealed that despite appropriate maternal methylation at the shared *PLAGL1*/*HYMAI* promoter, there was a loss of correlation between *PLAGL1* and *HYMAI* expression in IUGR. This incongruity was due to higher *HYMAI* expression in IUGR gestations, coupled with *PLAGL1* down-regulation in placentas from IUGR girls, but not boys. The PLAGL1 protein is a zinc-finger transcription factor that has been shown to be a master coordinator of a genetic growth network in mice. We observe PLAGL1 binding to the *H19*/*IGF2* shared enhancers in placentae, with significant correlations between *PLAGL1* levels with *H19* and *IGF2* expression levels. In addition, PLAGL1 binding and expression also correlate with expression levels of metabolic regulator genes *SLC2A4*, *TCF4* and *PPARγ1*. Our results strongly suggest that fetal growth can be influenced by altered expression of the *PLAGL1* gene network in human placenta.

INTRODUCTION

Intrauterine growth restriction (IUGR) in the western world most commonly results from an underlying placental insufficiency that prevents a fetus from achieving its normal growth potential. This situation is similar, but pathologically distinct, from small for gestational age (SGA) that describes a newborn that is below the 10th centile for gestational age of the pregnancy (1). Therefore SGA simply reflects small, but otherwise normal babies. The mechanisms leading to IUGR are not well understood, but manifest as anomalies in placental vasculature, which can be clinically identified by increased umbilical or uterine artery resistance in prenatal Doppler measures. Often pregnancies complicated by IUGR require early elective delivery if the fetus is suspected to be in distress, resulting in additional complications associated with prematurity (2). In addition, IUGR babies not only have immediate medical problems but are at increased risk of hypertension, type 2-diabetes and heart

*To whom correspondence should be addressed at: Neonatal Service, Hospital Sant Joan de Déu, Passeig Sant Joan de Déu, 2, 08950, Esplugues de Llobregat, Barcelona, Spain. Tel: +34 932532100; Fax:+34 932033959; Email: iiglesias@hsjdbcn.org (I. I.-P.); Tel: 0034 93 260 7500; Fax: 0034 93 260 7219; Email: dmonk@idibell.cat (D. M.)

disease in adult life through the effects of fetal programming (3–5), which is commonly referred to as the 'fetal origins of adult disease'.

To date, despite much effort, the genes involved in IUGR are largely unknown. The contribution of genetic inheritance towards birth weight has been estimated to be between 30 and 70% (6). Common variations in genes known to be involved in hormonal pathways influencing fetal growth or later metabolic disease have been investigated in relation to birth weight with mixed success and reproducibility (7–9). For example, mutations in the glucokinase gene produce an increased or reduced weight of newborns when the aberration is present in the mother or the baby, respectively (10). Recently, genome-wide scans have highlighted associations for the non-coding RNA (ncRNA) *LINC00881* (AK311218) and *ADCY5* genes, both mapping to chromosome 3, in determining fetal growth (11). The latter transcript has also been identified as a risk allele for type 2 diabetes and other measurements of the efficiency of adult glucose metabolism.

The lack of molecular mechanisms has hampered the identification of useful molecular markers for IUGR and of individuals with longer-term health risks. Changes in epigenetic modifications, including DNA methylation and histone modifications at regulatory regions, may contribute to stable alterations to gene expression and are ideal candidates for markers. These aberrations may then be maintained long after the initial insult during development and influence metabolic phenotypes later in life (12).

In mammals, imprinted genes are known to regulate placental development and fetal growth (13). These genes, of which there are ~100 in humans, are expressed in a parent-of-origin manner and are believed to have co-evolved with placentation (14). Imprinted genes are associated with multiple layers of epigenetic regulation, including DNA methylation and histone tail modifications that results in their monoallelic expression (15). Numerous mouse models that involve targeted deletions of imprinted transcripts have revealed specific effects on growth of the placenta and embryo, providing important links between imprinting and IUGR. For example, deletions of the active alleles of the paternally expressed genes *Igf2*, *Peg1* and *Peg3* result in reduced placental size and IUGR (16–18). Targeted deletions of maternally expressed genes, including *Grb10* and *Phlda2* result in fetal over-growth (19,20), highlighting the overall physiological effects of oppositely imprinted alleles (21–23). Several studies in humans have investigated changes in expression and regulation of imprinted genes in placentas from cohorts with heterogeneous clinical characteristics (healthy pregnancies, preeclampsia, SGA and IUGR), suggesting that deregulation of this group of genes might play a role in prenatal growth [*PHLDA2* (24); *MEST*, *MEG3*, *GNAS* and *PLAGL1* (25); *H19* and *IGF2* (26–29)].

The paternally expressed gene *PLAGL1* (previously known as *ZAC1*) encodes a zinc finger transcription factor (seven fingers of C_2H_2 class) that induces apoptosis and cell-cycle arrest (30). Over-expression of *PLAGL1*, and *HYMAI*, a ncRNA derived from the same promoter, is associated with Transient Neonatal Diabetes Mellitus (TNDM) (31), a rare form of neonatal diabetes that presents at birth and undergoes remission in infancy. All TNDM babies are severely growth restricted at birth, suggesting that either *PLAGL1* or *HYMAI* influence fetal growth. Interestingly, *Plagl1* deficient mice present with embryonic growth restriction (32), indicating that both under- or over-expression of *Plagl1*/*PLAGL1* is associated with an IUGR phenotype. Meta-analysis of microarray data revealed that *Plagl1* is a member of a network of co-regulated genes comprising other imprinted transcripts involved in fetal growth (33), the coordinated effects presumably due to the coactivator effect of this transcription factor (34,35).

We postulate that alterations in the epigenetic control mechanisms and the concomitant changes in placental expression of *PLAGL1* may affect fetal growth and lead to IUGR. In this study we present a comprehensive analysis of the expression and DNA methylation profiling of both *PLAGL1* and *HYMAI* in normal and IUGR placental tissues. Furthermore, we confirm an evolutionary conserved gene network influenced by the transcriptional activator *PLAGL1*.

RESULTS

Characterization of the sample sets

One hundred placental samples were obtained from 90 pregnancies (81 singletons, 8 sets of twins, 1 set of triplets) (Table 1). Most mothers were of Caucasian or Hispanic (91.2%) origin and were healthy before pregnancy. Mean age at delivery was 31.7 ± 5.5 years, mean height 162.3 ± 7.0 cm and mean pre-pregnancy weight was 64.8 ± 14.3 kg. Over half of the newborn babies were girls and the mean gestational age was under 36 weeks, as 49% of them were born before term age (30 were late or moderate preterms and 19 very preterm babies). Mean birthweight was 2.300 ± 887 g. The mean gestational age of the boys was significantly lower (34.8 ± 4.5 weeks versus 36.5 ± 3.9, $P = 0.046$) which reflected a higher proportion of boys among the very premature group (13 boys and 6 girls). Singleton pregnancies accounted for 81% of the cohort. Due to the high-risk obstetric characteristics of our population (IUGR, prematurity, complications of pregnancy), there was a high rate of cesarean section (48%).

Forty-three of the fetuses were diagnosed with IUGR during pregnancy. For the purpose of this analysis, we classified them according to the most severe ultrasound abnormality present at any time during scheduled controls. Mothers of IUGR babies delivered more often by elective cesarean section (44.2 versus 15.8%, $P = 0.002$), had a higher prevalence of preeclampsia (30.9 versus 8.8%, $P = 0.005$) or chronic hypertension and a trend to a higher prevalence of self-reported smoking habit (26.5 versus 10.5%, $P = 0.077$). IUGR and normally growing fetuses were comparable in their remaining clinical characteristics except, by definition, size at birth both in absolute terms and relative to gestational age.

Seven couples conceived with help from assisted reproduction technologies (ART) that resulted in 12 children. In this group, maternal age was higher (36.6 ± 6.8 years versus 31.5 ± 5.3, P 0.004) and multiple pregnancies were more frequent (57.1 versus 6.0%, $P = 0.001$), as would be expected. Also, most of the ART babies recruited were premature (8.6% of them had been born at term as compared with a 56.8% of the spontaneously conceived group, $P = 0.002$) and, consequently, gestational age (32.2 ± 4.9 weeks versus 36.2 ± 3.9) and weight, length and head circumference at birth were

Table 1. Clinical characteristics of the study group

	n (%) AGA (*n* = 57)	IUGR (*n* = 43)
Gender		
Boys	24 (42)	22 (51)
Girls	33 (58)	21 (49)
Gestational age group (w = weeks)		
Term (≥37 w)	32 (56)	19 (44)
Late/moderate premature (>32, <37 w)	13 (23)	17 (40)
Very premature (≤32 w)	12 (21)	7 (16)
Multiple gestation	8 (14)	11 (26)
Conception by ART	8 (14)	4 (9)
Preeclampsia	5 (9)	13 (30)
Delivery by cesarean section	23 (40)	25 (58)
	Mean ± SD	
Gestational age (weeks)	36.0 ± 4.7	35.4 ± 3.4
Anthropometric data at birth		
Birth weight (g)	2633 ± 921	1858 ± 614
Birth weight SDS	0.25 ± 0.80	−1.50 ± 0.73
Length (cm)	46.4 ± 5.4	42.6 ± 4.5
Length SDS	0.36 ± 0.64	−1.08 ± 1.0
Head circumference (HC, cm)	32.1 ± 3.9	30.2 ± 2.9
HC SDS	0.17 ± 0.68	−1.03 ± 0.92
Placental weight (g)	592 ± 174	387 ± 137

smaller. Nevertheless, there were no significant differences regarding *z*-scores for these parameters and the incidence of IUGR was comparable in both groups.

Up-regulation of the ncRNA *HYMAI* in IUGR placentae

We analyzed the relative mRNA transcript levels for *HYMAI* and the four alternative splice forms of *PLAGL1* in human placentae by qRT-PCR in comparison to the endogenous *RPL19* gene. We have previously shown that P1, P3 and P4 promoters give rise to paternally expressed transcripts, with the majority of expression originating from the P1 promoter (36).

HYMAI expression was higher in the placentas of the IUGR group (mean Log10 expression 0.991 versus −0.0012, *t*-test $P = 0.018$) in a univariate analysis (Fig. 1A). Although this was true for the sample as a whole, the effect was much more pronounced in male babies. *HYMAI* expression did not relate to maternal age, size or parity, gestational age or anthropometric parameters of the baby (weight, length or head circumference at birth), in either absolute terms or adjusted by gestational age (*z*-scores) (Supplementary Material, Table S2), but related specifically to the presence of IUGR defined by obstetric criteria. Levels of expression did not correlate with the variables that differed between AGA and IUGR pregnancies (cesarean section, preeclampsia, smoking). We performed a multivariate analysis (direct logistic regression, dependent variable: IUGR yes/no), adjusting for other variables known to influence low size at birth (maternal weight, parity, gestational age and gender of the baby). None of the additional variables made a statistically significant contribution to the model. The only factor independently associated with IUGR was the level of *HYMAI* expression, with an odds ratio of 18.30 (95% CI: 1.5–224.4, $P = 0.023$).

Expression of *PLAGL1* shows gender differences in IUGR placentae

The expression of *PLAGL1* did not correlate with the presence of IUGR in our cohort in the regression analysis. However, when boys and girls were analyzed separately, total *PLAGL1* expression was significantly lower in the placenta of IUGR girls (Log10 PLAGL1_All: 0.12 versus −0.08, $P = 0.023$) (Fig. 1B). This was not the case in boys. The lower expression levels of total and *PLAGL1*-P1 isoform in girls were associated with IUGR after adjusting for maternal age, weight and parity, length of gestation and use of assisted reproduction (Log10 PLAGL1_All, β −0.308, $P = 0.018$. Log10 PLAGL1_P1, β −0.257, $P = 0.040$). Western blot analysis of samples with extreme *PLAGL1* mRNA levels revealed similar protein profiles (Fig. 1C).

IUGR-dependent changes in correlation between *PLAGL1*-P1 and *HYMAI* despite maintained imprinted expression

We have previously shown that *PLAGL1*-P1 is the predominant isoform and that *HYMAI* originates from the same promoter region embedded in a maternally methylated DMR. In placentas of control babies (spontaneously conceived, normally grown), there was a correlation between the expression of both transcripts (r 0.479, $P = 0.002$). However, in IUGR pregnancies this correlation was lost, due to increased *HYMAI* expression without concomitant changes in *PLAGL1*-P1 (r 0.023, $P = 0.823$) (Fig. 1D).

Expression differences in ART placenta samples

Altered levels of expression of imprinted genes have been reported in both mouse and human after the use of ART (37) and placental epigenetic profiles seem particularly vulnerable (38). Levels of expression of *PLAGL1,* as determined using PCR primer in common exons (PLAGL1_All PCR) and by assessment of individual isoforms, were lower in placentas of babies conceived by ART compared with spontaneous conceptions. These differences were not attributable to unequal prevalence of IUGR, which was similar amongst ART and non-ART pregnancies (33 versus 44%, P 0.547). There were no significant differences regarding *HYMAI* levels (Fig. 1E). Due to the differences between these two groups regarding maternal age, prevalence of multiple pregnancy, parity, gestational age and size of the newborn, the relationship was analyzed by hierarchical multiple regression, controlling for the aforementioned factors and gender of the baby. The model remained significant (R^2 0.223, $P = 0.007$) and ART was the only factor independently associated with variance of the expression of *PLAGL1* (R^2 change 0.130, $P = 0.001$; standardized β 0.451, $P = 0.001$).

No changes in methylation and imprinted expression of *PLAGL1* or *HYMAI* in IUGR placental biopsies

To ensure that *PLAGL1* and *HYMAI* maintained imprinting, with expression solely from the paternal allele, we determined the allelic origin of transcription in all heterozygous individuals. In total 12 samples were heterozygous for the *PLAGL1* rs2076684 single nucleotide polymorphisms (SNPs) and 20 for

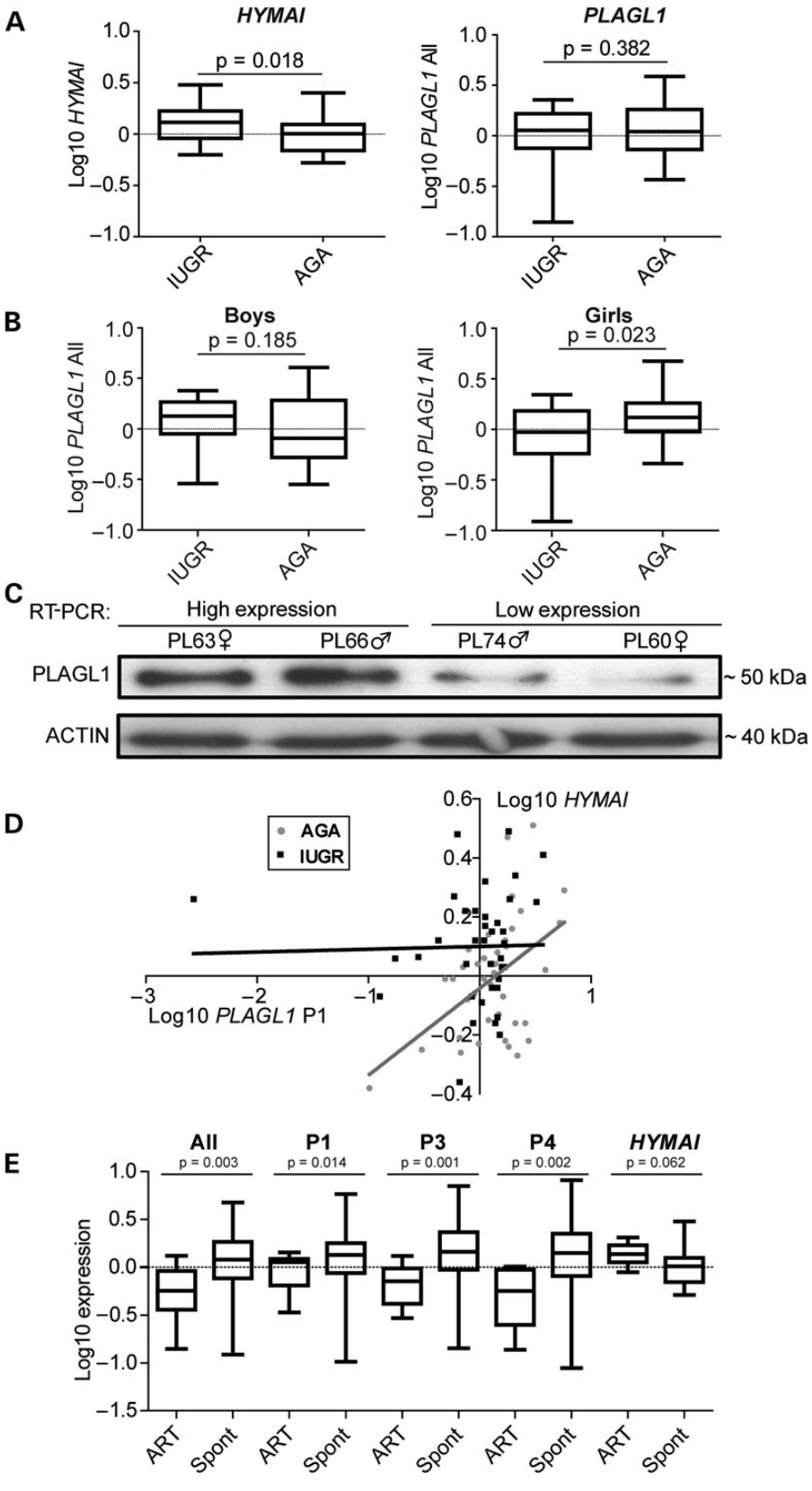

Figure 1. Expression analysis of *PLAGL1* and *HYMAI* in placental biopsies. (**A**) Univariant analysis of *HYMAI* (left) and *PLAGL1* (right) expression in IUGR placentae (AGA $n = 57$; IUGR $n = 43$). (**B**) The gender-specific expression difference for *PLAGL1* boys (left) and girls (right) in IUGR placental biopsies. The boxes represent the interquartile range (25th to 75th percentiles) and the horizontal line corresponds to the median. The whiskers extend to the 5th and 95th percentiles of the data set. (**C**) Western blot confirmation of expression differences in high and lowly transcribed placental samples. (**D**) Pearson correlation analysis of expression for the PLAGL1-P1 and HYMAI transcripts in IUGR placenta. The gray data points represent AGA and the black IUGR placental samples. (**E**) The significant lower expression ofPLAGL1, but not HYMAI, following assisted reproduction technologies (ART) when compared to spontaneous conceptions (Spont).

the *HYMAI* rs2281476 polymorphism. This represented all groups of gestational age, intrauterine growth and mode of conception. Monoallelic expression was observed in all cases (Fig. 2A), and origin was confirmed as paternal in the cases with homozygous mothers (one for *PLAGL1* and three for *HYMAI*).

DNA methylation acquisition abnormalities at imprinted DMRs in babies conceived by ART have previously been reported (39,40). To determine if the abnormal expression we observe in IUGR and following ART is due to abnormal DNA methylation at the *PLAGL1* DMR we performed pyrosequencing to quantitate methylation levels. Consistent with our observation of maintained imprinted expression of *PLAGL1* and *HYMAI* in all heterozygous samples, we observed no extreme deviation from the expected ~50% methylation in any sample. Levels of methylation were not significantly different when comparing AGA to IUGR samples nor when comparing ART versus spontaneous conceptions. The appropriate pattern of allelic methylation was also maintained (Fig. 2B and C). This suggests that loss of methylation at the *PLAGL1* DMR is not responsible for the variation in expression levels. This is in agreement with other studies that have shown that ART-associated decreased expression of imprinted genes is not attributable to methylation defects (41).

Correlation between *PLAGL1* expression levels and the genes of the growth network

Previous transfection over-expression assays and expression analyses in the *Plagl1*$^{+/1pat}$ mice have revealed an imprinted gene network affiliated to *Plagl1* expression (32). In addition, Plagl1 has been reported to regulate *Tcf4*, *Slc2a4* (also known as *Glut4*) and *Pparγ1* expression via binding to their proximal promoter intervals, with the latter gene being a downstream candidate for the potent antiproliferation function of PLAGL1 (42–44).

To determine whether human *PLAGL1* also functions as a master transcriptional regulator for both imprinted and non-imprinted genes, we performed qRT-PCR for *H19*, *IGF2*, *CDKN1C*, *SLC2A4* and *PPARγ1* on all placental biopsies. The resulting expression levels were compared with total *PLAGL1* revealing high correlation coefficients for *IGF2* (r 0.423, $P <$ 0.0001), *H19* (r 0.506, $P < 0.0001$), *SLC2A4* (r 0.436, $P <$ 0.0001) and lower, but still significant for *CDKN1C* (r 0.347, $P = 0.001$) and *PPARγ1* (r 0.257, $P = 0.02$) (Fig. 3A–E). These correlations were found in both AGA and IUGR and boys' and girls' placentas. Interestingly *Tcf4* has been shown to contribute to the enhanced expression of *Cdkn1c* (42). We therefore determined the abundance of total *TCF4* and specifically the β isoform, a transcript known to have a PLAGL1 binding site in its proximal promoter region (42,45). We observed highly correlated *PLAGL1* and *TCF4* expression (*TCF4* r 0.738, $P <$ 0.0001; *TCF4* –β r 0.739, $P < 0.0001$) (Fig. 3F) in our placental samples, but surprisingly no correlation between *CDKN1C* and *TCF4* (r −0.038, $P = 0.729$) (Fig. 3G).

PLAGL1 binds to the *H19* 3′ enhancers shared by *IGF2* and *H19*

Given the similar correlations for *H19* and *IGF2* expression and the levels of *PLAGL1* transcription, we wished to determine whether *PLAGL1* directly regulates these reciprocally imprinted genes by binding to the shared 3′ *H19* enhancer region. This conserved regulatory element regulates the expression of both transcripts, but on opposite chromosomes, with the allelic utilization determined by the paternally methylated *H19*-ICR insulator (46,47).

We performed chromatin immunoprecipitation using a *PLAGL1* specific antibody in a term placenta sample. Five PCR amplicons were designed to the orthologous human sequences reported for endodermal and mesodermal enhancers in mouse and one to the *H19*-ICR. No PLAGL1 binding was observed in the *H19*-ICR, however significant binding at two regions within the enhancer region was observed (Fig. 4A and B). The SNPs rs217718/rs1635152 were heterozygous in the placenta chromatin preparation, and subsequent sequencing of the immunoprecipitated (IP) fraction revealed that PLAGL1 binds to both alleles (Fig. 4C). A bioinformatics search for the known Plagl1 consensus binding sequences GGGGCCCC (48) and GCCGCCG (49) revealed two GCCGCCG sites near amplicon 2 and two G_4C_4 sites immediately adjacent to amplicons 3 and 4. These regions, separated by only ~2 kb, overlap with the known conserved enhancer sequence CS1, CS3 and CS4 (50) and are located ~500 bp from the reported Plagl1 binding sites within the mouse *H19* 3′ enhancer interval (32). These results suggest that PLAGL1 plays a conserved role in regulating expression levels of both *H19* and *IGF2*.

To confirm that the correlated expression we observe between the non-imprinted genes and *PLAGL1* is due to the transcription factor function of the latter gene, we performed ChIP. We utilized quantitative PCRs on the IP fractions to determine PLAGL1 binding within the proximal promoter regions of *SLC2A4*, *PPARγ1* and *TCF4* –β isoform (Fig. 4C–E). In all cases we observed robust binding near PLAGL1 consensus sequences, suggesting PLAGL1 is involved in the regulation of transcriptional output at these loci.

DISCUSSION

The data from this study highlights the role of imprinted transcripts from the human chromosome 6q locus in fetal growth via a placental mediated mechanism. We have shown increased *HYMAI* expression in IUGR placentas after adjustment for variables shown to influence size at birth, like maternal size, parity and age. Expression of this ncRNA was not influenced by weight, length or head circumference at birth, suggesting a specific association with mechanisms of abnormal fetal growth *in utero* and not with anthropometric parameters at birth. The role of *HYMAI* in fetal development is unknown. Emerging evidence supports a role of this ncRNAs in regulating *PLAGL1* expression by possibly recruiting chromatin-modifying enzymes associated with active transcription (51). In support of this mechanism, we observe that transcriptional levels of *HYMAI* and *PLAGL1* were significantly and positively correlated in control samples. Abnormalities in regulation lead to a loss of this correlation in IUGR. We propose that the uncoupling of expression is probably not related to changes in transcription factor binding or DNA methylation within the shared promoter region, as this would presumably effect transcription of both genes in parallel. Instead we favor a post-transcriptional mechanism that either lowers the expression of *PLAGL1*, for example the up-regulation

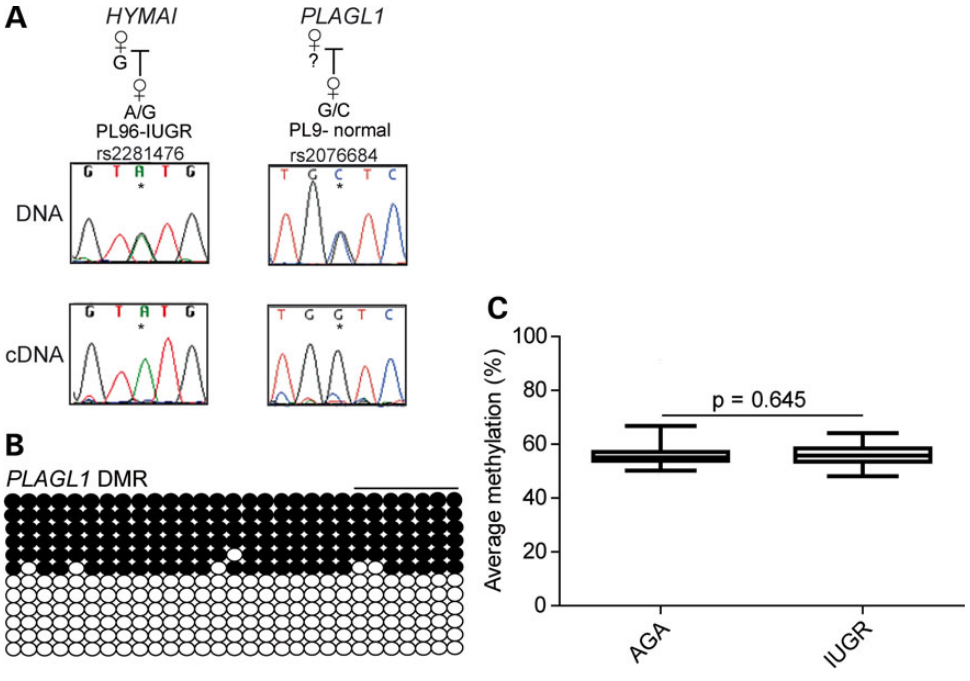

Figure 2. Analysis of *PLAGL1* allelic DNA methylation and expression in placental samples. (**A**) Sequence traces showing allelic expression using standard RT-PCR and direct sequencing in placental samples. Asterisk (*) mark the polymorphic nucleotide (SNP rs2076684 for *PLAGL1* and rs2281476 for *HYMAI*). (**B**) An example of the placental methylation profile of the *PLAGL1* DMR as determined by cloning and direct sequencing. Each circle represents a single CpG dinucleotide on the strand, (•) a methylated cytosine, (O) unmethylated cytosines. The CpG dinucleotides targeted by the pyrosequencing assay are indicated. (**C**) The methylation profile for the *PLAGL1* DMR as determined by pyrosequencing of AGA and IUGR placental biopsies.

of a microRNA (miRNA). Interrogation of the predicted miRNA binding sites within the *PLAGL1* 3′UTR revealed 20 conserved alignments (http://microRNA.org), including miR-16, miR-34, miR-139 and miR-424, all of which have previously been shown to be expressed in placenta and involved in growth (52–54). In addition, the difference in expression could be attributed to changes in RNA stability, since we have previously shown that *HYMAI* is a nuclear retained unstable transcript with a short half-life comparable with other ncRNAs, whereas the *PLAGL1* mRNA is stable and exported to the cytoplasm for translation (51).

The expression of *PLAGL1*, both total and from the predominant P1 promoter was significantly lower in the placenta of IUGR girls but not boys. Decreased expression of *PLAGL1* in IUGR placentae with maternal vascular hypoperfusion has previously been suggested in a microarray screening study (25). However gender was not specified or taken into consideration in that study. Gender differences in expression of imprinted and non-imprinted genes have recently been shown to be a frequent feature in somatic tissues in the mouse (55–57), with tissue-specific sex-dependent effects of *Plagl1* noted in two studies (56,57). The gender expression differences in placenta could be due to transient exposure to fetal testosterone or by the sex chromosome complement, which can influence autosomal gene expression in a hormone-independent fashion (58). Further work is required to decipher the mechanism responsible for these observations. We recommend that fetal gender should be taken into account when determining placental expression profiles as differences between male and female placental gene expression and function are progressively being uncovered (59).

The increased abundance of *HYMAI* in IUGR placentas and lower levels of placental *PLAGL1* from babies conceived following ART were not paralleled by loss or gain of methylation at the *PLAGL1* DMR, respectively. Other authors have also found expression levels differences of imprinted genes without obvious methylation changes in the corresponding imprinted DMRs. This presumably reflects transcriptional deregulation by other *trans*-acting mechanisms, such as transcription factor binding (60). In support of this, expression of *Plagl1* during postnatal life in the mouse is down-regulated in a process that does not involve changes in methylation at the DMR (51).

Finally our results suggest that the growth gene network described in pre- and postnatal mice (32) is also present in the human placenta. We confirm that *PLAGL1* acts as an activating transcription factor for expression of *TCF4* (42), *Pparγ1* (44) and *Slc2a4* (43) in cell lines by direct binding to consensus sequences found in their promoters. In our samples, expression of *PLAGL1* correlated with expression of imprinted and non-imprinted genes, with strong association with *H19* and *IGF2* for which we confirm PLAGL1 binding to the shared H19 3′ enhancer region. We observe high correlation between *PLAGL1* expression and that of *TCF4*–β isoform, but curiously a lack of correlation between *TCF4* and *CDKN1C*. This suggests that PLAGL1 can bind directly to either the *CDKN1C* proximal promoter or enhancer regions rather than influencing transcriptional output via transactivation of *TCF4* as has been observed during mouse neuronal differentiation (39). Therefore in

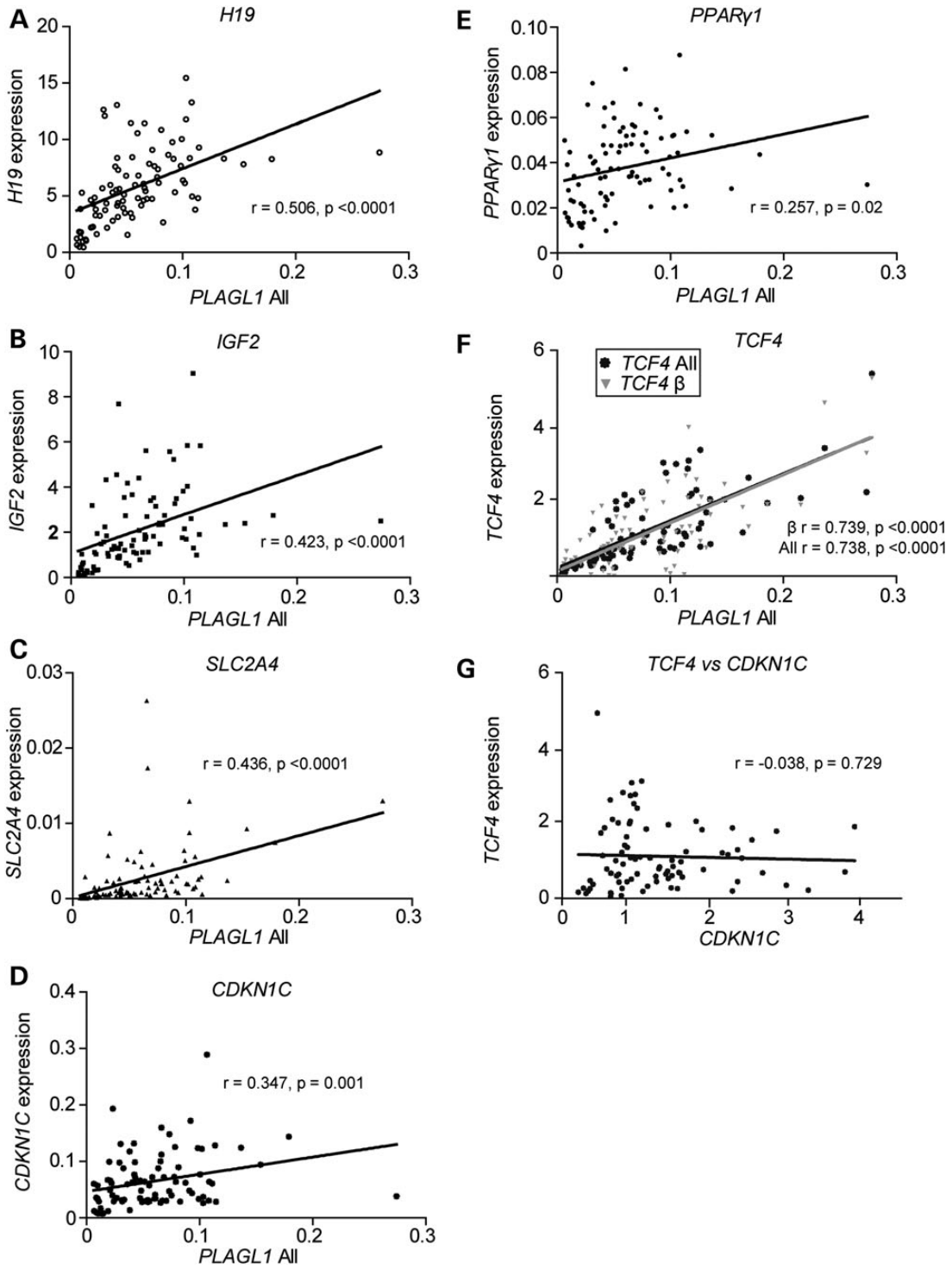

Figure 3. Expression analysis of the *PLAGL1* gene network in placental samples. Correlations between the expression of *PLAGL1* and (**A**) *H19*, (**B**) *IGF2*, (**C**) *SLC2A4,* (**D**) *CDKN1C,* (**E**) *PPARγ1* and (**F**) *TCF4* (total transcription shown in black; β isoform in gray) from the co-regulated gene network. Panel (**G**) reveals a lack of correlation between *TCF4* and *CDKN1C* in the same placental biopsies.

human placenta, PLAGL1 co-regulates the expression of a number of important growth genes by acting as a transcription factor. It would be intriguing to compare PLAGL1 binding and transactivation in pancreas, a key metabolic tissue directly involved in TDNM, to identify both tissue-specific and ubiquitously bound loci.

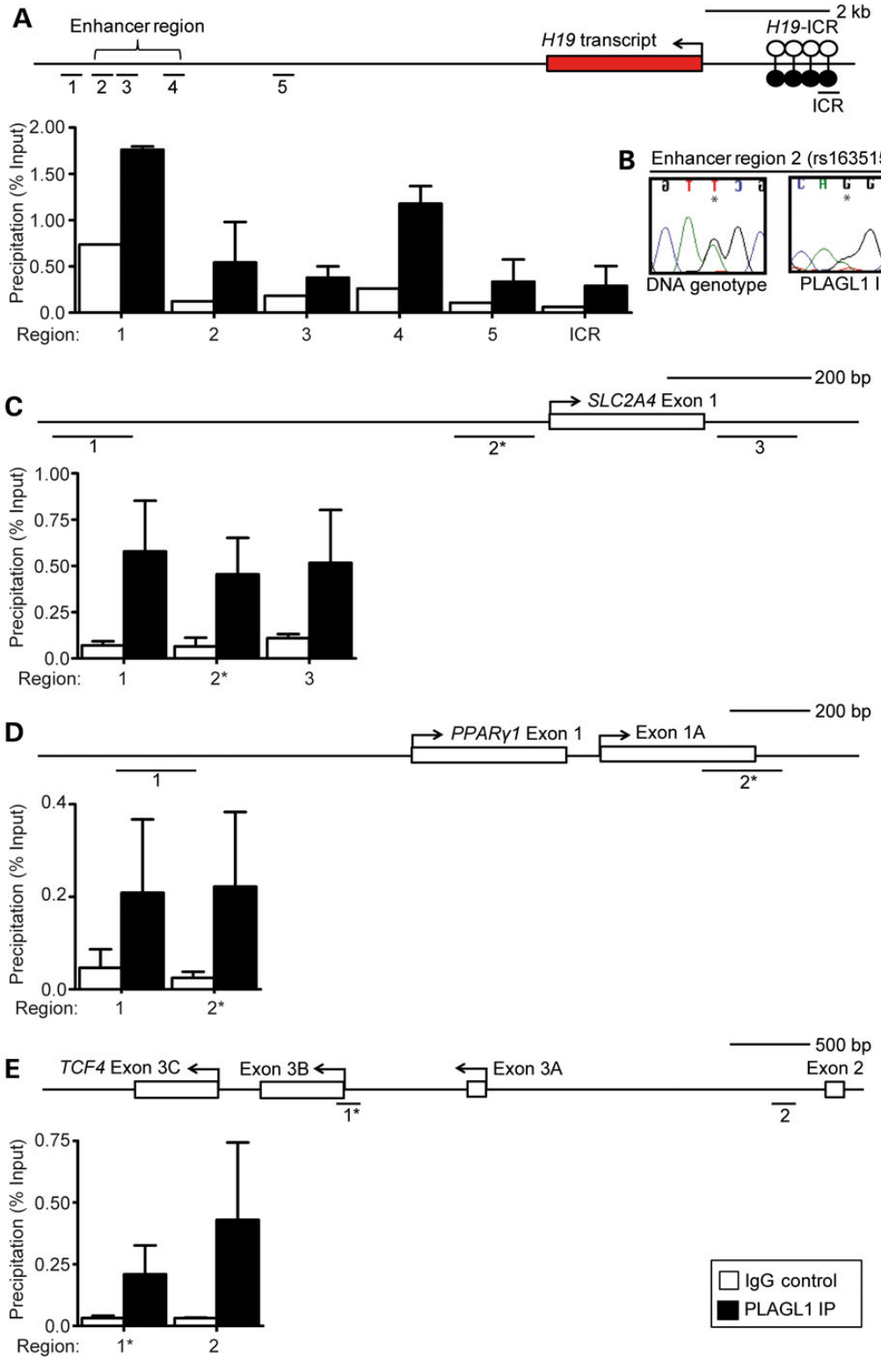

Figure 4. PLAGL1 ChIP reveals binding to enhancers and promoters in human placenta. (**A**) The map of the *H19* loci, showing the position of the *H19* ICR, the *H19* transcriptional unit and the shared enhancer region. The approximate locations of the PCR primers are indicated. The graph represents the enrichment, as determined by qPCR on PLAGL1 ChIP material, from a normal term placenta sample at various regions across the *H19* region. (**B**) Sequence trace showing the biallelic precipitation of PLAGL1 at the *H19* enhancer region. Quantitative PCR on IP fractions was also used to determine PLAGL1 binding in the *SLC2A4* (**C**), *PPARγ1* (**D**) and *TCF4* promoter regions. * Denotes previously reported PLAGL1 binding sites.

CONCLUSION

Our results show DNA methylation independent differences of *PLAGL1* and *HYMAI* expression in IUGR and ART placentas. Our results support the idea that gender-specific mechanisms could apply to IUGR placentae with *PLAGL1* acting as an upstream regulator for a network of genes influencing placental and consequently, fetal growth.

MATERIAL AND METHODS

One hundred babies from 90 participants were recruited among pregnant women delivering their babies in Sant Joan de Déu Hospital, Barcelona. The protocol was approved by both the Sant Joan de Déu Hospital and IDIBELL Research and Ethics Committees (PI35/07 and PR006/08) and individual informed consent was obtained. Upon delivery, the placentae were weighed and biopsies from the fetal side adjacent to the umbilical cord insertion site were excised. The tissue was thoroughly rinsed in saline and snap frozen in liquid nitrogen. Maternal blood samples were collected in ethylenediaminetetraacetic acid tubes and frozen at -20°C until processed. Clinical information for the anonymized samples on pregnancy course was recorded. For the purpose of the study, subgroups were established according to the characteristics of the pregnancy and of the newborn (term or preterm, IUGR or non-IUGR, conceived spontaneously or by assisted reproduction). According to length of gestation, newborns were classified in: term ($\geq$37 weeks), late and moderate preterm ($>$32 and $<$37 weeks) and very preterm ($\leq$32 weeks). Intrauterine growth restriction was defined as a weight below the third percentile for gestational age or below the 10th percentile when accompanied by fetal Doppler flow abnormalities (1).

Nucleic acid extraction

Genomic DNA from placenta was prepared by sodium dodecyl sulphate/Proteinase K lysis followed by phenol/chloroform extraction and ethanol precipitation. DNA from blood was extracted using a commercial kit (QIAamp DNA Blood Midi Kit®, QIAGEN), following the suggested spin protocol. RNA was extracted using Trizol® (Invitrogen), and double-treated with DNase (DNase I, Invitrogen and TurboDNase®, Ambion). Reverse transcription was performed with MMLV retrotranscriptase (Promega) and random primers (Promega) following the manufacturer's instructions.

Quantitative polymerase chain reaction

Expression of the transcripts of interest was analyzed by quantitative real-time RT-PCR with a fluorochrome (SYBR® Green) assay and normalized against *RPL19*. This housekeeping gene was selected because of optimal expression stability in placental tissue (data not shown). Primer sequences are listed in Supplementary Material, Table S1. The assays were run in triplicate in 384-well plates in 7900HT Fast Real-time PCR System (Applied Biosystems). Dissociation curves were obtained at the end of each reaction to rule out the presence of primer dimers or unexpected DNA species in the reaction. Non-template controls, an interplate control and standard curves from the same serial dilutions of cDNA obtained from pooled normal placental tissue were included in each assay. Results were analyzed with the SDS 2.3 software (Applied Biosystems). Amplification plots and automatic baseline and threshold values were individually checked and adjusted where necessary according to guidelines (61). The DataAssist v2.0® software (Applied Biosystems) was used for exclusion of outlier replicates and for interplate comparisons. Only samples with two or more valid readings per triplicate were included. Analysis of the results was performed using the comparative $\Delta\Delta$CT method (62). All expression measurements were expressed in logarithmic scale for normalization and subsequently analyzed against clinical values.

Assessment of imprinted expression

Analysis of SNP was performed to determine allelic expression of the transcripts of interest. We used rs2076684 and rs2281476 for assessment of allelic expression of *PLAGL1* and *HYMAI*, respectively. PCR primers were designed to incorporate the SNPs within the amplicons. All placental DNA samples were genotyped to identify heterozygous individuals. Expression was then analyzed in heterozygous samples by RT-PCR and sequencing of the resulting amplicons. Imprinting was suggested only if a single base peak was observed at the SNP site in the RT-PCR product of a heterozygous sample. In these samples, the parental origin of expression was determined, when possible, by assessing the maternal genotype. Primers for RT-PCR were located in different exons, so that the PCR product crossed a splice site. In addition, RT-PCR was performed on RT-positive and negative samples in order to rule out genomic contamination.

Methylation analysis by bisulfite pyrosequencing

Bisulfite treatment of 1 μg of DNA was performed with the EZ Gold in a 96-well plate format (EZ-96 DNA Methylation-GoldTM® Kit, Zymo Research), following the manufacturer's protocol. A commercial control was used as reference for bisulfite-converted fully methylated DNA (EpiTect Control DNA®, methylated, Qiagen). Pyrosequencing was selected for the quantitative assessment of DNA methylation at the *PLAGL1* DMR overlapping the shared *PLAGL1*-P1 and *HYMAI* promoters was performed a previously described (63) (see Supplementary Material, Table S1 for primer sequences). Bisulfite PCR was performed with a biotin-labeled reverse primer. Immobilization of the PCR products for purification was achieved by streptavidin-coated sepharose beads (Qiagen) with the use of the PyroMark Q24 Vacuum Prep Workstation® according to the manufacturer's instructions. The PCR product incorporated 29 CpG dinucleotides, but subsequent pyrosequencing analysis was limited to 6 CpG dinucleotides within the amplicon given the limited length of the sequence reads.

Chromatin immunoprecipitation (ChIP)

One hundred micrograms of snap frozen placental tissue was reduced to powder with a pestle and mortar under liquid nitrogen. The pulverized placenta sample was cross-linked with 1% formaldehyde for 7 min at room temperature and the reaction was blocked by adding glycine to a final concentration of 0.125 M.

Approximately 70 μg chromatin was used for each immunoprecipitation reaction with Protein G magnetic beads (Millipore, 16–157) and specific antibody. The antibody against PLAGL1 (Ab129063 Lot: GR99015-1) was obtained from Abcam. For each ChIP, a fraction of the input chromatin (5%) was also processed for DNA purification and a mock immunoprecipitation with a neutral, unrelated IgG (Millipore PP64B Lot: 1968270) antiserum was carried out in parallel.

Levels of immunoprecipitated chromatin at specific regions were determined by quantitative PCR using SYBR Green (Applied Biosystems) on an Applied Biosystems 7900 Fast real-time PCR machine (see Supplementary Material, Table S1 for primer sequences). The ChIP was performed three times in duplicate with each PCR run in triplicate and protein binding was quantified as a percentage of total input material.

Statistical analysis

Clinical and molecular data were introduced in a Statistical Package for Social Sciences (SPSS®, IBM) software v17.0 database. Comparisons between groups were evaluated with χ^2 for categorical variables and Student *t*-test (for two groups) or analysis of variance (ANOVA) (more than two groups) for continuous variables. Non-parametric tests were applied where indicated. Relationships between variables were explored by Pearson's correlation and subsequently introduced in multiple regression models to adjust for possible interactions or confounding factors. Results were considered significant if the *P*-value was under 0.05; values under 0.1 were considered as a trend. This study was sufficiently powered to detect differences in expression of 0.7 SDs between groups (statistical power 80%) and correlations with a Pearson's r coefficient >0.3 between gene expression and clinical variables (statistical power 86%).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We would like to thank all the families that participated in this study and the clinical staff involved in their care, specially the perinatal nurses at Hospital Sant Joan de Déu who contributed to the collection of samples.

FUNDING

This work was supported by Spanish Ministerio de Educacion y Ciencia (SAF2008-1578 to D.M.); Fundació La Marató de TV3 (101130 to D.M.); Ayuda Merck Serono- Fundación Salud 2000 de Investigación en Endocrinología 2009 (to D.M. and I.I.P.); beca 'Bombers amb causa' 2012 (to I.I.P.). D.M. is a Ramon y Cajal research fellow. Research in the Neonatal Unit in HSJD is partially funded by an unrestricted grant from BBDue Spain.

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