A genome-wide approach reveals novel imprinted genes expressed in the human placenta

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Genomic imprinting characterizes genes with a monoallelic expression, which is dependent on the parental origin of each allele. Approximately 150 imprinted genes are known to date in humans and mice, but though computational searches have tried to extract intrinsic characteristics of these genes to identify new ones, the existing list is probably far from being comprehensive. We used a high-throughput strategy by diverting the classical use of genotyping microarrays to compare the genotypes of mRNA/cDNA vs. genomic DNA to identify new genes presenting monoallelic expression, starting from human placental material. After filtering of data, we obtained a list of 1,082 putative candidate monoallelic SNPs located in more than one hundred candidate genes. Among these, we found known imprinted genes, such as IPW, GRB10, INPP5F and ZNF597, which contribute to validate the approach. We also explored some likely candidates of our list and identified seven new imprinted genes, including ZFAT, ZFAT-AS1, GLI3, NTM, MAGI2, ZC3H12C and LIN28B, four of which encode zinc finger transcription factors. They are, however, not imprinted in the mouse placenta, except for Magi2. We analyzed in more details the ZFAT gene, which is paternally expressed in the placenta (as ZFAT-AS1, a non-coding antisense RNA) but biallelic in other tissues. The ZFAT protein is expressed in endothelial cells, as well as in syncytiotrophoblasts. The expression of this gene is, moreover, downregulated in placentas from complicated pregnancies. With this work we increase by about 10% the number of known imprinted genes in humans.

Introduction

In mammalian cells, both alleles of autosomal genes are assumed to contribute equally to the overall expression level. However, genes submitted to genomic imprinting do not follow this rule of thumb. These genes are characterized by a monoallelic expression that is dependent on the parental origin of the expressed allele, meaning that some genes are expressed only from the maternally inherited copy whereas others are expressed only from the allele derived from the father. Imprinted genes present a series of common, though not essential, features: localization within clusters, complex epigenetic regulation, preferential expression in placenta and brain, non-coding antisense RNAs, etc.1 Up to now, about 150 imprinted genes are known in mice and humans (http://igc.otago.ac.nz; www.geneimprint.com; www.mousebook.org/catalog.php?catalog=imprinting).

As mentioned above, most of the known imprinted genes are highly expressed in the placenta, and they often play important roles in placental and fetal development. They are therefore interesting candidates for understanding mother/fetus exchanges and placental pathologies. Imprinted genes participate to an intertwined regulation network where they contribute to control fetal growth.2 This network structure is quite important to regulate the system leading to nutrient exchanges. Furthermore, it has been postulated that deregulations or mutations of a so far unknown imprinted gene could be responsible for some cases of preeclampsia, a pregnancy specific disease caused by a dysfunctional placenta.3 In these perspectives, other preeclampsia candidate genes,
Attempts to identify new imprinted genes in humans and mice by computational means or using parthenogenetic tissues have failed to identify numerous new imprinted genes. Nevertheless, approaches to confirm the imprinted status of these computed genes have globally failed to identify numerous new imprinted approaches to confirm the imprinted status of these candidates such as gene organization or presence of repeated elements, to provide with lists of candidate genes. However, validation approaches to confirm the imprinted status of these computed genes have globally failed to identify numerous new imprinted genes. Nevertheless, it appears crucial to obtain an exhaustive list of human imprinted genes.

In order to identify new genes that could be imprinted or monoallelic in the human placenta, we designed a strategy using high throughput genotyping arrays. This strategy is decomposed in a first high-throughput genomic search for monoallelic expression followed by a second step of validation where (1) the monoallelic expression is confirmed and (2) the maternal or paternal transmission of the expression profile is explored. We describe here the identification of seven new imprinted genes expressed in the human placenta.

Results

Strategy for a global search for monoallelic expression in the human placenta. In order to identify new genes exhibiting a monoallelic expression profile (and, therefore, potentially imprinted) in the human placenta, we designed a genome-wide strategy using and “diverting” high-throughput genotyping microarrays, depicted in Figure S1. We hybridized in parallel two 250K genotyping Affymetrix arrays with either cDNA (cDNA) or genomic DNA (gDNA), both extracted from a single human placenta. We performed this approach for five unrelated placentas obtained from normal pregnancies. We called the genotypes for all SNPs of the arrays in order to compare the results obtained between cDNA and gDNA from each placenta. As expected, the quality of the genotypes obtained from cDNAs was lower than from genomic DNA (70% interpretable vs. 96%, respectively). We therefore performed a filtration step on the quality of the genotypes to keep only the most secure results (detailed in the Supplemental Material). Quality filtration removed about 88% of the genotypes. To demonstrate a monoallelic pattern, an informative SNP should present the following characteristics: (1) a necessary heterozygosity on gDNA and (2) an apparent homozygosity on the corresponding cDNA. We selected all SNPs meeting these criteria (15,125 SNPs). We made another selection to concentrate on SNPs where at least two placentas out of the five tested showed this potential monoallelic profile. This left us with 1,088 candidate SNPs. The next step was to associate genes to the selected SNPs, leading to 1,082 SNPs effectively connected to at least one gene (provided as Table S2) among which some known imprinted genes could be identified, such as CDKN1C (p57kip2), IPW, GRB10, INPP5F and ZNF597. In particular, ZNF597 is a transcription factor reported as imprinted in human white blood cells. Using rs2737 and/or rs37824, we confirmed that ZNF597 is expressed following a monoallelic pattern in all human placentas tested (n = 7) and that the active allele is the one inherited from the mother (n = 3 informative mothers) (Fig. S2). We explored this gene in the murine placenta using rs4152238 but discovered a biallelic expression in E14.5 and E17.5 total placentas as well as in E17.5 labyrinth and spongiosanphoblast.

The list was concentrated to a total of 171 genes that were called by at least two SNPs or by an exonic SNP.

Validation of candidate genes. A list of 20 genes was chosen for further validation, with a priority given to genes with monoallelic SNPs according to the following criteria: (1) redundancy (n > 2 SNP in the gene), (2) location in exonic sequences or (3) located in introns. For each gene, one or two exonic (coding or UTR) polymorphisms were chosen from dbSNP (NCBI database), likely to be frequent in Caucasian populations. Specific primers were designed, in order to genotype these SNPs, with specific amplifications, different for genomic DNA and cDNA, when possible (see Table S1). More than 30 placental genomic DNAs were genotyped to identify heterozygous individuals. We then sequenced the cDNA obtained from RNA extracted from the same placental tissue. For 12 genes, a biallelic signal was observed on cDNA despite the microarray result (Table S3), demonstrating false positives. However, for the other candidate genes, sequencing of placentual cDNAs from heterozygous individuals led to observe a consistent monoallelic expression. Table 1 summarizes the list of monoallelic genes identified (as exemplified for the ZFAT gene in Fig. 1). For all SNPs and genes, either one or the other known alleles could be observed as monoallelic among the studied samples, rejecting the hypothesis that the nucleotide variations per se or an associated haplotype could be responsible for the extinction of one allele. None of these genes localizes in close proximity to known imprinted genes or cluster of imprinted genes.

The new genes identified as monoallelic seem to be involved in various functions, including diabetes, growth and cancer. The transcription factor LIN28B (Lin28 C. elegans homolog B) together with its paralogous gene LIN28A, has been the subject of many publications in relation to miRNA regulation and cell transformation. However, it is worth noting that four are transcription factors harboring zinc finger domains: (1) LIN28B, (2) ZFAT (zinc finger gene in Autoimmune Thyroid disease or zinc finger and AT hombox domain containing or ZNF406), (3) GLIS3 (GLIS family zinc finger protein 3 or ZNF515) and (4) ZC3H12C. Two other genes are involved in neuronal cell adhesion [NTM (neurotrimin) and MAGI2 (membrane-associated guanylate kinase, WW and PDZ domain containing 2 or S-SCAM for synaptic scaffolding molecule)].

While most identified genes show a strict monoallelic expression in all cDNA samples tested (from 8 to 11 independent samples), two genes have a less stringent profile. For MAGI2 and NTM, about half of the heterozygous samples show a monoallelic expression while other samples maintain the expression of both alleles.

Genotyping of genomic DNAs from the respective mothers, when available, was also performed in order to determine the allelic parental transmission. For a given gene, all informative samples (a heterozygous child with an homozygous mother) gave the same parental inheritance. Though this strict parental origin cannot be formally demonstrated, the scores calculated in
Table 1 tend to validate this specific inheritance. Therefore, six genes (ZFAT, ZFAT-AS1, GLIS3, ZC3H12C, MAGI2 and LIN28B) were expressed only from the paternal copy while for two genes (NTM and ZNF597), the maternally inherited allele was the only active one (Table 1).

Therefore, we report the identification of seven new imprinted genes, matching both criteria of monoallelic expression and parental dependent expression.

**New imprinted genes are mainly specific to humans.** We explored the expression of the newly identified imprinted genes in placentas from F1 mice resulting from the mating of two distinct murine sub-species, *Mus musculus domesticus* and *Mus musculus molossinus*. Exonic SNP whose allelic versions are different between the parental strains were chosen for each gene from the dbSNP and MGI databases. Heterozygosity was first confirmed for all SNPs on genomic DNA extracted from each fetus whose placentas were studied. Sequencing cDNAs from the corresponding E17.5 total placentas (n = 2) produced a biallelic profile in all cases (Fig. 2A) and therefore an absence of imprinting in this species, as in mice.

Only the Magi2 gene seemed to be also imprinted in mice and cattle. F1 heterozygous placentas exhibited a monoallelic signal for cDNA for rs2956343, a SNP located within the UTR region of the longest isoform. When studying two other SNPs located within a central exon common to all 3 alternative isoforms (rs47464539 and rs48892605), a similar monoallelic signal was obtained, in total placental cDNA, as well as in labyrinth and spongiotrophoblast extracts. The expressed allele was the one inherited from the mother (Fig. 3), which is different from the paternal expression of the human MAGI2 gene. In the bovine species, genomic annotation describes two MAGI2 genes in close neighborhood. For the most upstream one (XM_595287), we could find a polymorphic SNP (rs42342806). In embryonic and extra-embryonic tissues of 18 d (cotyledon and brain) and 60 d (allantoid, amnios, muscle, brain, heart and intestine) heterogeneous animals, a monoallelic signal was observed in Magi2 cDNAs. The expressed allele was the one inherited from the mother, as in mice.

The ZFAT gene presents characteristics of classic imprinted genes. We demonstrated that the ZFAT gene presents both basic characteristics of an imprinted gene: a monoallelic expression pattern in placental tissue and a strict parental (paternal) origin of the expressed allele. ZFAT codes for a widely expressed transcription factor, mainly studied in lymphocyte signaling but strongly expressed in the placenta and was recently shown to be involved in capillary formation and organization of the endothelium.22 We analyzed more deeply other traits that are shared by reported imprinted genes.

A non-coding antisense RNA overlaps the ZFAT locus. A non-coding antisense RNA, overlapping the ZFAT genomic region and

### Table 1. Summary of newly identified imprinted genes

| Gene      | Chromosome localization | SNP Localization | SNP | Monoallelic heteroz. | Biallelic heteroz. | Informative mothers | Expressed allele | Murine imprinting | Score (p value) |
|-----------|-------------------------|------------------|-----|----------------------|-------------------|----------------------|-----------------|------------------|-----------------|
| ZFAT      | 8q23–24                 | rs3739423 syn    | 9   | 0                    | 2                 | paternal             | no              | 0.06             |
|           |                         | rs894344 syn     | 6   | 0                    | 2                 | paternal             | NA              | 0.125            |
| ZFAT-AS1  | 8q23–24                 | rs894343 exonic   | 10  | 0                    | 3                 | paternal             | NA              | 0.01             |
| LIN28B    | 6q21                    | rs221635 3'UTR   | 9   | 0                    | 2                 | paternal             | no              | 0.125            |
|           |                         | rs56042804 3'UTR | 3   | 0                    | 2                 | paternal             | NA              | 0.03             |
| ZNF597    | 16p13                   | rs12737 3'UTR    | 7   | 0                    | 3                 | maternal             | no              | 0.125            |
| GLIS3     | 9p24                    | rs6415788 non syn| 11  | 0                    | 6                 | paternal             | no              | 0.00             |
| ZC3H12C   | 11q22                   | rs1026608 syn    | 8   | 0                    | 5                 | paternal             | no              | 0.03             |
| NTM       | 11q25                   | rs11634 3'UTR    | 3   | 1                    | 1                 | maternal             | -               | 0.5              |
| MAGI2     | 7q21                    | rs13438302 syn   | 4   | 3                    | 2                 | paternal             | yes             | 0.25             |

The last column represents a statistical score estimated by a binomial law assuming that probability of inheriting any allele is 0.5.
designed primers to amplify these regions from bisulfite-treated placental genomic DNAs. After direct sequencing, we could read 24 CpGs from the first region, 8 from the second and 23 from the third one. All non-CpG cytosines in the first two regions had been fully transformed by the bisulfite treatment and no trace of resistance due to methylation could be observed at any position and in any of the ten placental genomic DNAs tested, reflecting an unmethylated status. CpGs in the third region were however completely resistant to bisulfite treatment, showing a complete methylated status (data not shown). Therefore, we could not observe the specific differentially methylated profile of many genuine imprinted genes. This suggests that the imprinted status of the locus could be related to a differential methylation profile of other more distant CpG islands, or to other epigenetic mechanisms of regulation of monoallelic expression.

Imprinted expression of ZFAT is not observed in other human tissues.

We then wanted to check if the monoallelic pattern of ZFAT was also present in other human tissues. We explored lymphocytes of individuals genotyped as heterozygous for rs3739423 and/or rs894344. In six independent cases (five simple heterozygotes and a double heterozygote), ZFAT cDNAs showed a biallelic pattern (Fig. 1D). Therefore, we consider that the ZFAT gene is not imprinted in lymphocytes.

Endometrial tissues were also explored. In two samples heterozygous for either SNP of the ZFAT gene, a biallelic expression could be observed (data not shown) and, therefore, suggested that ZFAT is not imprinted in this cell type. We genotyped four patients affected with thyroid tumors for ZFAT and ZFAT-AS1 SNPs. Three of them were found concordantly heterozygous on both lymphocyte and tumor genomic DNAs, in order to exclude loss of heterozygosity in the tumor due to genic rearrangements. ZFAT and ZFAT-AS1 expression remained biallelic in the tumor. In conclusion, ZFAT monoallelic

Figure 1. Sequencing results of SNPs in the human ZFAT and ZFAT-AS1 genes. Imprinted expression was deduced by comparing genotypes of gDNA, cDNA and maternal gDNA, in placenta using SNPs rs3739423 (A), rs894344 (B) and rs894343 (C) and in lymphocytes (D).

Gene and CpG island localization, methylation levels. ZFAT is located on chromosome 8q24. The nearest known human imprinted genes are located distally at about 5 Mb (KCNK9), 8 Mb (LY6D) and 10 Mb (GPT). The murine Zfat gene is carried by chromosome 15 in a region syntenic to its human equivalent and is also relatively distant from validated imprinted genes (Kcnk9, Peg 13 and Sla38a4). It is interesting to note the presence of two miRNA coding genes, MIR30B and MIR30D, near ZFAT (Fig. S3).

We searched the ZFAT genomic sequence for CpG-rich regions using the newcpgreport, cpgplot and newcpgseek softwares. They were consistently located in three regions: (1) near the miRNA genes about 120 kb upstream of ZFAT, (2) at the beginning of the gene in the transcription start region and (3) at the end of the gene. These three CpG rich regions are approximately 1.5 kb (162 CpGs), 1.5 kb (143 CpGs) and 3.5 kb (215 CpGs) long, respectively, and are conserved in mammals. We
expression is not ubiquitously distributed in the body.

**ZFAT expression in pathological placentas.** The expression of ZFAT was estimated by real-time RT-PCR in placental samples from pregnancies complicated by either preeclampsia, preeclampsia associated with IUGR, isolated IUGR or vascular IUGR (n = 14, n = 4, n = 9 and n = 7, respectively), and compared with placentas from uncomplicated pregnancies (n = 16). Interestingly, ZFAT appears to be under-expressed in all pathological placentas, particularly in preeclampsia where the average level is at least 3 times lower than in controls (p = 0.002) (Fig. 4).

The expression of ZFAT-AS1 was also challenged, but this antisense gene seems to be expressed at a much lower level (about 1,000 times less) than the sense ZFAT gene and was therefore too close to the detection threshold to conclude.

**ZFAT protein expression in placentas.** We used an anti-ZFAT antibody to reveal ZFAT expression profile on human placenta sections. Labeling was strong in endothelial cells, in both 19 and 32 weeks of amenorrhea placentas, with a cytoplasmic localization of the protein (Fig. 5A and B). A fainter labeling was sometimes present in the syncytiotrophoblast layer, as a thin border. As a positive control, fetal lung also harbored an endothelial expression pattern (Fig. 5C). A western blot using various human and murine tissues exhibited multiple bands compatible with the theoretical molecular masses of the main isoforms of ZFAT (139, 137 and 94 kDa for proteins Q9P243-1, -2 and -3, respectively, and 128 kDa for another isoform), although some slight differences in isoform expression can be observed between tissues and species (Fig. 5D).

**LIN28B.** The LIN28B gene, a regulator of miRNA expression, was also more deeply analyzed. LIN28B is surrounded by CpG islands, and we chose to analyze the one co-localizing with its promoter. Direct sequencing of bisulfite-treated genomic DNA from 10 normal human placentas revealed double peaks at the 19 CpG positions available (data not shown). This suggested the presence of a differentially methylated region (DMR) characteristic of imprinted genes. To further characterize it, we cloned and sequenced this fragment for four individuals. Figure 6 shows the analysis of this region for one representative placenta, heterozygous for two SNPs and whose mother was moreover informative. We observed that CpGs in this region are completely methylated on the maternal allele, whereas the paternal allele shows a global unmethylated state. This is coherent with the extinction of the maternal copy of LIN28B and the expression of its paternal allele. This region therefore behaves as a differentially methylated region (DMR).

In agreement with an expression profile mainly embryonic, we could not amplify the LIN28B cDNA in lymphocytes or endometrial tissue and therefore could not investigate a putative imprinting status in other cell types than placenta.

Expression analysis of the LIN28B gene in pathological placentas did not show any significant variation in comparison to samples from normal pregnancies (data not shown).

**Discussion**

By diverting high-throughput genotyping arrays, we explored the putative imprinted status of candidate human genes. Historically, the first maps of imprinted genes have been established thanks to the use of mice harboring uniparental disomies. These large and complex chromosomal rearrangements together with ad hoc crosses allowed the production of
mice carrying a large genomic region in the expected double copies but inherited from only one or the other parent. The resulting phenotypes were severe and allowed the identification of large clusters of imprinted genes, controlled by an imprinting control region (ICR). Putative imprinted genes, disseminated across the genome in opposition to those located in clusters, might have been missed by this screen as they are unlikely to participate to such large phenotypic syndromes and as they are probably not under the control of a long range ICR. In addition, imprinted genes having a restricted expression profile, either spatially or temporally, or with an imprinted pattern limited to a precise developmental stage, organ, or isoform, are probably very difficult to identify, particularly in humans. For example, in spite of controversial reports suggesting or excluding an imprinted status for the well-studied retinoblastoma gene RB1, it is only very recently that it was shown to undergo genomic imprinting.25

Attempts to exhaustively identify imprinted genes have been developed, either using computational approaches or with high throughput strategies.9,10,13-15 If bioinformatical screenings have suggested that imprinted genes might be much more numerous than the actual known list, up to 600 in mice22 and 150 in humans,11 validation analyses to confirm the imprinted status of the candidate genes have not been very successful and the list has not increased much. A previous approach using genotyping arrays failed to reveal new imprinted genes.26 However, a similar screening led to the identification of the random monoallelic pattern of lymphocytes.27 Difficulties to identify new imprinted genes partially rely on the lack of strict characteristics of sequence, gene organization, etc., common to all imprinted genes and that could be used as criterion to identify new candidates.

High-throughput techniques as in this work allow the screening of a huge number of genes simultaneously, though the location of these SNPs, not gene-focused, is not ideal. Therefore, the background is high, particularly for the hybridization of cDNA and needs a careful selection of pertinent data. We used filtering steps to concentrate on the more robust set of data in terms of quality and reproducibility of the results and performed a complete validation step.

By sequencing the cDNA of placentas previously genotyped as heterozygous for SNPs located within exons, we could distinguish true or false candidates. Our validation step allowed the exclusion of some false positive genes. These might be due to a lack of sensitivity of the technique; indeed, the software is designed to provide the genetic composition of genomic DNA (heterozygosity/homozygosity) whereas cDNA signals might deviate from this clear-cut situation and lead to a misinterpretation. In addition, our validation approach concentrates on the analysis of the major isoform or all isoforms of a given gene and might therefore miss some imprinting effects that could be specific of a particular alternative isoform of the gene (potentially targeted by the array’s probe), as already observed for GRB10, for example.28

In our screen, ZFAT, the best candidate from the arrays, was targeted by six monoallelic SNPs, in at least two of the five placentas under study. It is noteworthy that these six SNPs are located in intronic regions of the ZFAT gene. Re-examination of
the complete data showed that six additional SNPs, also located in intronic sequences, presented a monoallelic pattern in the only informative of the five placentas. It is therefore likely that, as we hypothesized prior to undertaking this study and as already mentioned, a significant portion of pre-messengers is present in the RNA pool. This contributes efficiently to the hybridization process and produces consistent and robust results leading to the identification of monoallelic genes. This result strengthens the choice of the Affymetrix genotyping arrays that unfortunately harbor only 5% of coding SNPs, but that contain a large proportion of intronic SNPs.

This approach led to the confirmation of known imprinted genes such as INPP5F, IPW, GRB10 and ZNF597. This latter gene, a zinc finger transcription factor, had been cited as imprinted in lymphocytes and was recently identified by screens for differentially methylated regions in uniparental disomies and hydatidiform moles. We confirmed that this gene was also imprinted in the human placenta and that the expressed allele was the maternally inherited one, as recently reported. In the murine placenta, the Zpf597 gene displayed a biallelic expression. Homozygous mice invalidated for this gene (also known as HIT-4) are early embryonic lethal whereas heterozygous mice exhibit abnormal behaviors and abnormal structures in the brain. However, the precise role of this gene in both placental and brain function remains to be analyzed.

Besides, seven novel human imprinted genes were found, which represents an increase of about 10% relative to the today list of human imprinted genes. Little is known about the genes we identified and their precise role in the placenta generally remains to be characterized. Neurotrimin (NTM) is a cell adhesion molecule that seems to participate in synaptogenesis. MAGI2 is involved in infantile spasm. ZFAT is a widely expressed transcription factor from the zinc finger family that appears to play a role in immune functions and apoptosis. GLIS3 is implicated in neonatal diabetes and pancreatic development. This pathway has a considerable importance in placental physiology, since glucose transport and insulin regulation are considerably affected in growth restricted fetuses, as reviewed in references 37 and 38. In addition, GLIS3 is strongly expressed in endothelial cells, an issue of relevance for the normal placental physiology, also encountered for ZFAT. LIN28B controls the expression of the let-7 miRNA and is implicated in cancer progression and glucose metabolism. LIN28B was found associated with age at menarche in various populations. LIN28, the paralogous gene of LIN28B, has been shown to control IGF2 expression level at the post-transcriptional level. The expression of LIN28B is in addition correlated with IGF2 expression, at the mRNA and peptidic level in ovarian cancers, an interesting connection between imprinted genes. The ZC3H12C gene has been shown to regulate inflammatory pathways in mice. The balance between pro- and anti-inflammatory pathways is an issue of extreme importance in human placental diseases, where pro-inflammatory protein encoding genes are abnormally upregulated in pathological pregnancies, especially in preeclampsia. The imprinted status of inflammatory factors may thus be relevant to adjust the amount of protein synthesized. Some of these seven genes have a very strong expression in the placenta (ZFAT, LIN28B) while others are barely expressed in this tissue (NTM, MAGI2, ZNF597) (data obtained using Unigene and Nextbio).

A cerebral expression profile is also common. Population studies have highlighted SNPs in some of them in association with growth related phenotypes. Among several independent placenta samples, a perfectly monoallelic expression could be seen for five of the seven candidate genes, showing a strict imprinting. In these cases, either one or the other allele could be observed in the cDNAs, rejecting the hypothesis that one of the nucleotidic variations could be responsible for a functional effect resulting in the decrease of the

![Immunohistochemistry to detect ZFAT on sections of human placentas.](image)

Figure 5. Immunohistochemistry to detect ZFAT on sections of human placentas. (A and B) 19 and 32 weeks of amenorrhea respectively and (C) fetal lung. (+) shows the detection with the primary antibody sc-87510 and (-) the negative control of detection without this antibody. Plain arrows show the strong labeling of endothelial cells while dotted arrows point to the syncytiotrophoblast labeling. (D) A western blot of human and mouse tissues revealed by an anti-ZFAT antibody. PBL stands for peripheral blood leukocytes whereas JEG-3 is a human chorioncarcinoma cell line commonly used as a model of trophoblast.
expression level of one copy. For two genes (NTM and MAGI2), however, variability among individuals was observed, as some exhibited a strict monoallelic expression, while others maintained a biallelic profile of the same gene, tested under the same conditions. This phenomenon of polymorphic imprinting had already been observed for the IGF2R gene that is imprinted in mice but shows a variable pattern in humans.51 The mechanisms underlying this variability are so far unknown.

Genes identified by this screen are located away from known imprinted genes or clusters. As no other signals in their neighborhood could be deduced from the arrays, they could therefore a priori belong to the class of isolated imprinted genes. It is striking to find four out of seven new imprinted genes coding for transcription factors with zinc finger domains. Analysis of 3′ exons of genes from this family had highlighted the coexistence of different and contradictory histone marks that could be comparable to those of imprinted genes.52 The expression of a selection of ZF genes was however found biallelic by these authors and the presence of these marks was rather correlated with the repeats of ZF domains. In addition to the genes identified in this screen (ZFAT, GLIS3, ZC3H12C and LIN28B), other ZF genes are known to be imprinted: ZNF264, ZIM2, PLAGL1, PEG3, among others.

Conservation of the imprinted status is not strict between mammalian species. We explored murine placentas and observed a biallelic profile for most of the newly identified imprinted genes. Therefore, the placental imprinted profile of these genes seems to be specific to the human species. The L3MBTL1 gene, coding for a zinc finger transcription factor and potential tumor suppressor, is the only other case of an imprinted gene (also expressed from the paternal copy in human tissues) but escaping imprinting in mice.53 However, it is difficult to refute or affirm categorically the imprinted status of a gene, particularly in the human species, as this phenomenon can be very restricted in its temporal and/or spatial profile.

The Magi2/S-Scam gene, however, seems to be also imprinted in the murine and bovine species. This gene produces three alternative isoforms, all of which seem to be imprinted from our results. A partial knockout mouse was performed that lacks the expression of the longest isoform (S-SCAMα) but maintains the two others (S-SCAMβ and γ).54 No reproduction failure was reported but these mice die shortly after birth and seem to have abnormal signaling responses in dendrites. It is likely that this isoform, whose function is necessary in the brain, is not crucial in the murine placenta and that the presence of the two other isoforms is sufficient for the placental function of the Magi2 gene, whatever it is. In humans, deletion of this gene was associated with infantile spasms.55 It was a surprise to see that the human MAGI2 gene expressed the paternal copy while in murine placenta and bovine tissues, the active copy was the maternal one. This situation has already been described for the ZIM2 gene55 and some imprinted genes also show variable imprinting effects according to different isoforms and tissues.

Imprinted genes have in common a complex epigenetic regulation involving different mechanisms including antisense, miRNAs, differential methylation and histone modifications. Though not a strict requirement, the presence of antisense RNA is a hallmark of imprinted genes. ZFAT-AS1 is a 2-exon gene transcribed from the opposite strand and partially overlapping some ZFAT exons. We could show that this antisense gene also presents a monoallelic expression in the placenta and that the expressed copy is also of paternal origin. According to the ncRNAimprint database, all antisense RNAs described in the context of imprinted genes present this paternal specificity.56 The antisense ZFAT-AS1 RNA together with the described antisenses for GLIS3 and MAGI2 seem to be human specificities absent in other mammalian species, from available sequence data of these genomic regions. The nearest genes distal to ZFAT, located about 80 kb upstream, are two miRNAs. The ZFAT gene has been hypothesized to be a potential target of these miR30B and miR30D genes (http://services.bio.ifl.lmu.de/mirsel). These miR30 RNAs, however, putatively target other genes, including the known imprinted genes RB1, CSF2 and CDKN1C. The small size and the paucity of frequent SNPs within these sequences make it difficult to analyze the expression profile and the potential imprinted status of these miRNAs, whose interactions with ZFAT might be of great interest.
The LIN28B and ZFAT genes were further studied in order to characterize their potential role in placenta function, as an imprinted gene.

We then explored the methylation status of the regions surrounding ZFAT and LIN28B. If the epigenetic control of known imprinted genes, generally located within clusters under the control of an ICR, is difficult to fully characterize, it appears even more difficult to anticipate how an isolated imprinted gene is regulated and at which distance are the crucial elements responsible for the differential imprints affixed during gametogenesis. In spite of this apparent lack of differentially methylated regions in ZFAT CpG islands, binding sites for transcription factors known to regulate imprinted genes can be detected in the ZFAT promoter, such as CTCF and YY1. In addition, other mechanisms than DNA methylation could be involved in the regulation of the ZFAT expression profile. For LIN28B, the CpG island located around the promoter showed, on the contrary, a differentially methylated profile that is likely to be an important regulatory region, a DMR as observed in most imprinted genes.

We then explored the imprinted status of ZFAT and LIN28B in other human tissues but failed to observe a monoallelic expression in other tissues than the placenta: in lymphocytes and endometrial tissue, ZFAT is strictly biallelic while LIN28B is not expressed. Therefore, the imprinted profile of ZFAT and LIN28B seems to be rather specific and may be restricted to the placenta. However, ZFAT could be successfully amplified from different tissues and also seems expressed in many tissues including spleen, thymus and brain. Other genes are also known to have a restricted imprinted profile whereas others maintain their imprint within the complete body; even IGF2 escapes imprinting in the brain.

Null mice for Zfat present an embryonic lethality and do not survive after E8.5. The phenotype is not observed in heterozygous mice, in agreement with our finding of a biallelic expression of Zfat in mouse. A placental phenotype was observed, as the spongiotrophoblast does not develop correctly. At E8, Zfat is normally expressed in the endothelial and hematopoietic progenitors of blood islands but the authors show in null mice an impaired differentiation of hematopoietic progenitors in blood islands of the yolk sac. ZFAT is also suspected to play a role in angiogenesis and hematopoiesis. By immunohistochemistry we could find ZFAT expression in endothelial cells and syncytiotrophoblast. In addition, the gene was consistently downregulated in placentas from preeclampsia and/or IUGR, whereas this gene was described as stable along the human gestation period. As a loss of imprinting would rather be synonym of increased expression, this deregulation is likely due to a transcriptional mechanism in the pathological context. Very interestingly, a recent study revealed that SNP variants within the ZFAT gene are associated with hypertension. Our data, together with the literature coincidence to propose ZFAT as a regulator of the differentiation of endothelial cells within and outside the placenta. This is consistent with the role of this gene in the assembly of endothelial cells during the angiogenesis process, and the formation of capillary networks, particularly in HUVECs. Therefore, ZFAT appears as an interesting candidate to have a role in placental development that needs to be further characterized. Labeling of slides from pathological placentas might bring additional information on this abnormal expression profile.

In conclusion, in this work we could validate and identify new human imprinted genes whose function in the placenta remains to be explored. Increasing the number of identified imprinted genes and completing exhaustively their list will help to extract the specificities of this class of particular genes in terms of epigenetic regulation, physiological function and pathological alterations. These data are of particular interest in the present context, where approaches using new technologies such as RNA-seq to identify imprinted genes in mice tend to question some previously known imprinted genes and failed to identify many new imprinted genes. Indeed, some genes exhibiting a maternal expression could have been misinterpreted as imprinted after contamination of the murine placent al tissue by highly expressing maternal uterine cells. The exploration of human placental tissue circumvents this problem, as its size and volume make it easier to avoid maternal contaminations.

**Material and Methods**

**Tissue samples.** Human placentas were collected from caesarean sections of normal and pathological pregnancies. Protocols were approved by the local ethics committee and all patients signed an informed consent. Patients' description was previously reported. After removal of the maternal membranes, a small piece of villi was collected as previously described. Human lymphocytes were purified from blood samples of mothers, patients or anonymous donors. Endometrial tissues were collected as described. Thyroid tumors were also collected.

**Mouse placentas** were collected at embryonic day E14.5 and E17.5 after mating of a Mus musculus domesticus C57B6 female and a Mus musculus molossinus male. At E17.5, placentas were either kept complete or dissected to separate the labyrinth from the spongiotrophoblast layer, whereas E14.5 placentas were kept complete. Fetal material was also collected for DNA extraction and genotyping.

**Bovine placent al and fetal** (trophoblast, cotyledon, allantois, liver, brain and heart) samples were collected from crosses between a female Holstein and a male Charolais, at 18 or 60 d post-coitum.

Whatever the source, all collected samples were divided in two aliquots and stored frozen either dry or in Trizol, for further DNA and RNA extraction respectively.

**DNA and RNA extraction.** Extractions were performed under standard procedures. Briefly for DNA extraction, samples were first digested with proteinase K and SDS. Phenol-chloroform extractions were followed by alcohol precipitation of genomic DNA. Gel electrophoresis and OD measurement (Nanodrop) were performed to quantify the genomic DNA and check for its integrity. For RNA extraction, samples were lysed using beads in the Qiagen TissueLyser II apparatus. A Trizol extraction was then performed according to the manufacturer's recommendations (Invitrogen).

**Genotyping arrays.** After validation of the RNA quality with the Agilent Bioanalyzer 2100 (using Agilent RNA6000 nano...
required (Affymetrix); briefly, a first strand cDNA synthesis was performed using Superscript II and T7oligodT followed by a second strand cDNA synthesis using E.coli DNA ligase, E.coli DNA polymerase and RNase H. The double strand cDNA was then purified using cDNA clean up spin column, eluted and quantified with the Nanodrop ND1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Inc.). All cDNA samples were independently processed and final concentrations were adjusted to 100 ng/μl. The processing of cDNA samples was then identical to the processing of genomic DNA samples. Genotyping analysis with Affymetrix NspI chip was performed for both sample types following manufacturer’s guidelines. Briefly either 250 ng of double stranded cDNA or 250 ng of genomic DNA were restricted with NspI. NspI adaptors were then ligated to restricted fragments and subjected to PCR using the universal primer PCR002 provided by the kit. PCR fragments were then purified and 90 μg used for fragmentation and end-labeling with biotin using Terminal Transferase. Labeled targets were then hybridized overnight to Genechip® human 250K NspI array (Affymetrix) at 49°C. Chips were washed on the fluidic station FS450 following specific protocols (Affymetrix) and scanned (Affymetrix) at 49°C. Chips were washed on the fluidic station FS450 following specific protocols (Affymetrix) and scanned (Affymetrix) at 49°C. Chips were washed on the fluidic station FS450 following specific protocols (Affymetrix) and scanned (Affymetrix) at 49°C. Chips were washed on the fluidic station FS450 following specific protocols (Affymetrix) and scanned (Affymetrix) at 49°C.

PCR, RT-PCR, sequencing. cDNA synthesis was performed using the MMLV cDNA or the SuperScript II synthesis kit (Invitrogen) from 2 μg of RNA, previously treated with DNase I to eliminate putative DNA contaminations. Primers were designed using the PRIMER3 software (http://frodo.wi.mit.edu). The list, sequences and conditions of use of the primers are in Table S1. Classically, PCR was performed on 1/10 of the RT product or on 200 ng of genomic DNA, for 35 cycles on a GeneAmp 7500 thermocycler (Applied Biosystems) with Platinum Taq polymerase. Sequencing was performed on an Applied Biosystems 3130XL sequencer by the local platform.

Methylation analysis. Various softwares were used to locate CpG rich regions (newcpgreport, Cpgplot Cpgseek, CpGprod) at http://mobile.pasteur.fr. Aliquots of genomic DNA were treated with sodium bisulphite using the EZ DNA Methylation-Gold kit (Zymo Research). These DNAs were used as templates for amplification and direct sequencing of specific CpG islands and subsequent cloning into the pJET vector (Fermentas). Positive clones were checked by PCR and sequenced.

Real-time PCR. Real time PCR was performed using the LightCycler 480, the corresponding LC480 SYBRGreen Master kit and 96-well plates (Roche). Conditions were as follows: 95°C for 5 min, and 40 cycles of three temperature steps (95°C for 10 sec, 55°C for 10 sec and 72°C for 10 sec). Finally, samples were submitted to a progressive temperature ramping resulting in a melting curve, validating the specificity of the amplification. Amplification products were also checked by agarose gel electrophoresis and sequencing. The threshold cycle numbers (Ct) were obtained using the LightCycler 480 software (Roche) and the second derivate maximum method. Data from target genes were normalized using the succinate dehydrogenase subunit A (SDHA) used as reference gene and shown previously to be stable and highly expressed in the human placenta.70 Data were analyzed using the delta Ct method.71 Experiments were conducted in triplicates.

Immunoblotting and immunohistochemistry. Placenta, peripheral blood lymphocytes (PBL) or human choriocarcinoma JEG-3 cells were solubilized in RIPA buffer for 1h at +4°C. Fifty μg of each sample were loaded per well and separated on 4–12% NuPage Bis-Tris precast gels (Invitrogen) in MOPS SDS running buffer and then electro-transferred to PVDF membrane under standard conditions. The ZFAT protein was detected with a goat anti-ZFAT IgG (sc-87510, obtained from Santa Cruz Biotechnology) at 1:750 and donkey anti-goat peroxidase conjugated IgG at 1:7500 revealed by the enhanced Chemiluminescence (ECL) detection system (Millipore).

Human placental and fetal lung slides were hybridized with the same antibody (1:100) and revealed using the Universal LSAB + HRP Detection Kit (Dako). A counterstaining of slides was performed with Methyl Green.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/epigenetics/article/21495

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