Re-investigation and RNA sequencing-based identification of genes with placenta-specific imprinted expression

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Within the vertebrate groups, only mammals are subject to a specialized epigenetic process termed genomic imprinting in which genes are preferentially expressed from one parental allele. Imprinted expression has been reported for >100 mouse genes and, for approximately one-quarter of these genes, the imprinted expression is specific to the placenta (or extraembryonic tissues). This seemingly placenta-specific imprinted expression has garnered much attention, as has the apparent lack of conserved imprinting between the human and mouse placenta. In this study, we used a novel approach to re-investigate the placenta-specific expression using embryo transfer and trophoblast stem cells. We analyzed 20 genes previously reported to show maternal allele-specific expression in the placenta, and only 8 genes were confirmed to be imprinted. Other genes were likely to be falsely identified as imprinted due to their relatively high expression in contaminating maternal cells. Next, we performed a genome-wide transcriptome assay and identified 133 and 955 candidate imprinted genes with paternal allele- and maternal allele-specific expression. Of those we analyzed in detail, 1/6 (Gab1) of the candidates for paternal allele-specific expression and only 1/269 (Ano1) candidates for maternal allele-specific expression were authentically imprinted genes. Imprinting of Ano1 and Gab1 was specific to the placenta and neither gene displayed allele-specific promoter DNA methylation. Imprinting of ANO1, but not GAB1, was conserved in the human placenta. Our findings impose a considerable revision of the current views of placental imprinting.

INTRODUCTION

Genomic imprinting is an essential mechanism in mammalian development that regulates the preferential expression of the paternally or maternally inherited allele of a subset of genes. Within vertebrate lineages, imprinting appears to be restricted to eutherian mammals and marsupials (1). Imprinting arose during mammalian evolution and could thus be linked to placental development and function (2,3). Various theories have been proposed to explain the biological and evolutionary significance of this phenomenon (4,5).

One of the key defining features of eutherian mammals is the chorioallantoic placenta. This structure, which infiltrates the maternal uterus, has a pivotal role in embryonic growth and development through regulating the transport of nutrition, gas and waste between fetal and maternal blood (6,7). A large number of imprinted genes are expressed in the placenta and it has been proposed that some of these control the supply of...
nutrients to the fetus (8–11). In contrast, imprinted genes expressed in the embryo may determine nutritional demand by regulating the growth rate of fetal tissues (3). Importantly, low birth weight has implications for postnatal development and has been linked to the development of certain diseases later in life (12), highlighting the critical role of the placenta both in the neonatal period and, more perniciously, in the adult.

In the mouse, X chromosome inactivation does not occur randomly in extraembryonic lineages and genes subject to X-inactivation in female mice display tissue-specific imprinted expression in the placenta but are expressed mosaically in the embryo and adult (13,14). Similarly, there are ~30 autosomal genes, which have been reported to show imprinted expression only in the placenta (or extraembryonic tissues) (15–17). Remarkably, almost all of these genes specifically imprinted in the placenta are active on the maternal allele and repressed on the paternal allele.

The mechanisms for maintaining imprinted expression in the embryo may differ from those found in the placenta as loss of the maintenance DNA methyltransferase, Dnmt1, results in the relaxation of imprinting of some genes preferentially in the embryo (18,19). For some genes with placenta-specific imprinted expression, imprinted gene expression depends on H3-K9 dimethylation (H3K9me2) and/or H3-K27 trimethylation (H3K27me3) and is impaired in the absence of G9a and EED, which may be a consequence of lineage-specific, temporal dependence on long non-coding RNAs (9,15,16). A comprehensive, whole genome analysis aimed at identifying genes with placenta-specific imprinted expression may provide a clearer picture regarding the requirement of imprinted gene expression in the placenta versus the embryo. However, the mouse placenta consists of contributions from both the mother and the fetus (6) raising the possibility that strategies aimed at identifying novel imprinted genes may be confounded by contaminating material. There are several potential sources of contamination as complete removal of the decidua from the placenta is difficult, maternal cells are also known to exist in the spongiotrophoblast and labyrinth layers (20–22) and the ectoplacental cone is already invaded by maternal blood at embryonic day (E) 6.5 (15).

To assess the importance of this issue, we re-investigated the imprinted status of genes previously reported to show placenta-specific imprinted expression first using an embryo transfer procedure to identify the maternal cell contribution and then using trophoblast stem (TS) cells grown in culture away from the maternal environment. In addition, we performed genome-wide screen to identify all the genes that might fall into this same category, either as contaminants or imprinted genes.

RESULTS

Imprinted gene expression in the placenta without maternal decidua

The expression level of 27 genes, previously reported to show placenta-specific maternal allele expression, was first determined by quantitative polymerase chain reaction (QPCR) in the maternal decidua of E13.5 placenta after dissection (Fig. 1A). Of the 27 genes examined, 6 (Ctnm3, Klrb1f, Art5, Cmah, Drd1a, Fbxo40) were expressed at negligible levels in the placenta. Low expression of these genes was also confirmed in the whole transcriptome sequencing as described below. Ten of 21 genes with placenta-specific imprinted expression showed more than 10 times higher expression in the decidua than in the placenta (Fig. 1A). The preferential expression in the decidua was also confirmed using in situ hybridization for Gatm, Tfp2 and Ampd3 (Fig. 1B).

In order to determine whether there was any remaining maternal contamination after surgical removal of the maternal decidua, we employed an embryo transfer strategy. C3H/HeJ (C3H) embryos were transferred into pseudopregnant C57BL/6 (B6) mice. Placentas derived from this mating strategy are composed of a C3H embryonic component and a B6 maternal component. E13.5 placentas were again collected and the decidua was carefully removed. After removal of the decidua, the remaining material was subjected to genomic DNA amplification over a polymorphism between the C3H and B6 strains spanning the Gapdh gene. This revealed that most of maternal cells were removed when dissecting away the decidua (Fig. 2A).

Single nucleotide polymorphisms (SNPs) between C3H and B6 were used to examine the expression of three genes highly expressed in the decidua and previously reported to be imprinted (Wt1, Gatm and Qpct). When Wt1 was amplified from genomic DNA obtained from the placenta after removal of the decidua, the peak of the B6 allele was near background level, consistent with the very small level of maternal contamination in this dissected material (Fig. 2B). However, analysis of the cDNA from dissected material revealed predominant expression of the Wt1 B6 allele (Fig. 2B). A similar pattern was obtained with Gatm and Qpct (Supplementary Material, Fig. S1A). These data demonstrated that, even after the careful removal of the decidua, there was still sufficient maternal cell contamination to significantly impact expression studies.

The analyses of the placenta-specific imprinting using embryo transfer and TS cells

As described above, maternal cell contamination was a significant factor in the analysis of imprinted gene expression in the mouse placenta. We therefore set up an experiment to ask how many of the genes previously reported to show maternal allele expression in the placenta might have been falsely identified. To distinguish between maternal allele-specific expression and maternal contamination, embryos obtained by crossing Japanese fancy 1 (JF1) females and B6 males ([JF1xB6]F1) were transferred to pseudopregnant B6 recipients. Placentas derived from this embryo transfer experiment were composed of [JF1xB6]F1 embryonic cells and B6 maternal cells. Genes expressed from the maternal allele would carry JF1 SNPs, while genes expressed in contaminating maternal cells would have the B6 SNPs. Genes expressed from the maternal allele also carry B6 SNPs. E13.5 placentas were collected, the decidua was carefully removed as before and the allelic expression was determined. We could confirm that 6 out of
the 18 genes for which there were SNPs were authentically expressed from the maternal allele (Ppp1r9a, Ascl2, Th, Tssc4, Slc22a3, and Slc22a2) (Table 1 and Supplementary Material, Fig. S2), while 11 genes were potentially falsely identified as imprinted due to their relatively high expression in maternal material (Wt1, Tfpi2, Pon3, Pon2, Ampd3, Osbpl5, Dhcr7, Mst1r, Dcn and Scin are shown in Supplementary Material, Fig. S2; Tspan32 is shown in Fig. S2). Nap1l4 was biallelically expressed in the [B6xJF1]F1 and [JF1xB6]F1 placentas obtained by normal mating (Supplementary Material, Fig. S2).

For the 12 genes which did not show maternal allele-specific expression in the [JF1xB6]F1 placentas obtained by the embryo transfer, it was still possible that these genes showed maternal allele-specific expression but that this expression was obscured by the contaminating material. TS cells can differentiate into all cell types of the placenta and do not contain contaminating maternal cells (23). First, we confirmed the faithful expression of 10 well-known imprinted genes (Igf2r, H19, Meg3, Grb10, Phlda2, Cdkn1c, Peg10, Solec, Snrpn, Mest) in [B6xF1]F1 and [F1xB6]F1 TS cells (Supplementary Material, Fig. S2). Using undifferentiated and differentiated TS cells, we were able to confirm imprinting for only 2 of the 12 genes (Tspan32 and Tfpi2) (Fig. 2C and Supplementary Material, Fig. S3B). Similar results were obtained in at least two [B6xF1]F1 and [F1xB6]F1 TS cell lines. Furthermore, we analyzed [F1xB6]F1 and [C3HxB6]F1 placentas at E9.0 and confirmed that Wt1, Gatm, Pon3, Pon2, Ampd3, Osbpl5, Dhcr7, Mst1r, Dcn, Scin and Qpct did not show maternal allele-specific expression (Supplementary Material, Figs S1B and S2B).

Whole transcriptome sequencing analysis of placental imprinting
To determine how significant this issue of maternal contamination might be to the identification of novel imprinted genes, we applied whole transcriptome sequencing to the dissected F1 material obtained by normal mating. About 300 million reads were sequenced and SNPs with biased allelic expression were identified (see Materials and Methods for details). Preferential expression from paternal and maternal alleles were observed at 323 and 1930 SNP sites, respectively, equivalent to 133 and 955 candidate imprinted genes with paternal allele- and maternal allele-specific expression (Supplementary Material, Tables S1 and S2). Expression of 49 genes...
previously reported to be imprinted were at sufficient level to assess allelic expression. Of these, 36 showed imprinted expression (Supplementary Material, Table S3). The success rate of imprinted gene identification was comparable with the previous work (24–27).

Identification of novel genes with placenta-specific imprinted expression

Among the novel genes identified, 6 and 269 candidate genes with paternal allele- and maternal allele-specific expression, which contain more than two SNP sites with biased allelic expression, were chosen for further analysis. The allelic expression of the six candidate genes with paternal allele expression was analyzed by Sanger sequencing. 

**Gab1** was confirmed to be imprinted (Fig. 3A). Gab1 also showed paternal allele-specific expression in TS cells, but biallelically expressed in the embryo and yolk sac at E13.5 and in adult tissues (Fig. 3A). The other five genes were biallelically expressed in the placenta (Supplementary Material, Fig. S4A).

The allelic expression of 269 candidate genes with maternal allele-specific expression was analyzed in the [JF1xB6]F1 placentas obtained by the embryo transfer strategy (Supplementary Material, Table S5). All these candidate genes showed a higher or equal level of expression from B6 allele, implying that the maternal allele-specific expression identified in the natural mating strategy could be attributed to maternal cell contamination. But again, it was possible that the maternal allele-specific expression was obscured by the contaminating material. To address this possibility, we examined the allelic expression of 269 genes in the TS cells. Only Anol was found to be expressed from the maternal allele (Fig. 3B). Anol was biallelically expressed in the embryo, yolk sac and adult tissues (Fig. 3B).

Epigenetic modification of Gab1, Anol and Sfmbt2

As imprinted genes tend to be clustered, the allelic expression of neighboring genes for Gab1 and Anol was analyzed in the [B6xF1]F1 and [JF1xB6]F1 TS cells. None was found to be imprinted in the TS cells (Supplementary Material, Fig. S4B). We also analyzed the Sfmbt2 locus. Sfmbt2 shows placenta-specific paternal allele expression and does not map to a known imprinted gene clusters (28). The neighboring genes of Sfmbt2 were also biallelically expressed in the TS cells (Supplementary Material, Fig. S4B).

The DNA methylation patterns of Gab1, Anol and Sfmbt2 were analyzed in E13.5 placenta by bisulphite sequencing, but no differentially methylation was found (Fig. 4A–C). Furthermore, the paternal allele-specific expression of Gab1 and Sfmbt2 was not affected in E9.5 placenta obtained from Dnmt3l-deficient or oocyte-specific Dnmt3a/3b-deficient female mice (Fig. 4D). This indicates that the establishment of imprinting of Gab1 and Sfmbt2 does not require maternal germline methylation. Because Anol was mainly expressed...
Table 1. Summary of genes with placenta-specific imprinted expression

<table>
<thead>
<tr>
<th>Chr.</th>
<th>Imprinting status in the mouse</th>
<th>Placenta</th>
<th>TS cells</th>
<th>Imprinting status in the human placenta</th>
</tr>
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<tr>
<td>2</td>
<td><strong>Sfmbt2</strong></td>
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<td>Imprinted (P)</td>
<td>Non-imprinted</td>
</tr>
<tr>
<td>2</td>
<td><strong>Wt1</strong></td>
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<td>Non-imprinted</td>
<td>Imprinted (polymorphic) (59)</td>
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<tr>
<td>2</td>
<td><strong>Gatm</strong></td>
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<td>Non-imprinted (48)</td>
</tr>
<tr>
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<td><strong>Tfpi2</strong></td>
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<td><strong>Imprinted (M)</strong></td>
<td>Imprinted (polymorphic) (30)</td>
</tr>
<tr>
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<td><strong>Ppp1r9a</strong></td>
<td>Imprinted (M)</td>
<td>Imprinted (M)</td>
<td>Imprinted (polymorphic) (60)</td>
</tr>
<tr>
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<td><strong>Pen3</strong></td>
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<td>Non-imprinted</td>
<td>Non-imprinted (30)</td>
</tr>
<tr>
<td>6</td>
<td><strong>Pen2</strong></td>
<td>Non-imprinted</td>
<td>Non-imprinted</td>
<td>Non-imprinted (30)</td>
</tr>
<tr>
<td>6</td>
<td><strong>Cntn3</strong></td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td><strong>Kleb1f</strong></td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td><strong>Art5</strong></td>
<td>Non-imprinted</td>
<td>Non-imprinted</td>
<td>Non-imprinted (49)</td>
</tr>
<tr>
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<td><strong>Ampd3</strong></td>
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<td>Imprinted (M)</td>
<td>Non-imprinted (48)</td>
</tr>
<tr>
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<td><strong>Th</strong></td>
<td>Imprinted (M)</td>
<td><strong>Imprinted (M)</strong></td>
<td>Non-imprinted (48)</td>
</tr>
<tr>
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<td><strong>Ascl2</strong></td>
<td>Imprinted (M)</td>
<td>Imprinted (M)</td>
<td>Non-imprinted (48)</td>
</tr>
<tr>
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<td><strong>Tspan32</strong></td>
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<td>Non-imprinted</td>
<td>Non-imprinted (48)</td>
</tr>
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<td>NA</td>
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<tr>
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<td><strong>Tssc4</strong></td>
<td><strong>Imprinted (M)</strong></td>
<td><strong>Imprinted (M)</strong></td>
<td>Non-imprinted (48)</td>
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<tr>
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<td><strong>Imprinted (M)</strong></td>
<td>Non-imprinted (48)</td>
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<tr>
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<td><strong>Oshp15</strong></td>
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<td>Non-imprinted (61)</td>
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<tr>
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<td><strong>Dhcr7</strong></td>
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<td>Non-imprinted (49)</td>
</tr>
<tr>
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<td><strong>Ano1</strong></td>
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<td><strong>Imprinted (M)</strong></td>
<td>Imprinted (polymorphic)</td>
</tr>
<tr>
<td>8</td>
<td><strong>Gab1</strong></td>
<td>Imprinted (P)</td>
<td>Imprinted (P)</td>
<td>Non-imprinted</td>
</tr>
<tr>
<td>8</td>
<td><strong>Mst1r</strong></td>
<td>Non-imprinted</td>
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<td>NA</td>
</tr>
<tr>
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<td><strong>Dcn</strong></td>
<td>Non-imprinted</td>
<td>ND</td>
<td>Non-imprinted (48)</td>
</tr>
<tr>
<td>12</td>
<td><strong>Scin</strong></td>
<td>Non-imprinted</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td><strong>Cmah</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td><strong>Dr3l1a</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td><strong>Fbxo40</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>17</td>
<td><strong>Slc22a3</strong></td>
<td><strong>Imprinted (M)</strong></td>
<td><strong>Imprinted (M)</strong></td>
<td>Imprinted (polymorphic) (48)</td>
</tr>
<tr>
<td>17</td>
<td><strong>Slc22a2</strong></td>
<td><strong>Imprinted (M)</strong></td>
<td>ND</td>
<td>Imprinted (polymorphic) (48)</td>
</tr>
<tr>
<td>17</td>
<td><strong>Qpct</strong></td>
<td>Non-implemented</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Genes where paternal and maternal allele expression was confirmed in the placenta and/or TS cells are shown in bold. ‘M’, maternal allele-specific expression; ‘P’, paternal allele-specific expression; ND, not detected; NA, not analyzed.

from contaminated maternal cells, the allelic expression was not analyzed in those mutant mice.

Allele-specific expression of some genes with placenta-specific imprinted expression is reported to be regulated by the histone methylation (18,29,30). We analyzed the allelic histone modifications in E13.5 [B6xJF1]F1 placenta or [B6xJF1]F1 TS cells by chromatin immunoprecipitation (ChIP) analyses (Fig. 4). Following antibodies were used: dimethylated H3-Lys4 (H3K4me2), trimethylated H3-Lys4 (H3K4me3), H3K9me2, H3K27me3, H3K4me2 and H3K4me3 are markers of active genes and H3K9me2 and H3K27me3 are repressive markers. At the Ano1 transcription start site, H3K4me2 and H3K4me3 were enriched on the maternal allele in the placenta and TS cells. Allelic enrichment of H3K9me2 or H3K27me3 was not observed (Fig. 4B). At Sfmbt2, maternal enrichment of H3K9me2 and H3K27me3 and paternal enrichment of H3K4me2 and H3K4me3 were observed (Fig. 4C). The histone modification around the Gab1 transcription start site could not be analyzed as no SNPs were found between B6 and JF1. Maternal enrichment of H3K9me2 and paternal enrichment of H3K4me3 were observed at the intron 1 of Gab1 (Fig. 4A). The levels of H3K9me2 and H3K27me3 at Sfmbt2 and Gab1 were comparable with those at Tssc4 and Slc22a3, which are reported to be regulated by H3K9me2 and/or H3K27me3 (31,32) (Supplementary Material, Fig. S5).

**Preferential expression of ANO1 from the maternal allele in the human placenta**

We next examined the imprinting status of ANO1, GAB1 and SFBMT2 in the human placenta. Preferential expression of ANO1 from the maternal allele was observed in one of the two term placenta samples where we had informative parental genotyping (Fig. 5A and Supplementary Material, Fig. S6). The maternal allele expression of ANO1 was also confirmed by restriction fragment length polymorphism (RFLP) analysis (Fig. 5B). Importantly, we were also able to show monoallelic expression in three of five term placenta samples where both the mothers and the fetuses were heterozygous for the SNP, which formally excludes maternal contamination (Supplementary Material, Fig. S6). GAB1 and SFBMT2 were biallelically expressed in four human term placenta samples (Fig. 5A and Supplementary Material, Fig. S6). ANO1, GAB1 and SFBMT2 were biallelically expressed in one or two first trimester placenta samples (Supplementary Material, Fig. S6). Biallelic expression of SFBMT2 in the human placenta was quite recently reported (33).

**DISCUSSION**

The key finding from this study is that maternal contamination is a confounding factor when analyzing imprinted gene
expression in the mouse placenta. As a result of our analysis, we can provide a new map of genes with placenta-specific imprinted expression, shown in Figure 6. We have also identified two novel genes with placenta-specific imprinted expression, Ano1 and Gab1. Our data provide a more accurate picture of imprinting in the placenta, which will help in understanding of the function of imprinting in the mammalian placenta and evolution of genes with placenta-specific imprinted expression.

Re-examination of genes with placenta-specific imprinted expression

We have shown that genes highly expressed in the maternal decidua can be falsely identified as showing placenta-specific imprinted expression. For Dcn and Osbp15, the predominant expression from the decidua has already been reported (34,35) and we provide a list of additional genes that fall into this category. The expression level in the decidua may be a good indicator of the risk that the allelic expression is affected by maternal cell contamination. We have shown that, for genes highly expressed in the decidua, maternal allele-specific expression in the placenta can be explained by maternal cell contamination.

Although maternal cell contamination can be misleading, it does not exclude the possibility that some genes, in fact, show maternal allele-specific expression. For example, Tfpi2 and Tspan32 were found to be expressed from contaminated maternal cells in the placenta but were also found to show maternal allele expression in TS cells. Maternal contamination can therefore result both in the false identification of imprinted expression and also obscure genuine imprinted expression. TS cells, which do not contain any maternal cells, are useful model systems for studying genomic imprinting in the placenta. However, this approach may not be full proof because it is possible that some genes are biallelically expressed in TS cells despite definitive maternal allele-specific expression in the placenta. In addition, it is already known that in vitro fertilization and embryo culture could disturb the imprinted expression of some genes (36,37), and this might also affect our analysis because in vitro fertilized embryos were used for the embryo transfer experiments. For Gatm, Pon3, Pon2 and Osbp15, which are not confirmed to be imprinted in this study, preferential expression from the maternal allele was reported in the yolk sac, but the bias is very weak and should be carefully interpreted (35,38,39). One way to resolve these problems would be to utilize a knock-in reporter system where allele-specific expression can be finely assigned to specific tissues.

Identification of novel genes with placenta-specific imprinted expression

By the whole transcriptome analysis of the placenta, >1000 genes were identified as showing supposedly allele-specific differences. We identified 19 genes with paternal allele-specific expression, which contain more than two SNP sites with biased allelic expression. Among them, 13 were known
imprinted genes and the other 6 candidate genes were analyzed in detail. Gab1 was confirmed to show imprinted expression, but the other five genes were biallelically expressed in the placenta. It is possible that for the five genes, only some splicing variants show imprinted expression and others are biallelically expressed. Alternatively, it is also known that candidate genes identified by RNA sequencing involve some false positive genes. Of the 269 candidate genes with maternal allele expression, which we analyzed in [JF1xB6]F1 placenta obtained by embryo transfer, only Ano1 was confirmed to show maternal allele-specific expression, by the TS cell strategy. These data indicate that there are many genes highly expressed from contaminating maternal cells in the placenta.

Recently, Wang et al. (40) identified two paternally expressed and three maternally expressed novel imprinted genes using E17.5 mouse placenta samples from reciprocal cross F1 progeny of AKR/J and PW/D/PhJ. The two genes with paternal allele-specific expression are not included in our candidate imprinted genes and this may reflect the differences in developmental stages and mouse strains. For the three genes with maternal allele-specific expression, the possibility of maternal cell contamination was not considered. Importantly, Brideau et al. (17) reported 10 novel imprinted genes using very similar samples to those used by Wang et al. (40), but we failed to confirm the imprinting of the 10 genes. Among them, four genes (Wil, Mst1r, Scin, Qpct) were predominantly expressed by the contaminating maternal cells and the other six (Ctnm3, Klrb1f, Art5, Cmah, Drd1a, Fbxo40) were not detected in E13.5 placenta. It is possible that the maternal cell contamination is greater at E17.5 and the six genes are detectable at E17.5 but not at E13.5. These data suggest that E17.5 placenta obtained by natural mating is not suitable for the identification of imprinted genes because of significant maternal cell contamination.

In this study, Gab1 was found to show paternal allele-specific expression. Recently, Gab1 was reported to show lower expression in parthenogenetic blastocysts than in fertilized embryos, consistent with our data. Ano1 and Gab1 appear to be genes with placenta-specific imprinted expression because we have shown that they are not imprinted in the embryo, yolk sac or adult tissues. Including these two genes, there are now 11 confirmed genes with placenta-specific imprinted expression (Fig. 6). While most imprinted genes

Figure 4. Epigenetic modification of Gab1, Ano1 and Sfmbt2. (A–C) DNA methylation in [B6xJF1]F1 placenta was analyzed by bisulphite sequencing. Black and white circles indicate methylated and unmethylated residues. Histone modifications in [B6xJF1]F1 placenta and TS cells were analyzed using ChIP and SNuPE. The allele-specific histone modification was expressed as a percentage of maternal (red bars) or paternal (blue bars) alleles in the total immunoprecipitate. The error bars indicate the means + SD from two replicates. The genomic structure of Gab1, Ano1 and Sfmbt2 is shown and arrows indicate transcription start sites. (D) The allelic expression of Gab1 and Sfmbt2 in the E9.5 placentas obtained from Dnmt3l-deficient and oocyte-specific Dnmt3a/3b-deficient females. Dnmt3a/3b and Dnmt3l knockout female mice were crossed with WT JF1 male mice.
with placenta-specific maternal allele expression are included in known imprinted gene clusters, Ano1, which was imprinted in the mouse and human, is unlikely to be included in the Kcnq1 imprinting cluster because the distance between ANO1 and KCNQ1 is over 67 Mb. Gab1 and Sfmbt2 are not included in any known imprinted gene clusters. For the maintenance of allele-specific expression of most imprinted genes with placenta-specific maternal allele expression, H3K9me2 and/or H3K27me3 are known to play important roles while maintenance of DNA methylation seems to be less important (18,29,30). Differentially methylated regions (DMRs) were not identified at the promoter region of Ano1, Gab1 or Sfmbt2, but allelic enrichment of histone modifications was detected. Recently, we reported a genome-wide screening of DMRs using mouse TS cells (42) and no DMR was found near Ano1, Gab1 or Sfmbt2. This suggests that perhaps DNA methylation is not required for inducing allelic expression of Ano1, Gab1 or Sfmbt2. We showed that for the establishment of imprinting of genes with placenta-specific paternal allele expression, Gab1 and Sfmbt2, maternal germline methylation was not required. It remains possible that methylation in the paternal germline is required for their imprinted expression at a DMR not identified in our genome-wide screen, but it is also possible that the establishment of imprinting of these genes does not need DNA methylation at all.

Among two novel imprinted genes we identified, Gab1 is already known to be important for placental development (43,44). Deficiency of Gab1 results in the reduction in the number of trophoblast cells in the labyrinth region. Gab1 functions as a signaling mediator of various receptor tyrosine kinases and regulates multiple signaling effectors, such as phosphatidylinositol 3-kinase and Shp2 (45). While the placentas of heterozygous Gab1+/− have not been analyzed, we predict that placental abnormalities will be present as the inactive allele is inherited from their father. Ano1 is a subunit of calcium-activated chloride channels and Ano1 knockout mice are reported to exhibit severe defects in tracheal development and death within 1 month of birth, while no overt phenotype has been observed for heterozygous Ano1+/− mice (46,47). The role of Ano1 in placental development has not been determined, but our data suggest this will merit investigation.

Human ANO1 showed preferential expression from the maternal allele in the term placenta. We find that, for most of the genes authentically expressed from the maternal allele in the mouse placenta, there is evidence of allele-specific expression in the human placenta, albeit polymorphic in most cases (Table 1). Polymorphic imprinting might imply that imprinting in the human placenta is stage or cell type specific. Nonetheless, our data indicate that, contrary to previous suggestions (48,49), imprinting in the placenta is well conserved between the mouse and human. The only exception to this is the proximal region of Kcnq1 imprinting cluster where Th, Asc12, Tspan32 and Tssc4 all show imprinted expression in either the mouse placenta or TS cells but not in the human placenta.

Mouse Gab1 and Sfmbt2 are two imprinted genes with placenta-specific paternal expression, which were not imprinted in the human placenta. Sfmbt2 is located in the proximal chromosome 2, of which maternal duplication is known to result in placental growth retardation (50). Currently, Sfmbt2 is the only known imprinted gene with paternal allel- specific expression in the proximal chromosome 2 and this suggests that Sfmbt2 might be important for placental development. Gab1 is already known to be required for placental development (43,44). Both Gab1 and Sfmbt2 may therefore positively regulate placental growth. We cannot say whether the mouse Gab1 and Sfmbt2 genes have gained imprinted expression in mice or whether the human homologues have lost their imprints, but it is interesting to speculate that these genes are not imprinted to increase the capacity of the human placenta to support fetal growth.

**MATERIALS AND METHODS**

**Whole transcriptome sequencing and data analysis**

B6 females were mated with JF1 (51) males to generate [B6xJF1]F1 mice and reciprocally crossed to generate [JF1xB6]F1 mice. Total RNA was extracted from four [B6xJF1]F1 and four [JF1xB6]F1 placentas at E13.5, respectively, and pooled. Twelve micrograms of total RNA was used for rRNA depletion (Riboflavin Eukaryote Kit for RNA-seq, Invitrogen, CA, USA) and RNA-seq library construction (SOLiD Whole Transcriptome Analysis Kit, Life Technologies, CA, USA) according to the manufacturer’s protocol. Libraries were cloned amplified on SOLiD P1 DNA Beads by emulsion PCR and sequenced using SOLiD3Plus System (Life Technologies). All SOLiD3+ reads were aligned with AB WT Analysis Pipeline (Applied Biosystems, CA, USA) against mouse NCBI genome build 37 (mm9) and against RefSeq Genes to capture alignment to splice sites. Reads aligned to rRNA, tRNA or sequence-adapted were filtered. Among total 319 701 254 reads obtained ([B6xJF1]F1: 154 500 642 and [JF1xB6]F1: 165 200 612), 144 406 747 (45.2%) were
aligned and passed filter. AB WT Analysis Pipeline aligns reads with up to five colorspace mismatches and provides mapping quality for each read. In order to collect highly reliable SNP candidates in each [B6xJF1]F1 and [JF1xB6]F1 on forward and reverse strand, we applied SAMtools 'pileup' software (52) with 15 over coverage and Phred-scaled likelihood SNP quality over 20 (as accuracy of SNP call 99%) to divided reads. Finally, we detected 128 837 candidate SNPs in transcriptome. Allele counts were tallied independently by transcript coordinates. To exclude minor alignment bias against sequences, we set the coverage threshold as 15 for each SNP. SNPs with biased allelic expression was determined using the following criteria: the ratio of maternal or paternal reads to total reads was 65% both in [B6xJF1]F1 and [JF1xB6]F1 samples. Unless otherwise indicated, 'transcripts' in this study comprise mouse UCSC known genes, RefSeq genes and Ensemble genes. Transcripts mapping to the sex chromosomes and mitochondrial chromosome were not considered.

Preparation of DNA and RNA

Production of mice with the conditional alleles, referred to as Dnmt3a2lox and Dnmt3b2lox, was described previously (53,54). To disrupt the conditional alleles in growing oocytes, the mice were crossed with those carrying a Zp3-Cre gene (55). The precise timing of conditional deletion of Dnmt3a and Dnmt3b by Zp3-Cre is described elsewhere (56). By crossing (Dnmt3a2lox/2lox, Dnmt3b2lox/2lox, Zp3-Cre) females with wild-type JF1 male mice, we obtained (Dnmt3a+/−, Dnmt3b+/−) E9.5 embryos. Dnmt3l knockout female mice (57) were also crossed with wild-type JF1 male mice to obtain Dnmt3l−/− E9.5 embryos.

For embryo transfer experiments, in vitro fertilized embryos were transferred to pseudopregnant recipients. The mating between B6 and JF1 does not efficiently occur even if superovulation is used, and we used in vitro fertilization to obtain enough embryos for the embryo transfer. [B6xJF1]F1 TS cells and [JF1xB6]F1 TS cells were derived and cultured in the absence of mouse embryonic fibroblasts (MEF) as previously described (23). For differentiation, TS cells were cultured in the absence of MEF conditioned medium and FGF4 for 5 days. Total RNA was prepared using ISOGEN (Nippon Gene, Tokyo, Japan) and further purified using RNeasy mini Kit and RNase-free DNase (Qiagen, CA, USA). For human polymorphic analyses, human samples were obtained following informed consent at Yoshida Lady’s Clinic, Sendai, Japan. DNA was prepared from umbilical cord blood after delivery and from the mothers’ peripheral blood using standard protocols.

In situ hybridization analysis

cDNA probes for Gata4, Tphi2 and Amdp3 were generated by PCR and used to prepare sense and antisense riboprobes by in vitro transcription using the DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). The primer sets are shown in Supplementary Material, Table S4. Sagittal sections of paraffin embedding mouse placentas at E13.5 were used for in situ hybridization as described previously (42).

Real-time RT–PCR

First-strand cDNA was synthesized from total RNA using PrimeScript II (Takara Bio, Shiga, Japan). Real-time PCR reaction was done with SYBR Premix Ex Taq II (Takara Bio). The amount of target mRNA was determined from the appropriate standard curve and normalized to the amount of β-actin mRNA. The primer sets are shown in Supplementary Material, Table S4.

Analysis of allelic expression

PCR amplification was performed using KOD FX (TOYOBO, Osaka, Japan). PCR products were Sanger-sequenced and the sequence chromatograms were analyzed with Sequencing Analysis Software v5.4 (Applied Biosystems). Multiple sequence alignments were done using GENETYX ver. 10.0.3 (GENETYX, Tokyo, Japan). For RFLP analysis, the PCR
products were digested and electrophoresed, and the band intensity was measured with ImageJ (National Institutes of Health, Bethesda, MD, USA). In all experiments, similar results were obtained in at least two independently collected tissues and cell lines. The primer sets are shown in Supplementary Material, Tables S4 and S5.

**Bisulphite sequencing**

DNA sample was treated with sodium bisulphite using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) and PCR amplified using Ex taq Hot Start DNA Polymerase (Takara Bio). The PCR products were cloned into the pGEM-T Easy vector (Promega, Wisconsin, USA) and individual clones were sequenced. Primers used are listed in Supplementary Material, Table S4.

**ChIP and single nucleotide primer extension (SNuPE)**

ChIP analysis was performed using Magna ChIP G Chromatin Immunoprecipitation Kit (Millipore, Temecula, CA, USA) according to the manufacturer’s protocol. We used the following antibodies: dimethylated H3-Lys4, trimethylated H3-Lys4, dimethylated H3-Lys9 and trimethylated H3-Lys27 (Millipore). The precipitated DNA was PCR amplified and DNA Methylation Kit (Zymo Research, Orange, CA, USA) was used to treat the DNA sample. The bisulphite converted DNA was amplified using Taq DNA polymerase (Takara Bio). The PCR products were cloned into the pGEM-T Easy vector (Promega, Wisconsin, USA) and individual clones were sequenced. SNuPE analysis was performed using SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. The peak height was determined by GeneMapper v4.1 (Applied Biosystems) as described previously (58). Primers used are listed in Supplementary Material, Table S4.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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