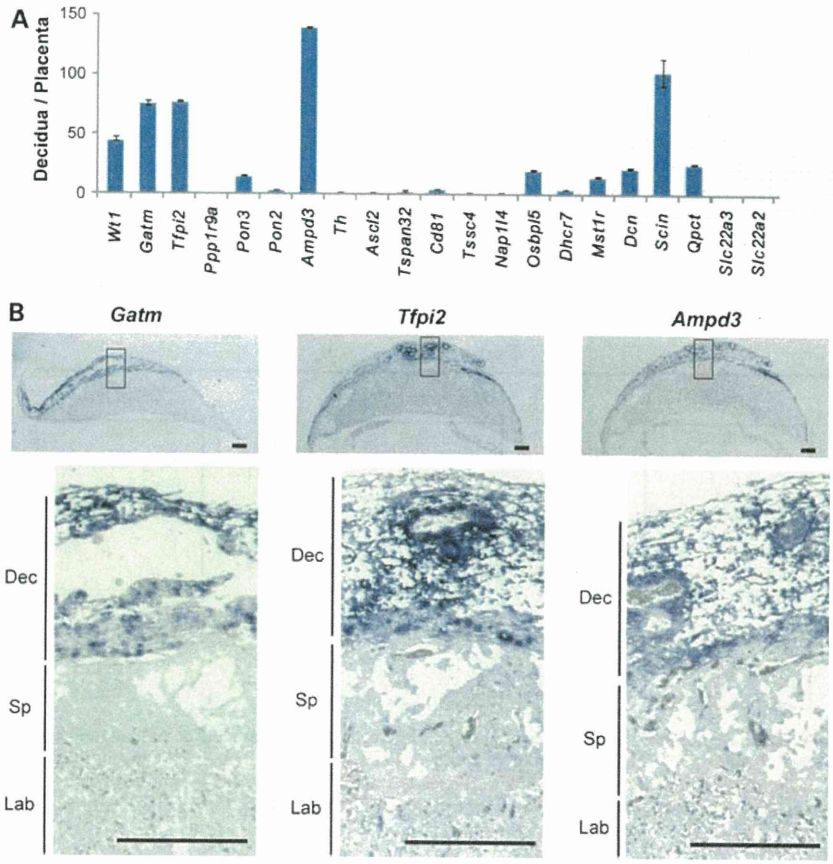

Figure 1. Expression of genes with placenta-specific imprinted expression in the decidua. (**A**) Real-time RT–PCR analysis of genes with placenta-specific imprinted expression in the decidua. Messenger RNA levels in the decidua were normalized to those observed in the placenta from which the decidua was dissected. The bars indicate the means ± standard deviation (SD) from two replicates. (**B**) Expression analysis of *Gatm*, *Tfpi2* and *Ampd3* in sagittal sections of E13.5 placentas by *in situ* hybridization. The lower panels show enlarged views of the boxed areas. Scale bars indicate 400 μm. 'Dec', decidua; 'Sp', spongiotrophoblast layer; 'Lab', labyrinth layer.

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. 2C). *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*, *Sgce*, *Snrpn*, *Mest*) in [B6xJF1]F1 and [JF1xB6]F1 TS cells (Supplementary Material, Fig. S3A). 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 [B6xJF1]F1 and [JF1xB6]F1 TS cell lines. Furthermore, we analyzed [JF1xB6]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

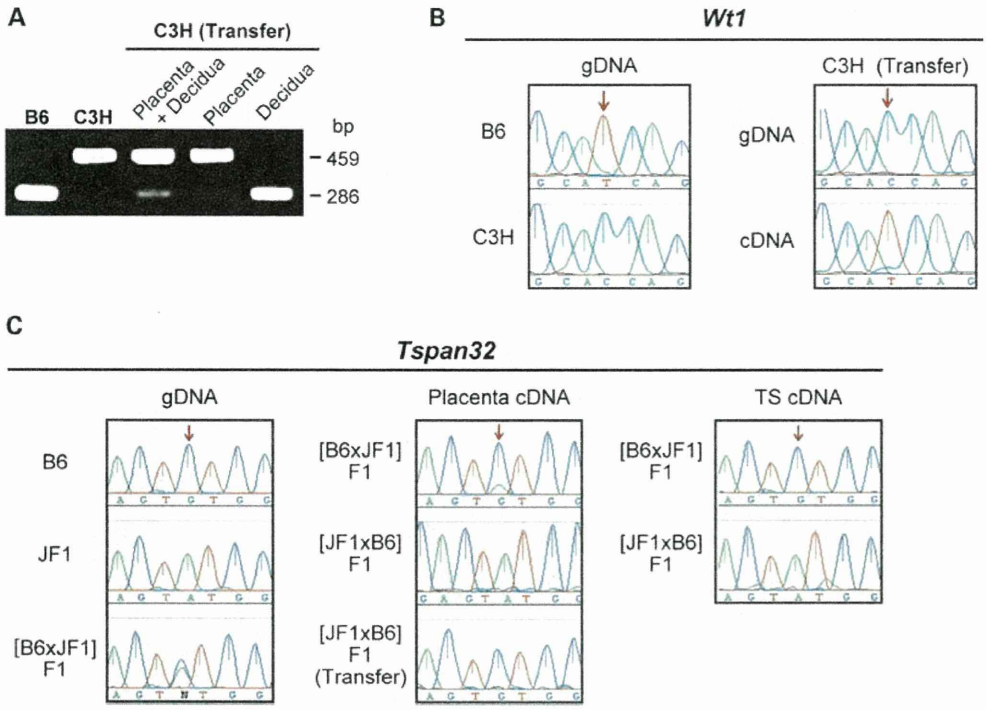

Figure 2. Analyses of the placenta-specific imprinting using embryo transfer and TS cells. (**A**) The proportion of maternal cells in the placenta. E13.5 placentas were obtained from C3H embryos transferred to recipient B6 mice [C3H (Transfer)]. Genomic DNA was PCR amplified with *Gapdh*-specific primers and digested with AflII. (**B**) Predominant expression of *Wt1* from maternal cells. cDNA and gDNA were PCR amplified with *Wt1*-specific primers and sequenced. (**C**) The allelic expression of *Tspan32* in [JF1xB6]F1 placentas transferred to recipient B6 mice ([JF1xB6]F1 (Transfer)) and TS cells. The SNP site between B6 and JF1 is indicated by arrows.

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 *Ano1* was found to be expressed from the maternal allele (Fig. 3B). *Ano1* was biallelically expressed in the embryo, yolk sac and adult tissues (Fig. 3B).

Epigenetic modification of *Gab1*, *Ano1* and *Sfmbt2*

As imprinted genes tend to be clustered, the allelic expression of neighboring genes for *Gab*1 and *Ano1* was analyzed in the [B6xJF1]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*, *Ano1* 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 *Ano1* was mainly expressed

Table 1. Summary of genes with placenta-specific imprinted expression

Chr.	Imprinting status in the mouse			Imprinting status in the human placenta
	Gene	Placenta	TS cells	
2	***Sfmbt2***	**Imprinted (P)**	**Imprinted (P)**	Non-imprinted
2	*Wt1*	Non-imprinted	Non-imprinted	Imprinted (polymorphic) (59)
2	*Gatm*	Non-imprinted	NA	Non-imprinted (48)
6	***Tfpi2***	**Non-imprinted**	**Imprinted (M)**	Imprinted (polymorphic) (30)
6	***Ppp1r9a***	**Imprinted (M)**	**Imprinted (M)**	Imprinted (polymorphic) (60)
6	*Pon3*	Non-imprinted	Non-imprinted	Non-imprinted (30)
6	*Pon2*	Non-imprinted	Non-imprinted	Non-imprinted (30)
6	*Cntn3*	ND	ND	NA
6	*Klrb1f*	ND	ND	NA
7	*Art5*	ND	ND	NA
7	*Ampd3*	Non-imprinted	Non-imprinted	Non-imprinted (49)
7	***Th***	**Imprinted (M)**	**ND**	NA
7	***Ascl2***	**Imprinted (M)**	**Imprinted (M)**	Non-imprinted (48)
7	***Tspan32***	**Non-imprinted**	**Imprinted (M)**	Non-imprinted (48)
7	*Cd81*	NA	NA	Non-imprinted (48)
7	***Tssc4***	**Imprinted (M)**	**Imprinted (M)**	Non-imprinted (48)
7	*Nap1l4*	Non-imprinted	ND	Non-imprinted (48)
7	*Osbpl5*	Non-imprinted	Non-imprinted	Imprinted (61)
7	*Dhcr7*	Non-imprinted	Non-imprinted	Non-imprinted (49)
7	***Ano1***	**Non-imprinted**	**Imprinted (M)**	Imprinted (polymorphic)
8	***Gab1***	**Imprinted (P)**	**Imprinted (P)**	Non-imprinted
9	*Mst1r*	Non-imprinted	Non-imprinted	NA
10	*Dcn*	Non-imprinted	ND	Non-imprinted (48)
12	*Scin*	Non-imprinted	ND	NA
13	*Cmah*	ND	ND	NA
13	*Drd1a*	ND	ND	NA
16	*Fbxo40*	ND	ND	NA
17	***Slc22a3***	**Imprinted (M)**	**Imprinted (M)**	Imprinted (polymorphic) (48)
17	***Slc22a2***	**Imprinted (M)**	**ND**	Imprinted (polymorphic) (48)
17	*Qpct*	Non-imprinted	NA	NA

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 *SFMBT2* 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 *SFMBT2* were biallelically expressed in four human term placenta samples (Fig. 5A and Supplementary Material, Fig. S6). *ANO1*, *GAB1* and *SFMBT2* were biallelically expressed in one or two first trimester placenta samples (Supplementary Material, Fig. S6). Biallelic expression of *SFMBT2* 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

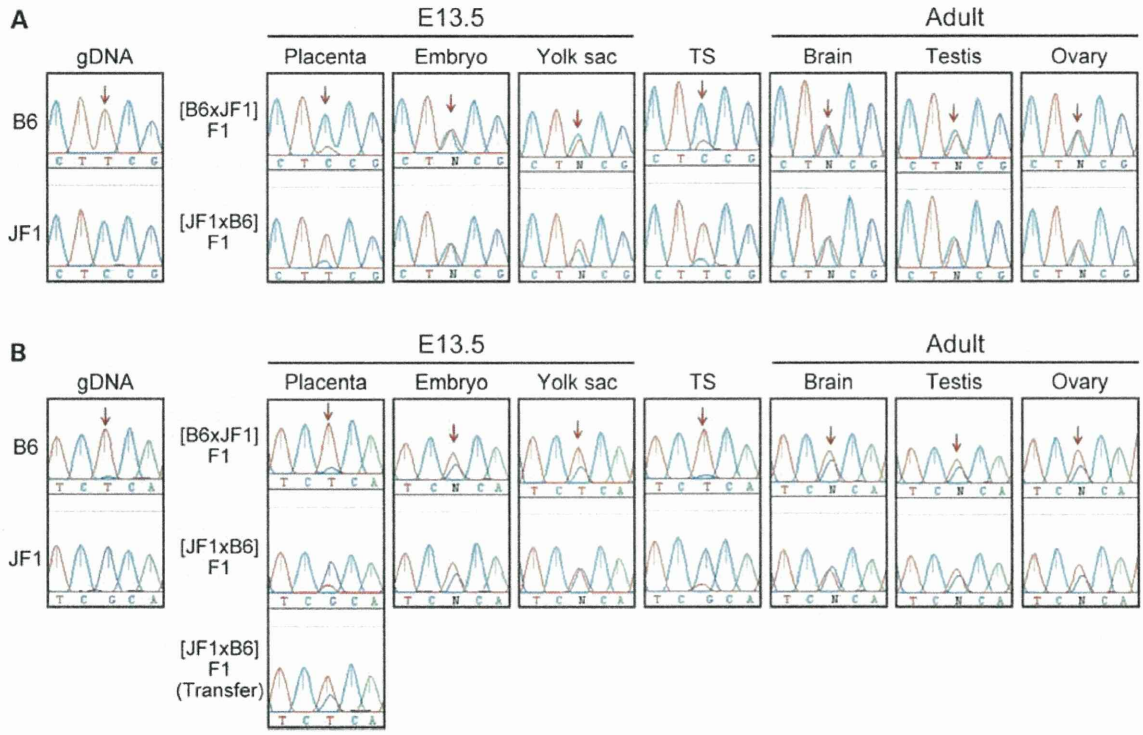

Figure 3. Allelic expression of novel imprinted genes identified by transcriptome sequencing. (**A**) Allelic expression of *Gab1*. *Gab1* showed paternal allele-specific expression in the placenta and TS cells but not in the embryo and adult tissues. (**B**) Allelic expression of *Ano1*. In the placenta, *Ano1* was predominantly expressed by contaminated maternal cells. *Ano1* showed maternal allele-specific expression in TS cells but not in the embryo, yolk sac and adult tissues.

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 *Osbpl5*, 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 *Osbpl5*, 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

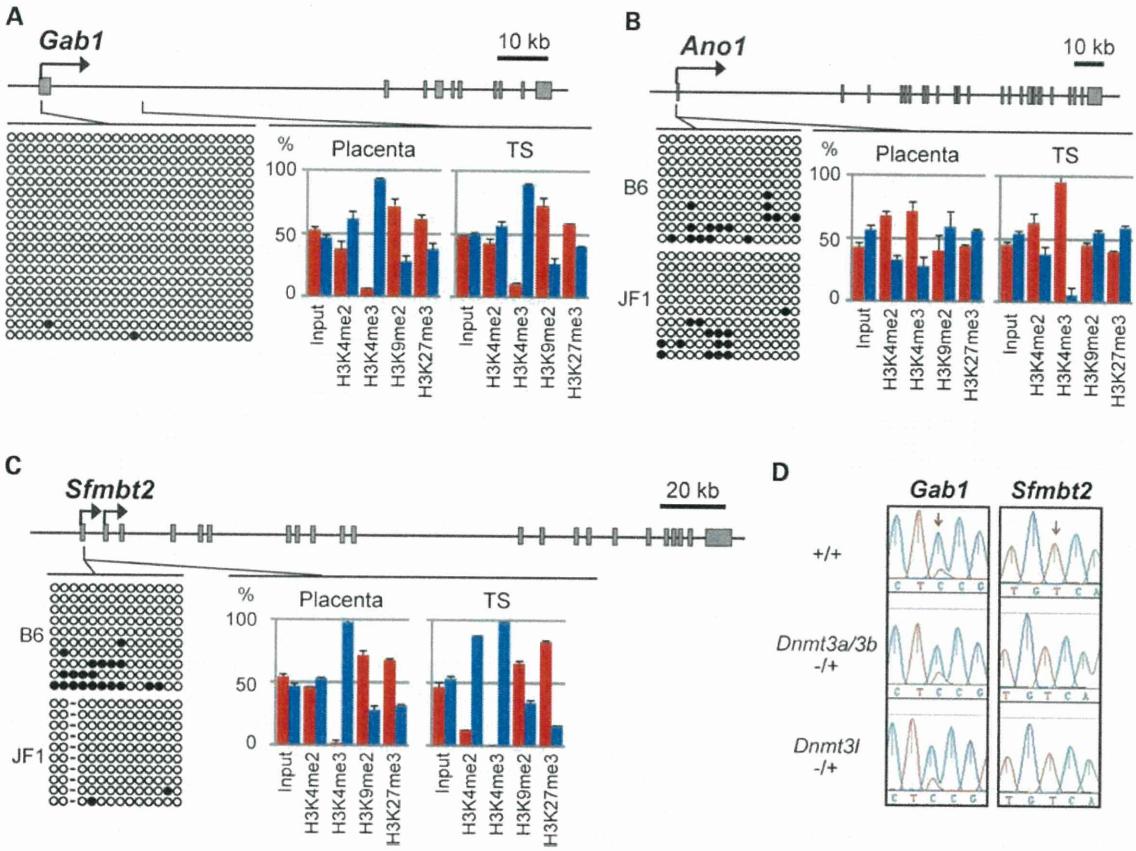

Figure 4. Epigenetic modification of *Gab1*, *Ano1* and *Sfmbt2*. (**A**–**C**) DNA methylation in [B6xJF1]F1 placenta was analyzed by bisulphite sequencing. Black and white circles indicate methylated and unmethylated residues. Histone modifications in [B6xJF1]F1 placenta and TS cells were analyzed 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.

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 (24). 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 PWD/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 (*Wt1, Mst1r, Scin, Qpct*) were predominantly expressed by the contaminating maternal cells and the other six (*Cntn3*, *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 (41), 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

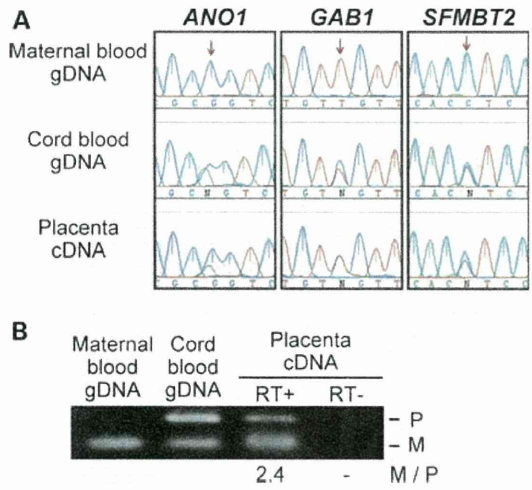

Figure 5. Allelic expression of *ANO1*, *GAB1* and *SFMBT2* in the human term placenta. (**A**) The A/G polymorphic site (SNP ID: rs2276067) in *ANO1*, T/G polymorphic site (SNP ID: rs1397529) in *GAB1* and C/G polymorphic site (SNP ID: rs2692756) in *SFMBT2* are indicated by the arrows. (**B**) Preferential expression of *ANO1* from the maternal allele was confirmed by the RFLP analysis. cDNA and gDNA were PCR amplified and digested with *Bst*UI. The ratio between maternal and paternal band intensity is represented.

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* and *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*, *Ascl2*, *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 allele-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 (RiboMinus 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 clonally 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-adapter 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

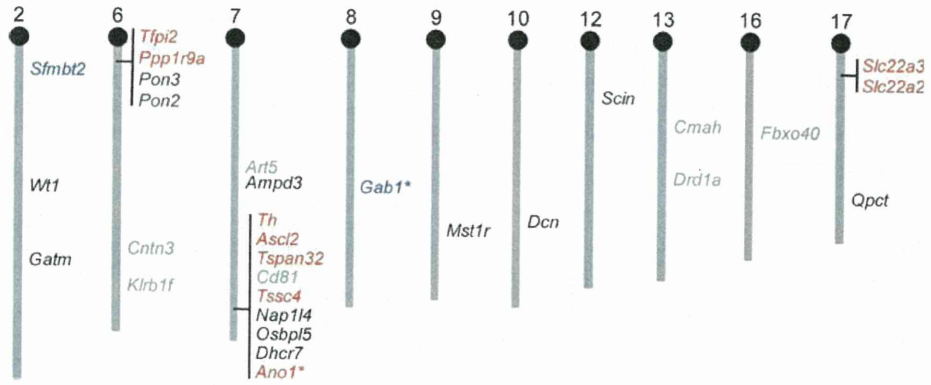

Figure 6. Chromosome map of genes with placenta-specific imprinted expression. Genes, of which paternal allele- and maternal allele-specific expression were confirmed in this study, are shown in blue and red, respectively. For genes shown in black, imprinted expression was not confirmed. Genes which do not contain SNPs (*Cd81*) or are poorly expressed in the placenta (*Cntn3*, *Klrb1f*, *Art5*, *Cmah*, *Drd1a* and *Fbxo40*) are shown in gray. Novel genes are marked by an asterisk (*).

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 *Dnmt3a*^{2lox} and *Dnmt3b*^{2lox}, 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 (*Dnmt3a*$^{2lox/2lox}$, *Dnmt3b*$^{2lox/2lox}$, *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 *Gatm*, *Tfpi2* and *Ampd3* 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 the allelic histone modifications were analyzed using single nucleotide primer extension (SNuPE). SNuPE analysis was performed using SNaPshot Multiplex kit (Applied Biosystems) 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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Conflict of Interest statement. None declared.

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REFERENCES

1. John, R.M. and Surani, M.A. (2000) Genomic imprinting, mammalian evolution, and the mystery of egg-laying mammals. *Cell*, **101**, 585–588.
2. Ferguson-Smith, A.C. and Surani, M.A. (2001) Imprinting and the epigenetic asymmetry between parental genomes. *Science*, **293**, 1086–1089.
3. Reik, W., Constancia, M., Fowden, A., Anderson, N., Dean, W., Ferguson-Smith, A., Tycko, B. and Sibley, C. (2003) Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. *J. Physiol.*, **547**, 35–44.
4. Jaenisch, R. (1997) DNA methylation and imprinting: why bother? *Trends Genet.*, **13**, 323–329.
5. Tilghman, S.M. (1999) The sins of the fathers and mothers: genomic imprinting in mammalian development. *Cell*, **96**, 185–193.
6. Rossant, J. and Cross, J.C. (2001) Placental development: lessons from mouse mutants. *Nat. Rev. Genet.*, **2**, 538–548.
7. Adamson, S.L., Lu, Y., Whiteley, K.J., Holmyard, D., Hemberger, M., Pfarrer, C. and Cross, J.C. (2002) Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Dev. Biol.*, **250**, 358–373.
8. Tycko, B. (2006) Imprinted genes in placental growth and obstetric disorders. *Cytogenet. Genome Res.*, **113**, 271–278.
9. Wagschal, A. and Feil, R. (2006) Genomic imprinting in the placenta. *Cytogenet. Genome Res.*, **113**, 90–98.
10. Miri, K. and Varmuza, S. (2009) Imprinting and extraembryonic tissues-mom takes control. *Int. Rev. Cell Mol. Biol.*, **276**, 215–262.
11. Bressan, F.F., De Bem, T.H., Perecin, F., Lopes, F.L., Ambrosio, C.E., Meirelles, F.V. and Miglino, M.A. (2009) Unearthing the roles of imprinted genes in the placenta. *Placenta*, **30**, 823–834.
12. Waterland, R.A. and Jirtle, R.L. (2004) Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition*, **20**, 63–68.
13. Reik, W. and Lewis, A. (2005) Co-evolution of X-chromosome inactivation and imprinting in mammals. *Nat. Rev. Genet.*, **6**, 403–410.
14. Takagi, N. (2003) Imprinted X-chromosome inactivation: enlightenment from embryos in vivo. *Semin. Cell Dev. Biol.*, **14**, 319–329.
15. Hudson, Q.J., Kulinski, T.M., Huetter, S.P. and Barlow, D.P. (2010) Genomic imprinting mechanisms in embryonic and extraembryonic mouse tissues. *Heredity*, **105**, 45–56.
16. Frost, J.M. and Moore, G.E. (2010) The importance of imprinting in the human placenta. *PLoS Genet.*, **6**, e1001015.
17. Brideau, C.M., Eilertson, K.E., Hagarman, J.A., Bustamante, C.D. and Soloway, P.D. (2010) Successful computational prediction of novel imprinted genes from epigenomic features. *Mol. Cell Biol.*, **30**, 3357–3370.
18. Lewis, A., Mitsuya, K., Umlauf, D., Smith, P., Dean, W., Walter, J., Higgins, M., Feil, R. and Reik, W. (2004) Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. *Nat. Genet.*, **36**, 1291–1295.
19. Weaver, J.R., Sarkisian, G., Krapp, C., Mager, J., Mann, M.R. and Bartolomei, M.S. (2010) Domain-specific response of imprinted genes to reduced DNMT1. *Mol. Cell Biol.*, **30**, 3916–3928.
20. Vernochet, C., Caucheteux, S.M. and Kanellopoulos-Langevin, C. (2007) Bi-directional cell trafficking between mother and fetus in mouse placenta. *Placenta*, **28**, 639–649.
21. Proudhon, C. and Bourc'his, D. (2010) Identification and resolution of artifacts in the interpretation of imprinted gene expression. *Brief Funct. Genomics*, **9**, 374–384.
22. Rossant, J. and Croy, B.A. (1985) Genetic identification of tissue of origin of cellular populations within the mouse placenta. *J. Embryol. Exp. Morphol.*, **86**, 177–189.
23. Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A. and Rossant, J. (1998) Promotion of trophoblast stem cell proliferation by FGF4. *Science*, **282**, 2072–2075.
24. Wang, X., Sun, Q., McGrath, S.D., Mardis, E.R., Soloway, P.D. and Clark, A.G. (2008) Transcriptome-wide identification of novel imprinted genes in neonatal mouse brain. *PLoS ONE*, **3**, e3839.
25. Gregg, C., Zhang, J., Butler, J.E., Haig, D. and Dulac, C. (2010) Sex-specific parent-of-origin allelic expression in the mouse brain. *Science*, **329**, 682–685.
26. Gregg, C., Zhang, J., Weissbourd, B., Luo, S., Schroth, G.P., Haig, D. and Dulac, C. (2010) High-resolution analysis of parent-of-origin allelic expression in the mouse brain. *Science*, **329**, 643–648.
27. Babak, T., Deveale, B., Armour, C., Raymond, C., Cleary, M.A., van der Kooy, D., Johnson, J.M. and Lim, L.P. (2008) Global survey of genomic imprinting by transcriptome sequencing. *Curr. Biol.*, **18**, 1735–1741.

28. Kuzmin, A., Han, Z., Golding, M.C., Mann, M.R., Latham, K.E. and Varmuza, S. (2008) The PcG gene Sfmbt2 is paternally expressed in extraembryonic tissues. *Gene Expr. Patterns*, **8**, 107–116.
29. Umlauf, D., Goto, Y., Cao, R., Cerqueira, F., Wagschal, A., Zhang, Y. and Feil, R. (2004) Imprinting along the Kcnq1 domain on mouse chromosome 7 involves repressive histone methylation and recruitment of Polycomb group complexes. *Nat. Genet.*, **36**, 1296–1300.
30. Monk, D., Wagschal, A., Arnaud, P., Muller, P.S., Parker-Katiraee, L., Bourc'his, D., Scherer, S.W., Feil, R., Stanier, P. and Moore, G.E. (2008) Comparative analysis of human chromosome 7q21 and mouse proximal chromosome 6 reveals a placental-specific imprinted gene, TFPI2/Tfpi2, which requires EHMT2 and EED for allelic-silencing. *Genome Res.*, **18**, 1270–1281.
31. Nagano, T., Mitchell, J.A., Sanz, L.A., Pauler, F.M., Ferguson-Smith, A.C., Feil, R. and Fraser, P. (2008) The air noncoding RNA epigenetically silences transcription by targeting G9a to chromatin. *Science*, **322**, 1717–1720.
32. Terranova, R., Yokobayashi, S., Stadler, M.B., Otte, A.P., van Lohuizen, M., Orkin, S.H. and Peters, A.H. (2008) Polycomb group proteins Ezh2 and Rnf2 direct genomic contraction and imprinted repression in early mouse embryos. *Dev. Cell*, **15**, 668–679.
33. Wang, Q., Chow, J., Hong, J., Smith, A.F., Moreno, C., Seaby, P., Vrana, P., Miri, K., Tak, J., Chung, E.D. *et al.* (2011) Recent acquisition of imprinting at the rodent Sfmbt2 locus correlates with insertion of a large block of miRNAs. *BMC Genomics*, **12**, 204.
34. Singh, U., Fohn, L.E., Wakayama, T., Ohgane, J., Steinhoff, C., Lipkowitz, B., Schulz, R., Orth, A., Ropers, H.H., Behringer, R.R. *et al.* (2004) Different molecular mechanisms underlie placental overgrowth phenotypes caused by interspecies hybridization, cloning, and Esx1 mutation. *Dev. Dyn.*, **230**, 149–164.
35. Hudson, Q.J., Seidl, C.I., Kulinski, T.M., Huang, R., Warczok, K.E., Bittner, R., Bartolomei, M.S. and Barlow, D.P. (2011) Extra-embryonic-specific imprinted expression is restricted to defined lineages in the post-implantation embryo. *Dev. Biol.*, **353**, 420–431.
36. Mann, M.R., Lee, S.S., Doherty, A.S., Verona, R.I., Nolen, L.D., Schultz, R.M. and Bartolomei, M.S. (2004) Selective loss of imprinting in the placenta following preimplantation development in culture. *Development*, **131**, 3727–3735.
37. Maher, E.R. (2005) Imprinting and assisted reproductive technology. *Hum. Mol. Genet.*, **14**, R133–R138.
38. Sandell, L.L., Guan, X.J., Ingram, R. and Tilghman, S.M. (2003) Gatm, a creatine synthesis enzyme, is imprinted in mouse placenta. *Proc. Natl Acad. Sci. USA*, **100**, 4622–4627.
39. Ono, R., Shiura, H., Aburatani, H., Kohda, T., Kaneko-Ishino, T. and Ishino, F. (2003) Identification of a large novel imprinted gene cluster on mouse proximal chromosome 6. *Genome Res.*, **13**, 1696–1705.
40. Wang, X., Soloway, P.D. and Clark, A.G. (2011) A Survey for Novel Imprinted Genes in the Mouse Placenta by mRNA-seq. *Genetics*, **189**, 109–122.
41. Liu, N., Enkemann, S.A., Liang, P., Hersmus, R., Zanazzi, C., Huang, J., Wu, C., Chen, Z., Looijenga, L.H., Keefe, D.L. *et al.* (2010) Genome-wide gene expression profiling reveals aberrant MAPK and Wnt signaling pathways associated with early parthenogenesis. *J. Mol. Cell Biol.*, **2**, 333–344.
42. Hiura, H., Sugawara, A., Ogawa, H., John, R.M., Miyauchi, N., Miyanari, Y., Horiike, T., Li, Y., Yaegashi, N., Sasaki, H. *et al.* (2010) A tripartite paternally methylated region within the Gpr1-Zdbf2 imprinted domain on mouse chromosome 1 identified by meDIP-on-chip. *Nucleic Acids Res.*, **38**, 4929–4945.
43. Itoh, M., Yoshida, Y., Nishida, K., Narimatsu, M., Hibi, M. and Hirano, T. (2000) Role of Gab1 in heart, placenta, and skin development and growth factor- and cytokine-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation. *Mol. Cell Biol.*, **20**, 3695–3704.
44. Schaeper, U., Vogel, R., Chmielowiec, J., Huelsken, J., Rosario, M. and Birchmeier, W. (2007) Distinct requirements for Gab1 in Met and EGF receptor signaling in vivo. *Proc. Natl Acad. Sci. USA*, **104**, 15376–15381.
45. Nishida, K. and Hirano, T. (2003) The role of Gab family scaffolding adapter proteins in the signal transduction of cytokine and growth factor receptors. *Cancer Sci.*, **94**, 1029–1033.
46. Huang, F., Rock, J.R., Harfe, B.D., Cheng, T., Huang, X., Jan, Y.N. and Jan, L.Y. (2009) Studies on expression and function of the TMEM16A calcium-activated chloride channel. *Proc. Natl Acad. Sci. USA*, **106**, 21413–21418.
47. Rock, J.R., Futtner, C.R. and Harfe, B.D. (2008) The transmembrane protein TMEM16A is required for normal development of the murine trachea. *Dev. Biol.*, **321**, 141–149.
48. Monk, D., Arnaud, P., Apostolidou, S., Hills, F.A., Kelsey, G., Stanier, P., Feil, R. and Moore, G.E. (2006) Limited evolutionary conservation of imprinting in the human placenta. *Proc. Natl Acad. Sci. USA*, **103**, 6623–6628.
49. Schulz, R., Menheniott, T.R., Woodfine, K., Wood, A.J., Choi, J.D. and Oakey, R.J. (2006) Chromosome-wide identification of novel imprinted genes using microarrays and uniparental disomies. *Nucleic Acids Res.*, **34**, e88.
50. Cattanach, B.M., Beechey, C.V. and Peters, J. (2004) Interactions between imprinting effects in the mouse. *Genetics*, **168**, 397–413.
51. Koide, T., Moriwaki, K., Uchida, K., Mita, A., Sagai, T., Yonekawa, H., Katoh, H., Miyashita, N., Tsuchiya, K., Nielsen, T.J. *et al.* (1998) A new inbred strain JF1 established from Japanese fancy mouse carrying the classic piebald allele. *Mamm. Genome*, **9**, 15–19.
52. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G. and Durbin, R. (2009) The sequence alignment/map format and SAMtools. *Bioinformatics*, **25**, 2078–2079.
53. Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E. and Sasaki, H. (2004) Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature*, **429**, 900–903.
54. Dodge, J.E., Okano, M., Dick, F., Tsujimoto, N., Chen, T., Wang, S., Ueda, Y., Dyson, N. and Li, E. (2005) Inactivation of Dnmt3b in mouse embryonic fibroblasts results in DNA hypomethylation, chromosomal instability, and spontaneous immortalization. *J. Biol. Chem.*, **280**, 17986–17991.
55. de Vries, W.N., Binns, L.T., Fancher, K.S., Dean, J., Moore, R., Kemler, R. and Knowles, B.B. (2000) Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes. *Genesis*, **26**, 110–112.
56. Kaneda, M., Hirasawa, R., Chiba, H., Okano, M., Li, E. and Sasaki, H. (2010) Genetic evidence for Dnmt3a-dependent imprinting during oocyte growth obtained by conditional knockout with Zp3-Cre and complete exclusion of Dnmt3b by chimera formation. *Genes Cells*, **15**, 169–179.
57. Hata, K., Okano, M., Lei, H. and Li, E. (2002) Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development*, **129**, 1983–1993.
58. Norton, N., Williams, N.M., Williams, H.J., Spurlock, G., Kirov, G., Morris, D.W., Hoogendoorn, B., Owen, M.J. and O'Donovan, M.C. (2002) Universal, robust, highly quantitative SNP allele frequency measurement in DNA pools. *Hum. Genet.*, **110**, 471–478.
59. Jinno, Y., Yun, K.K., Nishiwaki, K., Kubota, T., Ogawa, O., Reeve, A.E. and Niikawa, N. (1994) Mosaic and polymorphic imprinting of the Wt1 gene in humans. *Nat. Genet.*, **6**, 305–309.
60. Nakabayashi, K., Makino, S., Minagawa, S., Smith, A.C., Bamforth, J.S., Stanier, P., Preece, M., Parker-Katiraee, L., Paton, T., Oshimura, M. *et al.* (2004) Genomic imprinting of PPP1R9A encoding neurabin I in skeletal muscle and extra-embryonic tissues. *J. Med. Genet.*, **41**, 601–608.
61. Higashimoto, K., Soejima, H., Yatsuki, H., Joh, K., Uchiyama, M., Obata, Y., Ono, R., Wang, Y., Xin, Z., Zhu, X. *et al.* (2002) Characterization and imprinting status of OBPH1/Obph1 gene: implications for an extended imprinting domain in human and mouse. *Genomics*, **80**, 575–584.

Characterization of DNA methylation errors in patients with imprinting disorders conceived by assisted reproduction technologies.

Running title: Widespread methylation errors of imprinted disorders.

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Abstract

BACKGROUND: There is an increased incidence of rare imprinting disorders associated with assisted reproduction technologies (ART). The identification of epigenetic changes at imprinted loci in ART infants has led to the suggestion that the techniques themselves may predispose embryos to acquire imprinting errors and diseases. However, it is still unknown at what point(s) these imprinting errors arise, or the risk factors. METHODS: In 2009 we conducted a Japanese nationwide epidemiological study of four well known imprinting diseases to determine any association with ART. We examined the DNA methylation status of 22 gametic differentially methylated regions (gDMRs) located within the known imprinted loci in patients with Beckwith-Wiedemann Syndrome (BWS, n=1 and also Silver-Russell Syndrome (SRS, n=5) born after ART, and compared these to patients conceived naturally, Bisulfite sequencing was used. RESULTS: We found a tenfold increased frequency of BWS and SRS associated with ART. The majority of ART cases showed aberrant DNA methylation patterns at multiple imprinted loci both maternal and paternal gDMRs (5/6), with both hyper- and hypomethylation events (5/6) and also mosaic methylation errors (5/6). Although our study may have been limited by small sample number, the fact that many of the changes were mosaic suggested that they occurred after fertilization. In contrast, few of the patients who were conceived naturally exhibited a similar pattern of mosaic alterations. The differences in methylation patterns between the patients who were conceived naturally or after ART did not manifest as differences in the disease phenotypes in these imprinting disorders. CONCLUSION: A possible association between ART and BWS/SRS was found, and

we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable.

Introduction

Human assisted reproduction technologies (ART) are used in the treatment of infertility and involve the manipulation of eggs and/or sperm in the laboratory. Several recent studies have identified an increased incidence of some normally very rare imprinting disorders after ART, including Beckwith-Wiedemann syndrome (BWS: ONIM 130650), Angelman syndrome (AS: ONIM 105830) and Silver-Russell syndrome (SRS: OMIM 180860) but not Prader-Willi syndrome (PWS: OMIM 176270) (DeBaun *et al.*, 2003; Gosden *et al.*, 2003; Svensson *et al.*, 2005). Additionally, there are several reports suggesting that epigenetic alterations (epimutations) at imprinted loci occur during the *in vitro* manipulation of the gametes, with both IVF and ICSI approaches implicated (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Moll *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Rossignol *et al.*, 2006; Bowdin *et al.*, 2007; Kagami *et al.*, 2007). However, some studies do not support a link between ART and imprinting disorders (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007).

Epigenetic marks laid down in the male or female germ lines, and which are inherited by the embryos, establish the imprinted expression of a set of developmentally important genes (Surani, 1998). Because imprinted genes are regulated by these gametic epigenetic marks, and by further epigenetic modifications in the somatic cell, they are particularly vulnerable to environmentally induced mutation. One of the best studied epigenetic marks is DNA methylation. DNA methylation is established in either the maternal or paternal germline at discrete genomic loci. This methylation is preserved in the fertilized embryo to generate differentially methylated regions (DMRs) which then

signal to nearby genes to establish domains of imprinted chromatin by mechanisms that are not fully understood (John and Lefebvre, 2011). These germline or gametic DMRs (gDMRs) can orchestrate the monoallelic expression of genes over megabases of DNA (Tomizawa *et al.*, 2011) and are reset with every reproductive cycle (Lucifero *et al.*, 2002; Obata and Kono, 2002).

The increased frequency of epimutation(s) at imprinted loci in ART infants has led to the suggestion that ART procedures may induce imprinting error(s). However, these studies are confounded because ART populations are, by their very nature, different from populations who were conceived without use of ART, with a low fertility rate, an increased frequency of reproductive loss and usually of advanced age, all of which are associated with increased occurrence of fetal and neonatal abnormalities. Furthermore, it is difficult to determine the causality of imprinting errors in any specific abnormality reported after ART. Both IVF and ICSI appear to be associated with an increased relative risk of imprinting disorders (Savage *et al.*, 2011). These procedures are often undertaken for unexpected infertility and require ovarian stimulation, oocyte collection and *in vitro* culture before the embryos are implanted. It has been suggested that infertility and any resulting ovarian stimulation may predispose to epigenetic errors (Sato *et al.*, 2007). Animal studies suggest that *in vitro* embryo culture may be associated with epigenetic alterations. In particular, the large offspring syndrome in cattle undergoing ART is associated with loss of maternal allele methylation at insulin-like growth factor 2 receptor (*IGF2R)* gDMR (Young *et al.*, 2001) and has phenotypic similarity to BWS. It is still unknown when these imprinting errors arise and what factors predispose to epigenetic changes. Previously, Chang *et al.* (2005) reported no phenotypic differences between BWS patients who were conceived after ART and

naturally . However Lim *et al.* (2009) reported that patients who were conceived after ART had a significantly lower frequency of exomphalos and higher risk of non-Wilms tumor neoplasia. Phenotypic differences between patients who were conceived after ART and naturally are largely unreported, while any changes to phenotype may be altered by the frequency and the degree of epimutations. Studies revealed that some patients with BWS born after ART presented with epimutations that were not restricted to the 11p15 region (Rossignol *et al.*, 2006; Bliek *et al.*, 2009; Lim *et al.*, 2009). Further analysis of abnormal methylation patterns in imprinting disorders may provide clues as to the cause of disease and identify the ART-related risk factor(s).

To address these questions in this study, we engaged in a nationwide epidemiological study of the Japanese population to determine the frequency of four imprinting disorders after natural conception and after ART. We then analyzed the DNA methylation status of 22 gDMRs in BWS and SRS patients conceived by the two routes. Finally, we compared the abnormal methylation patterns and the phenotypes reported for both sets of patients. As a result, we found that both BWS and SRS were more frequent after ART and that ART patients exhibited a higher frequency of aberrant DNA methylation patterns at multiple loci with, in some cases, mosaic methylation errors.

Materials and Methods

Nationwide investigation of imprinting disorders.

The protocol was established by the Research Committee on the Epidemiology of Intractable Diseases. The protocol consisted of a two-stage postal survey. The first-stage survey was used to estimate the number of individuals with any of the four imprinting diseases; BWS, SRS, PWS and AS. The second-stage survey was used to identify the clinico-epidemiological features of these syndromes.

In the first-stage survey, the pediatric departments of all hospitals were identified based on a listing of hospitals, as at 2008, supplied by the R&D Co. LTD (Nagoya Japan). Hospitals were classified into seven categories according to the type of institution and the number of hospital beds. The survey was mailed to a total of 3158 departments in October 2009 with letters of request for participation in recording these diseases. A simple questionnaire was used to ask about the number of patients with any of the four imprinting disorders. Diagnosis was determined by karyotype analyses, genetic analyses and clinical phenotypes by their clinical doctors. In December 2009, a second request was sent to departments that had not responded by the earlier deadline (the end of November 2009). Following the first-stage survey, we sent acknowledgement letters to departments that had responded.

The second questionnaires were forwarded to the departments that had reported patients with the imprinting disorders on the first questionnaires. Detailed clinical information for the patients with these imprinting disorders was collected, including the age, gender, growth and development pattern, the methods of the diagnosis, the presence of infertility treatment and the methods of ART where

applicable. Duplicate results were excluded using the information regarding the patient's age and gender where available. The study was approved by the Ethics Committee of Tohoku University School of Medicine.

Estimation of prevalence of imprinting disorders correct.

The number of patients who were diagnosed by genetic and cytogenetic testing, and by clinical phenotypes, was obtained from data from the departments who responded to the first survey. correct The 95% confidence interval (CI) was calculated as previously described (Wakai *et al.*, 1997). The prevalence was determined, based on the population of Japan in 2009 (127,510,000) with data from the Statistics Bureau of the Ministry of Internal Affairs and Communications.

DNA preparation.

Genomic DNA was obtained from blood or buccal mucosal cells samples from patients with one of the imprinting disorders using standard extraction methods (Kobayashi *et al.*, 2007). For control DNAs, DNA was prepared from sperm and cord blood samples from unaffected individuals. The study was performed after obtaining patient or their parents' consent.

Bisulfite-treatment PCR including the SNPs.

We first searched for single nucleotide polymorphisms (SNPs) within 22 previously reported human gDMRs (Kikyo *et al.*, 1997; Smith *et al.*, 2003; Kobayashi *et al.*, 2006; Wood *et al.*, 2007; Kobayashi *et al.*, 2009) using 20 control correct Japanese blood DNA samples. PCR primer sets were designed to span these SNPs

(**Supplementary Table I**) and human sperm DNA and blood DNA was used to confirm that these PCR assays detected the methylation status of the 22 DMRs. Paternal DMRs were shown to be fully methylated in sperm DNA, maternal DMRs were fully unmethylated and in blood DNA, both paternal and maternal DMRs showed approximately 50% methylation (**Supplementary Figure 1**). The human gDMRs and the non-imprinted repetitive long interspersed nucleotide element (*LINE1)* and *Alu* repetitive sequences were examined by bisulfite sequencing using established protocols (Kobayashi *et al.*, 2007). Briefly, PCR products were purified and cloned into the pGEM-T vector (Promega, Madison, WI, USA). Individual clones were sequenced using M13 reverse primer and an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). On average, 20 clones were sequenced for each sample.

Statistics

The frequency of the manifestation in patients who were conceived after ART was compared with that observed in patients naturally by Fisher's exact test.

Results

Frequency of four imprinting disorders and their association with ART.

We first investigated the nationwide frequency of four imprinting disorders (BWS, AS, PWS and SRS) in Japan in the year 2009. Of a total of 3158 departments contacted, 1602 responded to the first-stage survey questionnaire (50.7 %) . The total number of cases was calculated using a second-stage survey ensuring exclusion of duplicates (**Table I**). Using this information, and taking into account the number of patients with suspect clinical signs but without a formal diagnosis, we identified 444 BWS patients (95% CI 351 to 538), 949 AS patients (95% CI 682 to 1217), 2070 PWS patients (95% CI 1504 to 2636) and 326 SRS patients (95% CI 235 to 416). From these figures (and using the 2009 population of Japan: 127,510,000) we estimated the prevalence of these syndromes to be 1 in 287,000, 1 in 134,000, 1 in 62,000 and 1 in 392,000 respectively, for BWS, AS, PWS and SRS. Further details are given in **Supplementary Table II** and **Supplementary Figure 2**.

Between 1997 and 2008, the period during which the ART babies in this study were born, 0.64-0.98 % of the total number of babies born in Japan were born as a result of IVF and ICSI. We ascertained the frequency of ART procedures in the cases of BWS, AS, PWS and SRS via the questionnaire sent to doctors (**Table I, Supplementary Table III**). The numbers of patients with PWS and AS we identified was low, however, the frequency of ART in these cases was not dissimilar to that expected, based on the population rate of ART use, with 2/123 (1.6%) cases of AS and 4/261 (1.5%) cases of PWS born after ART. In contrast, for BWS and SRS the frequency of ART was nearly 10-fold higher than anticipated with 6/70 (8.6%) BWS and 4/42 (9.5%) SRS patients