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（作成上の留意事項）

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Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in *SLC25A13*

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ABSTRACT

Citrin deficiency is an autosomal recessive disorder caused by mutations in the *SLC25A13* gene and has two disease outcomes: adult-onset type II citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency. The clinical appearance of these diseases is variable, ranging from almost no symptoms to coma, brain edema, and severe liver failure. Genetic testing for *SLC25A13* mutations is essential for the diagnosis of citrin deficiency because chemical diagnoses are prohibitively difficult. Eleven *SLC25A13* mutations account for 95% of the mutant alleles in Japanese patients with citrin deficiency. Therefore, a simple test for these mutations is desirable. We established a 1-hour, closed-tube assay for the 11 *SLC25A13* mutations using real-time PCR. Each mutation site was amplified by PCR followed by a melting-curve analysis with adjacent hybridization probes (HybProbe, Roche). The 11 prevalent mutations were detected in seven PCR reactions. Six reactions were used to detect a single mutation each, and one reaction was used to detect five mutations that are clustered in a 21-bp region in exon 17. To test the reliability, we used this method to genotype blind DNA samples from 50 patients with citrin deficiency. Our results were in complete agreement those obtained using previously established methods. Furthermore, the mutations could be detected without difficulty using dried blood samples collected on filter paper. Therefore, this assay could be used for newborn screening and for facilitating the genetic diagnosis of citrin deficiency, especially in East Asian populations.

1. Introduction

Citrin deficiency is an autosomal recessive disorder that results from mutations in the *SLC25A13* gene [1] and causes two diseases: adult-onset type II citrullinemia (CTLN2; OMIM #603471) and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; OMIM#605814) [1–4]. The clinical appearance of these diseases is variable and ranges from almost no symptoms to coma, brain edema, and severe liver failure requiring transplantation [5–8]. In a study of patients with NICCD, only 40% of individuals were identified by newborn screenings to have abnormalities, such as hypergalactosemia, hypermethioninemia, and hyperphenylalaninemia [9]. Other patients were referred to hospitals with suspected neonatal hepatitis or biliary atresia, due to jaundice or discolored stool [9]. Hypercitrullinemia was not observed in all patients [9]. Mutation analysis of *SLC25A13* is indispensable because of the difficulties associated with the chemical diagnosis of citrin deficiency. The *SLC25A13* mutation spectrum in citrin deficiency is heterogeneous, and more than 31 mutations of *SLC25A13* have been identified to date [1,10–18]. However, there are several predominant mutations in patients from East Asia. As shown in Table 1, 6 prevalent mutations account for 91% of the mutant alleles in the Japanese population [12,19]. Five additional mutations also occur within a 21-bp cluster in exon 17 (Table 1 and Fig. 1D). The six prevalent mutations, together with the five mutations in exon 17, account for 95% of the mutant alleles in Japan [12,19].

Several different methods, such as direct sequencing, PCR restriction fragment length polymorphism (PCR-RFLP), and denaturing high performance liquid chromatography (DHPLC), are currently used for the detection of mutations in *SLC25A13* [1,10–14,19]. However, these methods are too complex for clinical use. Direct sequencing is a standard but cumbersome method. The PCR-RFLP method is

Abbreviations: CTLN2, adult-onset type II citrullinemia; FRET, fluorescence resonance energy transfer; HRM, high resolution melting; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; Tm, melting temperature.

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Table 1
Seven primer/probe sets and 11 targeted mutations of *SLC25A13*.

Primer/probe set	Mutation		Location	Nucleotide change	Effects of mutations	Allele frequency*[19]	References
A	Mutation [I]	:851del4	exon 9	c.851_854delGTAT	p.R284fs(286X)	33.2%	[1]
B	Mutation [II]	:g.IVS11+1G>A	intron 11	c.1019_1177del	p.340_392del	37.6%	[1]
C	Mutation [III]	:1638ins23	exon 16	c.1638_1660dup	p.A554fs(570X)	3.4%	[1]
D	Mutation [IV]	:S225X	exon 7	c.675C>A	p.S225X	5.3%	[1]
E	Mutation [V]	:g.IVS13+1G>A	intron 13	c.1231_1311del	p.411_437del	8.2%	[1]
F	Mutation [XIX]	:IVS16ins3kb	intron 16	c. aberrant RNA	p.A584fs(585X)	4.6%	[19]
G	Mutation [VI]	:1800ins1	exon 17	c.1799_1800insA	p.Y600X	1.3%	[10]
	Mutation [VII]	:R605X	exon 17	c.1813C>T	p.R605X	0.90%	[10]
	Mutation [VIII]	:E601X	exon 17	c.1801G>T	p.E601X	1.2%	[11]
	Mutation [IX]	:E601K	exon 17	c.1801G>A	p.E601K	0.30%	[11]
	Mutation [XXI]	:L598R	exon 17	c.1793T>G	p.L598R	0%	[15]
						Total 95.1%	

* The frequency of each mutant allele among Japanese patients with citrin deficiency.

complicated and can lead to genotyping errors, due to incomplete digestion by the restriction enzymes. DHPLC is time-consuming and requires expensive equipment. Thus, there is a strong need for the development of a simple test for these mutations.

The goal of this study was to establish a rapid and simple test for the detection of the 11 most common *SLC25A13* mutations. We adopted the HybProbe format (Roche) for the detection of the mutations using real-time PCR followed by a melting-curve analysis with adjacent hybridization probes [20,21]. This assay can be completed in less than 1 h and has the advantage of being a closed-tube assay. The fundamental process for detecting point mutations using the HybProbe assay is presented in Fig. 1A. The 11 prevalent mutations contain not only point mutations but also include a 4-bp deletion and insertions of 1-bp, 23-bp and 3-kb genomic fragments (Table 1 and Fig. 1). Careful design of the PCR primers and HybProbes enabled us to test for these various *SLC25A13* mutations.

2. Methods

2.1. Subjects

CTLN2 and NICCD were diagnosed, as previously described [9,10,19,22–24]. Genomic DNA of the patients was obtained from peripheral blood leukocytes using the DNeasy blood kit (Qiagen Inc., Valencia, CA, USA). Genomic DNA was purified from filter paper blood samples using the ReadyAmp Genomic DNA Purification System (Promega, Madison, WI, USA). Mutations in these DNA samples were analyzed at Kagoshima University using a combination of PCR with or without restriction enzyme digestion or by direct sequencing, as previously described [1,10–14,19]. Another set of samples was obtained from 420 healthy volunteers (mainly from Miyagi prefecture in the northeastern region of Japan) at Tohoku University. Genomic DNA from leukocytes was extracted, as described above.

2.2. Detection of seven prevalent mutations in SLC25A13 using the HybProbe assay

HybProbe probes comprise a pair of donor and acceptor oligonucleotide probes designed to hybridize adjacent to their target sites in an amplified DNA fragment [20,21]. The donor probes are labeled at their 3′ end with fluorescein isothiocyanate (FITC), whereas the acceptor probes are labeled at their 5′ end with LC Red640; these acceptor probes are phosphorylated at their 3′ end to prevent extension by the DNA polymerase. When two probes hybridize to the amplicon, the fluorescent dyes are located within 5 bases of each other, which allows fluorescence resonance energy transfer (FRET) between the excited FITC and the LC Red640; this process emits light that can be quantified by real-time PCR. Following PCR amplification, a melting-peak analysis is performed. The melting peak is produced by the reporter probe, which has a lower melting temperature (Tm) than the other probe, called the anchor probe. As the reporter melts from the target, the fluorophores are separated, and the FRET ceases. The Tm of the reporter probe determines the reaction

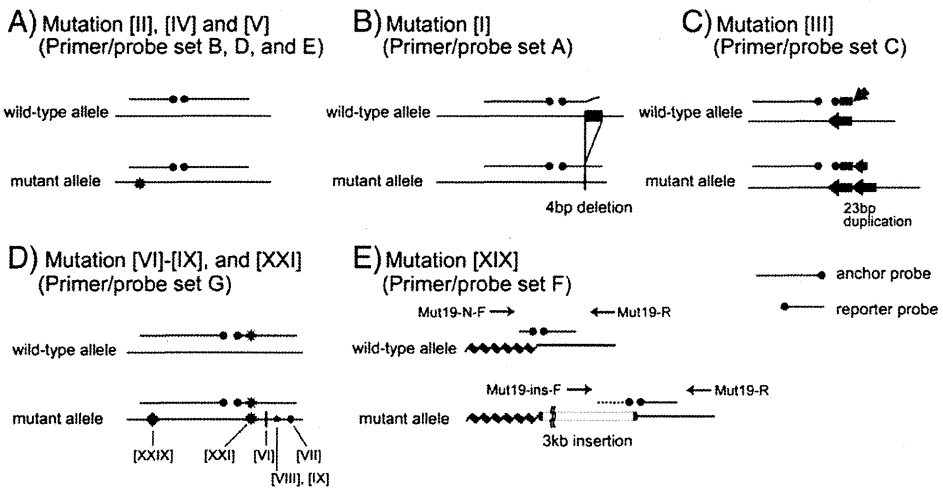

Fig. 1. Principle of *SLC25A13* mutation detection by melting-curve analysis with the HybProbe assay. In primer/probe sets A–E, and G, PCR was performed with a pair of primers, whereas in primer/probe set F, two forward primers and one common reverse primer were used for the amplification of both wild-type and mutant alleles. Note that mutation [XXIX], located on the anchor probe of primer/probe set G, is a non-target mutation.

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specificity (i.e., binding of the probe to a perfectly matched sequence rather than to regions with sequence mismatches).

Seven primer/probe sets were designed for this study. Fig. 1 shows a schematic diagram of the strategy for mutation detection using these primer/probe sets. Tables 1 and 2 list the primer/probe sets and corresponding sequences and primer concentrations that were used to target the 11 mutations. Primer/probe sets A, B, C, D, E, and F were designed to detect mutations [I], [II], [III], [IV], [V], and [XIX], respectively. Primer/probe set G was designed to detect the five mutations clustered on exon 17: mutations [VI], [VII], [VIII], [IX], and [XXI] (Fig. 1D). All primers and probes were synthesized based on the NCBI reference SLC25A13 gene sequence (GenBank accession no. **NM_014251**) with the exception of mutation [XIX]:IVS16ins3kb, which was designed according to [19].

Real-time PCR and subsequent melting curve analyses were performed in a closed tube using a 20-μL mixture on a LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan). The PCR mixture contained 2.0 μL of genomic DNA (10–50 ng), 0.5 μM of forward primer, 0.5 or 0.1 μM of reverse primer, 0.2 μM of each sensor and anchor probe, and 10 μL of Premix ExTaq™ (Perfect Real Time) reagent (TaKaRa Bio Inc., Otsu, Japan).

The thermal profile conditions were identical for all seven assays and consisted of an initial denaturation step (30 s at 95 °C), followed by 45 amplification cycles with the following conditions: denaturation for 5 s at 95 °C and annealing and extension for 20 s at 60 °C. The transition rate between all steps was 20 °C/s. After amplification, the samples were held at 37 °C for 1 min, followed by the melting curve acquisition at a ramp rate of 0.15 °C/s extending to 80 °C with continuous fluorescence acquisition.

Table 2
Primers, probes and target amplicon sequences, target mutation sites, and primer concentrations.

Primer/probe set	Name	Sequences of PCR products, primer locations, probe sequences, and mutation sites (5′ to 3′)	Concentration (μmol/L)
A		GGCTATACTGAAATATGAGAAatgaaaaagggatgtttttaaatttataatgtaaattgtaataaattggtatatttgttgcttgtgtttgtttttcccctacagac	
		gtatgaccttagcagacattgaacggattgctcctctggaagagggaactctgccCTTTAACTTGGCTGAGG (181 bp)	
	Mut1-F	GGCTATACTGAAATATGAGAA	0.5
	Mut1-R	CCTCAGCCAAGTTAAAG	0.5
	Mut1-UP	ATGTAAATTGTAATAAATTGGTATATTTGTTGCTTGTGTT-FITC	
	Mut1-DW	LC Red640-GTTTTTCCCCTACAGAC**GACC**-P	
B		GAATGCAGAACCAACGAtcaactggctcttttgtgggagaactcatgtataaaaacagctttgactgttttaagaaagtgctacgctatgaaggcttctt	
		tggactgtatagaggttagtgccacatgctcaatacctgttaggtgaaataacactcaaaggtttggtttctcatcttagtgcctGACATGAATTAGCAAGACTG (205 bp)	
	Mut2-F	GAATGCAGAACCAACGA	0.5
	Mut2-R	CAGTCTTGCTAATTCATGTC	0.1
	Mut2-UP	ACCTAACAGGTATTGAGCATGTG-FITC	
	MUt2-DW	LC Red640-CACTAA**C**CTCTATACAGTCCA-P	
C		GCAGTTCAAAGCACAGTTATTtttatatagtgagaatgtgaccagactgagatggtgttgtgtctctcctgcaggtatgcctgcagcatctttagtg	
		accccctgctgatgttatcaagacgagattacaggtg	
		gctgcccggg(**gagatta**caggtggctgcccggg)ctggccaaaccaCTTACAGCGGAGTGATAGAC (175 bp)	
	Mut3-F	GCAGTTCAAAGCACAGTTATT	0.5
	Mut3-R	GTCTATCACTCCGCTGTAAG	0.5
	Mut3-UP	ACCCCTGCTGATGTTATCAAGACGAGATTACAGGT-FITC	
	Mut3-DW	LC Red640-GCTGCCCGGG**GAGATTA**-P	
D		TCAATTTATTTGAGGCTGCtggaggtaccacatcccatcaagttagtttctcctattttaatggatttaattcgctccttaacaac	
		atggaactcattagaaagatctatagcactc	
		tggctggcaccaggaaagatgttgaagtGACTAAGGGTGAGTGAGAA (164 bp)	
	Mut4-F	TCAATTTATTTGAGGCTGC	0.5
	Mut4-R	TTCTCACTCACCCTTAGTC	0.5
	Mut4-UP	AATGGATTTAATT**C**GCTCCTTAACA-FITC	
	Mut4-DW	LC Red640-ATGGAACTCATTAGAAAGATCTATAGCACTC-P	
E		TGCACAAAGATGGTTCGgtcccacttgcagcagaaattcttgctggaggctgcgtaagtacctttgaagctctcttcattgaaaagacttgtttcac	
		atatatatcactaccatggtcaacaggtgtggactaaggcttctgttTAACCACAGATCCTGCA (162 bp)	
	Mut5-F	TGCACAAAGATGGTTCG	0.5
	Mut5-R	TGCAGGATCTGTGGTTA	0.5
	Mut5-UP	GTGAAACAAGTCTTTTCAATGAAGAGAGCTTC-FITC	
	Mut5-DW	LC Red640-AAGGTACTTA**C**GCAGCCTC-P	
F	normal allele	GGAGCTGGTGGTATGGAAatatgtgttcttaactaactctttggtatcaggtaaatttttaaaatatctaattatatctgtgatttctc	
		catttttttaaagctcgtgtatttcgatcctcaccccagtttggt	
		gtaactttgctgacttacgaattgctacagcgatggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaatttt	
		GGCTGCTGCTAATGCTC (244 bp)	
	insertion allele	CCATCTTCCTCCTCCCTTggcagccccgccccccgatttctccatttttttaaagctcgtgtatttcgatcctcaccccagtttggt	
		gtaactttgctgacttacgaattgctacagcgatggttctacattgatttt	
		ggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (196 bp)	
	Mut19-N-F	GGAGCTGGTGGTATGGAA	0.5
	Mut19-ins-F	CCATCTTCCTCCTCCCTT	0.5
	Mut19-R	GAGCATTAGCAGCAGCC	0.5
	Mut19-UP	ACCAAACTGGGGTGAGGATCGAAATACACGAGCTTTAAAAAAATG-FITC	
	Mut19-N-DW	LC Red640-AGAAATCACAGATATAATTAGATATTT-P	
	Mut19-ins-DW	LC Red640-AGAAATCGGGGGGCGGGG-P	
G		TCTTAACTAACTCTTTGGTATCAGGTaaatttttaaaatatctaattatatctgtgatttctccatttttttaaagctcg	
		tgtatttcgatcctcaccccagtttggtgtaactttg**ct**gactt**a(a)cg**aattgctacag**c**ga	
		tggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (217 bp)	
	Mut6-9, 21-F	TCTTAACTAACTCTTTGGTATCAGGT	0.5
	Mut6-9, 21-R	GAGCATTAGCAGCAGCC	0.5
	Mut6-9, 21-UP	TGTATTTCGATCCTCACCCCAGTTTGGTGTAACTT-FITC	
	Mut6-9, 21-DW	LC Red640-G**C**GGACTT**ACG**AATTGCTACAG**C**GA-P	

Upper case and underlined letters indicate the locations of primers and probes, respectively. Inserted DNA is shown in parenthesis. Nucleotides in boldface were used for mutation detection.
F: forward, R: reverse, UP: upstream, DW: downstream, N: normal allele, ins: insertion allele, FITC: fluorescein isothiocyanate, P: phosphate.

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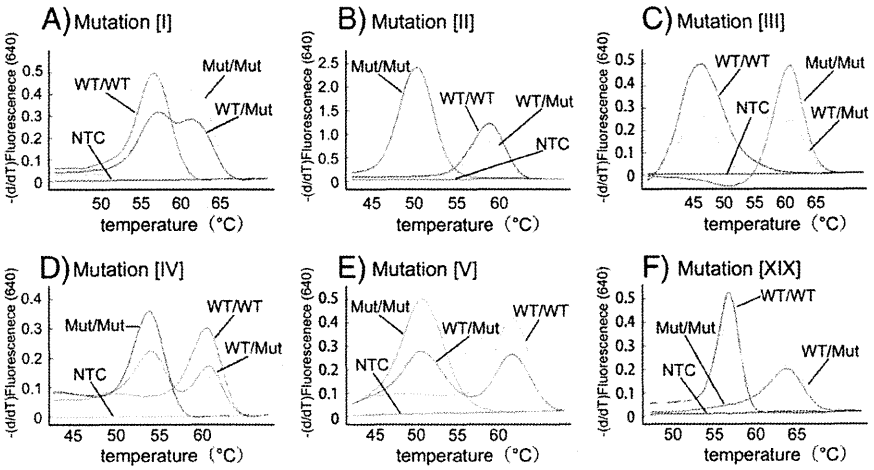

Fig. 2. Typical melting curves used in the detection of mutations [I–V] and [XIX]. Each assay using primer/probe sets A–F is displayed in a separate graph (A–F). WT: wild-type allele, Mut: mutant allele, NTC: no DNA template control.

2.3. Validation of the mutation detection system

After establishing the protocol for detecting the 11 prevalent mutations, 50 DNA samples from patients' blood were sent from Kagoshima University to Tohoku University for the validation of this system in a single-blind manner. Similarly, 26 DNA samples purified from paper-filter blood samples were analyzed in the same manner as the blood DNA samples.

2.4. Estimation of the carrier frequency

For the estimation of the heterozygous carrier frequency, 420 genomic DNA samples from healthy volunteers were screened using the HybProbe analysis for the 11 prevalent mutations. All detected mutations were confirmed by direct sequencing.

2.5. Ethics

This study was approved by the Ethical Committees of Tohoku University School of Medicine and Kagoshima University. Written informed consent was obtained from all participants or their guardians.

3. Results

3.1. Development of the mutation detection system

In primer/probe sets B, D, and E, the reporter probes were designed to be complimentary to the wild-type allele (Fig. 1A). To allow for an improved detection of the mutations, primer/probe sets A and C were designed to be complementary to the mutant allele (Figs. 1B, C). In the primer/probe set F, two forward PCR primers, which were specific to the wild-type and the mutant alleles, were used with a common reverse primer for the co-amplification of the wild-type and 3-kb insertion alleles (Fig. 1E). Two reporter probes, which had a common anchor probe, were used for the detection of the wild-type and mutant alleles. Because the two reporter probes had different melting temperatures, we were able to identify the allele that was amplified. Fig. 2 shows representative results of the melting curve analyses using the primer/probe sets A–F, in which all of the mutant alleles generated distinct peaks corresponding to the wild-type alleles.

In the primer/probe set G, we used a reporter probe that was complementary to the mutant [XXI] allele (Fig. 1D). All five mutations in exon 17 were successfully differentiated from the wild-type allele (Figs. 3A–E). The [XXIX] mutation is an additional mutation in exon

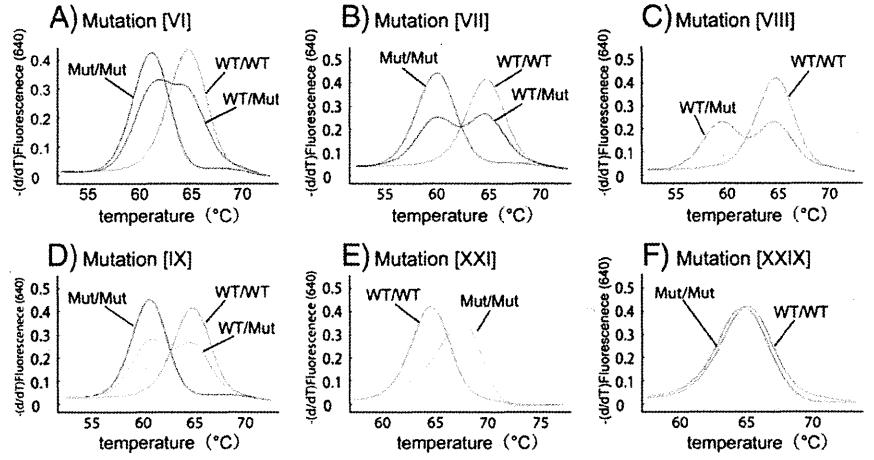

Fig. 3. Typical melting curves used in the detection of mutations [VI–XI], [XXI], and [XXIX] on exon 17. Genotyping was performed using primer/probe set G. Each melting curve for a target mutation is displayed in a separate graph (A–F). Note that mutation [XXIX] (F) is a non-target mutation on the anchor probe. WT: wild-type allele, Mut: mutant allele.

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17 that is not listed in Table 1. The [XXIX] mutation is located in the anchor-probe binding site and not on the reporter-probe binding site (Fig. 1D). To examine the effect of mutations on the anchor probe, we genotyped a patient with a heterozygous [XXIX] mutation using primer/probe set G (Fig. 3F). We found no change in the melting curves between the wild-type allele and the [XXIX] allele, thereby suggesting that point mutations within the anchor probe sequence have little effect on the melting curve analysis.

3.2. Validation

The genotypes determined at Tohoku University using the proposed method and those determined at Kagoshima University using a previously published method were identical for the 11 common mutations (Table S1 in supplementary material). We performed a similar test using DNA samples purified from filter-paper blood samples to determine if this method could be used for newborn screening. The genotypes determined in both laboratories were identical for all 26 DNA samples (Table S2 in supplementary material).

3.3. Frequency of eleven prevalent mutations

We found four heterozygous carriers of mutation [I], three of mutation [II], and two of mutation [V]. In addition, primer/probe set G detected one heterozygous mutation, which was confirmed as mutation [VIII] by direct sequencing. Altogether, 10 mutations were detected in 420 Japanese healthy controls.

4. Discussion

We developed a simple and rapid genetic test using real-time PCR combined with the HybProbe system for the 11 prevalent mutations in *SLC25A13*: mutations [I], [II], [III], [IV], [V], [VI], [VII], [VIII], [IX], [XIX], and [XXI]. This genetic test is a closed-tube assay in which no post-PCR handling of the samples is required. In addition, the genotyping is completed within 1 h. This test can utilize DNA samples purified from both peripheral blood and filter-paper blood. The reliability of the test was confirmed by genotyping 76 blind DNA samples from patients with citrin deficiency, including 50 peripheral blood and 26 filter-paper blood DNA samples. Because screening for the 11 targeted mutations would identify 95% of mutant alleles in the Japanese population [19], both, one, and no mutant alleles are expected to be identified in 90.4%, 9.3%, and less than 0.3% of patients, respectively. This genetic test would be useful not only in Japan but also other East Asian countries, including China, Korea, Taiwan and Vietnam, in which the same mutations are prevalent. Our test is expected to detect 76–87% of the mutant alleles in the Chinese population [12,19,25], 95–100% in the Korean population [12,19,26], 60–68% in the Taiwanese population [27,28], and 100% in the Vietnamese population [12,19]. If we were to prepare a primer/probe set for mutation [X]:g.IVS6+5G>A [12], which is prevalent in Taiwan, the estimated sensitivity would exceed 90% in the Taiwanese population [27,28].

Recently, the high resolution melting (HRM) method was reported to be suitable for the screening of mutations in the diagnosis of citrin deficiency [28]. HRM analysis is a closed-tube assay that screens for any base changes in the amplicons. The presence of SNPs anywhere on the amplicons can affect the melting curve, thereby suggesting that HRM is not suitable for screening for known mutations, but rather, is best suited to screening for unknown mutations. When we detected one heterozygous prevalent mutation, we performed HRM screening for all 17 exons of *SLC25A13*. After HRM screening, only the HRM-positive exons were subjected to direct sequencing analysis. Several mutant alleles were identified using this approach.

The frequency of homozygotes, including compound heterozygotes, presenting *SLC25A13* mutations in the population at Kagoshima (a prefecture in the southern part of Japan) has been calculated to be 1/17,000 based on the carrier rate (1/65) [19]. The prevalence of NICCD has been also reported to be 1/17,000–34,000 [29]. In this study, the carrier rate in Miyagi (a prefecture in northern Japan) was 1/42 (95% confidential interval, 1/108–1/26), thereby yielding an estimated frequency of patients with citrin deficiency of 1/7,100. Our result, together with the previous report [19], suggests that a substantial fraction of the homozygotes or compound heterozygotes of SLC25A13 mutations was asymptomatic during the neonatal period.

The early and definitive diagnosis of citrin deficiency may be beneficial for patients with citrin deficiency by encouraging specific dietary habits and avoiding iatrogenic worsening of brain edema by glycerol infusion when patients develop encephalopathy [30,31]. Because the screening of blood citrulline levels by tandem mass analysis at birth does not detect all patients with citrin deficiency, the development of a genetic test would be welcomed. In this study, we demonstrated that genomic DNA extracted from filter paper blood samples was correctly genotyped, thereby indicating the feasibility of newborn screening using this genetic test. If 100,000 babies in the northern part of Japan were screened by this method, we would detect 14 homozygotes or compound heterozygotes with *SLC25A13* mutations and 2400 heterozygous carriers. In 2400 heterozygous carriers, we would expect to observe only 1 to 2 compound heterozygotes with one target and one non-target mutation. The estimated frequency of babies with two non-target mutations is 0.04/100,000. Our genetic method would therefore allow us to screen newborn babies efficiently. If we performed this genetic test in a high-throughput real-time PCR system, such as a 384- or 1,536-well format, the cost per sample could be lowered.

In conclusion, we have established a rapid and simple detection system using the HybProbe assay for the 11 prevalent mutations in *SLC25A13*. This system could be used to screen newborns for citrin deficiency and may facilitate the genetic diagnosis of citrin deficiency, especially in East Asian populations.

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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 evolutional 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

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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 at work 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 (*Cntn3*, *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*, *Tfpi2* 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 deciduas (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 paternal 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

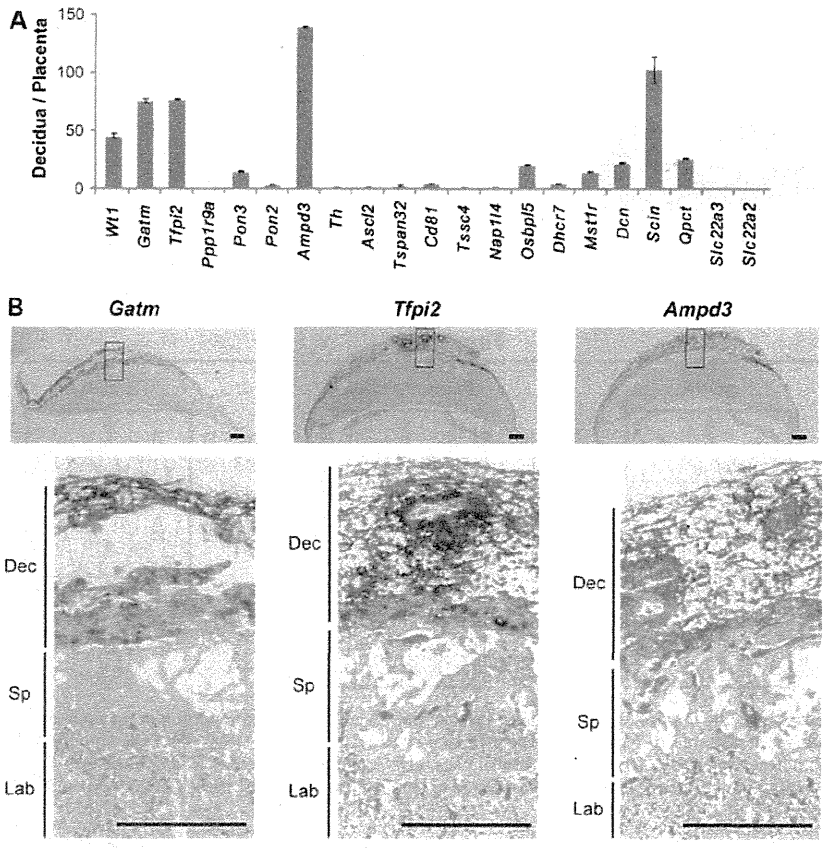

Figure 1. Expression of genes with placenta-specific imprinted expression in the decidua. (**A**) Real-time RT–PCR analysis of genes with placenta-specific imprinted expression in the decidua. Messenger RNA levels in the decidua were normalized to those observed in the placenta from which the decidua 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

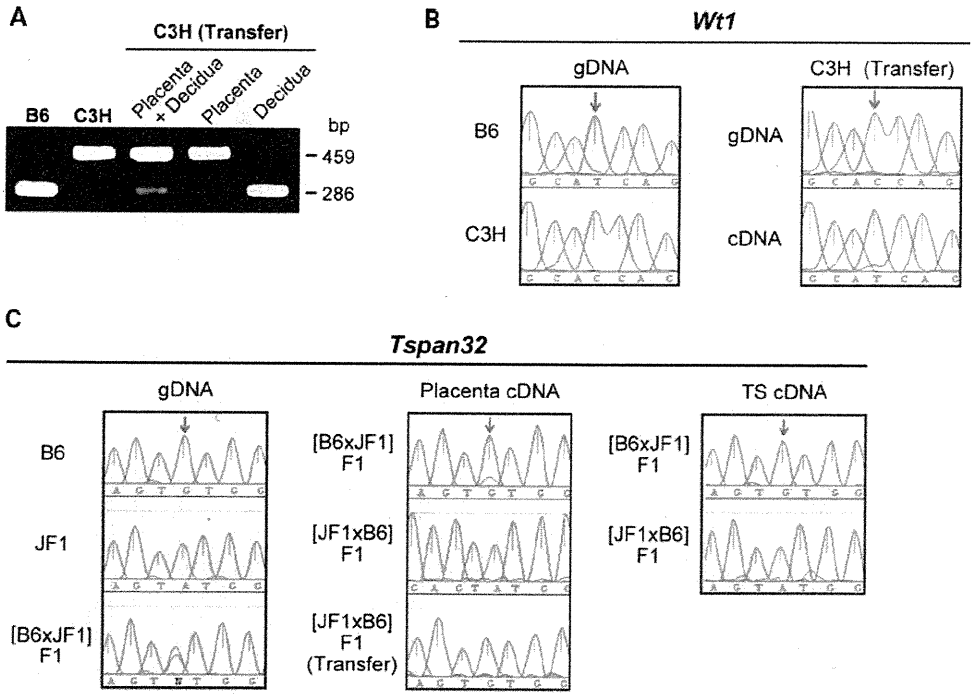

Figure 2. Analyses of the placenta-specific imprinting using embryo transfer and TS cells. (**A**) The proportion of maternal cells in the placenta. E13.5 placentas were obtained from C3H embryos transferred to recipient B6 mice [C3H (Transfer)]. Genomic DNA was PCR amplified with *Gapdh*-specific primers 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 *Gab1* 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

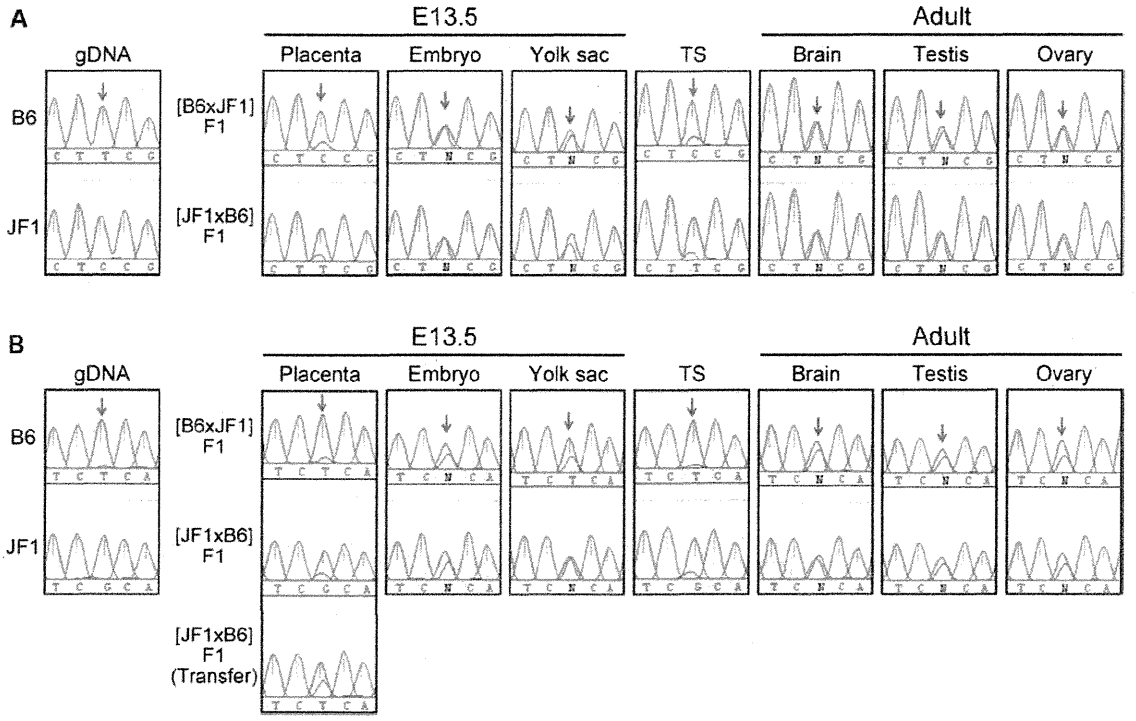

Figure 3. Allelic expression of novel imprinted genes identified by transcriptome sequencing. (**A**) Allelic expression of *Gab1*. *Gab1* showed paternal allele-specific expression in the placenta and TS cells but not in the embryo and adult tissues. (**B**) Allelic expression of *Ano1*. In the placenta,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

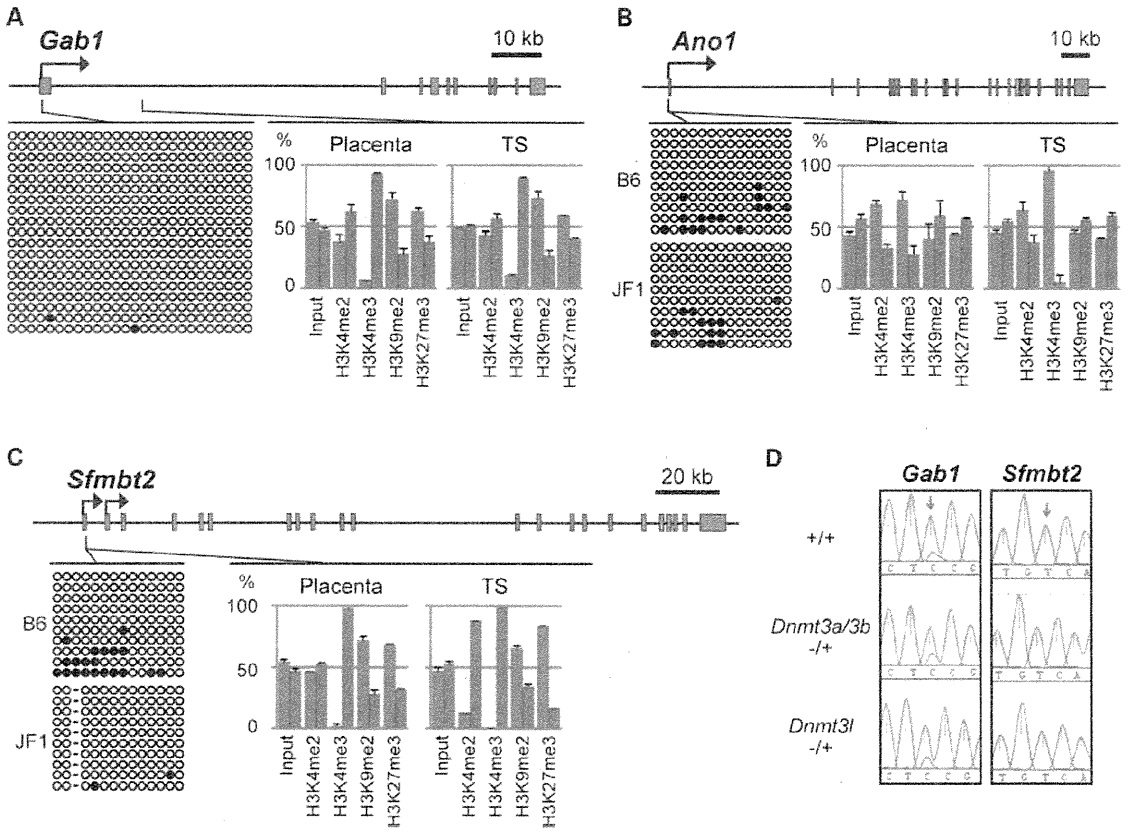

Figure 4. Epigenetic modification of *Gab1*, *Ano1* and *Sfmbt2*. (**A**–**C**) DNA methylation in [B6xJF1]F1 placenta was analyzed by bisulphite sequencing. Black and white circles indicate methylated and unmethylated residues. Histone modifications in [B6xJF1]F1 placenta and TS cells were analyzed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

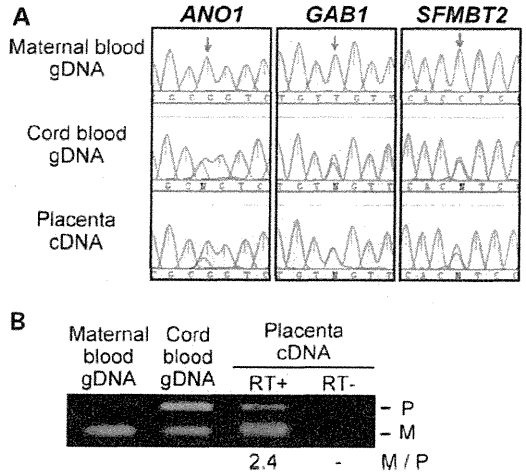

Figure 5. Allelic expression of *ANO1*, *GAB1* and *SFMBT2* in the human term placenta. (**A**) The A/G polymorphic site (SNP ID: rs2276067) in *ANO1*, T/G polymorphic site (SNP ID: rs1397529) in *GAB1* and C/G polymorphic site (SNP ID: rs2692756) in *SFMBT2* are indicated by the arrows. (**B**) Preferential 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

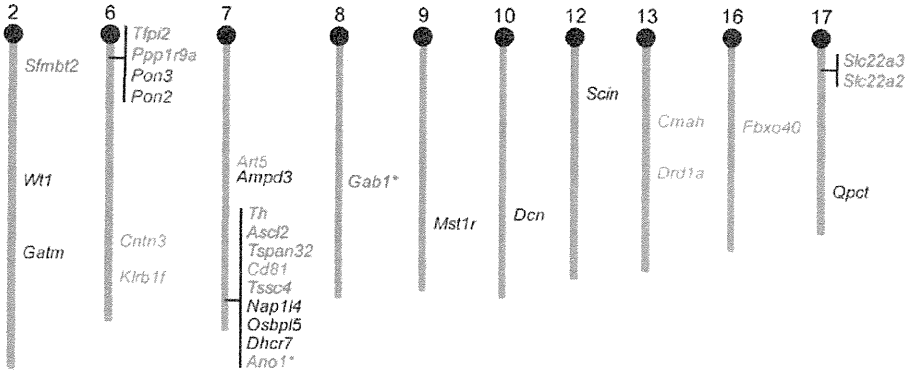

Figure 6. Chromosome map of genes with placenta-specific imprinted expression. Genes, of which paternal allele- and maternal allele-specific expression were confirmed in this study, are shown in blue and red, respectively. For genes shown in black, imprinted expression was not confirmed. Genes which 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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