Introduction

Human assisted reproduction technologies (ARTs) 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 the 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 the 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 to the earlier deadline (at 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

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. 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 cell samples from patients with one of the imprinting disorders using standard extraction methods (Kobayashi *et al.*, 2007). For control DNAs, DNA was prepared from the sperm and cord blood samples from unaffected individuals. The study was performed after obtaining patients 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, 2009; Wood *et al.*, 2007) using 20 control Japanese blood DNA samples. PCR primer sets were designed to span these SNPs (Supplementary data, Table SI) 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 ~50% methylation (Supplementary data, Fig. S1). 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 conceived naturally using 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 the 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: 351–538), 949 AS patients (95% CI: 682–1217), 2070 PWS patients (95% CI: 1504–2636) and 326 SRS patients (95% CI: 235–416). 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 data, Table SII and Supplementary data, Fig. S2.

Table I The 2009 frequency of four imprinting diseases in Japan in relation to use of assisted reproduction techniques (ART).

Imprinting disorders	Total estimated patient number (95% CI)	The total prevalence of the syndrome	The number of patients after ART/ total (%)
BWS	444 (351–538)	1 in 287 000	6/70 (8.6)
AS	949 (682–1217)	1 in 134 000	2/123 (1.6)
PWS	2070 (1504–2636)	1 in 62 000	4/261 (1.5)
SRS	326 (235–416)	1 in 392 000	4/42 (9.5)

Results of a nationwide epidemiological investigation of four imprinting disorders in Japan, under the governance of the Ministry of Health, Labor and Welfare of the Japanese government. Precise diagnosis was performed using fluorescence *in situ* hybridization and DNA methylation analyses. The type of ART, obtained from the questionnaires, was compared with the frequencies of these diseases and the epimutation rates. BWS, Beckwith-Wiedemann syndrome, AS, Angelman syndrome, PWS, Prader-Willi syndrome; SRS, Silver-Russell syndrome.

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 data, Table SIII). 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 born after ART.

After analyzing the second questionnaire, the blood or buccal mucosal cell samples were obtained from 15 individuals with BWS, 23 with SRS, 73 with AS and 29 with PWS. Using polymorphic bisulfite-PCR sequencing, we examined the methylation status of gDMRs within these samples at the imprinted regions implicated in these syndromes. For BWS we assayed *H19* and *KCNQ1OT1* (*LIT1*) gDMRs, for SRS we assayed the *H19* gDMR and for PWS and AS we assayed the *SNRPN* gDMR. For all patients (conceived naturally and with ART), the frequencies of DNA methylation errors (epimutations) corrected were 7/15 (46.7%) for BWS, 9/23 (39.1%) for SRS, 6/73 (8.2%) for AS and 2/29 (6.9%) for PWS. When looking at the ART cases exclusively, epimutation rates were 3/5 (BWS), 3/7 (SRS), 0/2 (AS) and 0/2 (PWS).

Abnormal methylation patterns in the ART and naturally conceived SRS patients with epimutations.

While hypomethylation of *H19* at chromosome 11 is known to be a frequent occurrence in SRS (Bliek *et al.*, 2006), various additional loci at chromosomes 7, 8, 15, 17 and 18 have been implicated as having a

role in this syndrome (OMIM 180860). We first identified SNPs in the previously reported 22 human DMRs using genomic DNA isolated from human sperm and blood from unaffected individuals, which could then be used in bisulfite-PCR methylation assays to assign methylation to the parental allele. We next collected a total of 15 SRS samples, including previously collected samples (ART: 2, naturally conceived: 4), which had DNA methylation errors at the paternal gDMR at *H19*. Five of these were born from ART and 10 were from natural conceptions. We analyzed and compared the DNA methylation status of the 3 other paternal gDMRs and the 19 maternal gDMRs (Supplementary data, Fig. S3, Table, Supplementary data, Table SIV). In four out of the five ART cases, DNA methylation errors were not restricted to the *H19* gDMR, and were present at both maternally and paternally methylated gDMRs. These four cases showed a mixture of hyper- and hypomethylation with mosaic (partial) patterns. In contrast, only 3 of the 10 naturally conceived patients showed DNA methylation errors at loci other than *H19* gDMR.

To determine whether DNA methylation errors occurred in patients at a broader level in the genomes, we assessed the methylation profiles of the non-imprinted *LINE1* and *Alu* elements. We examined a total of 28 CpG sites in a 413-bp fragment of *LINE1* and 12 CpG sites in a 152-bp fragment of *Alu* (Supplementary data, Table SIV), and no significant differences were found in the methylation ratios between patients conceived by ART and naturally.

The abnormal methylation pattern in BWS patients with epimutations

In BWS, hypermethylation of *H19* or hypomethylation of *KCNQ1OT1(LIT1)* at human chromosome 11 are both frequently reported (Choufani *et al.*, 2010). We collected seven BWS samples with DNA methylation errors of the *LIT1* gDMR, one of which was derived from ART patient and six from naturally conceived patients (Supplementary data, Fig. S3, Table II, Supplementary data, Table SIV). In the one ART (ICSI) case, we identified four additionally gDMR methylation errors, again present at both maternally and paternally methylated gDMRs and with mixed hyper- and hypomethylation patterns. Furthermore, the methylation error at the *NESPAS* DMR was mosaic in this patient. One of the six naturally BWS cases had similar changes. Although we had only one BWS case conceived by ART, widespread methylation errors were similar to those for the DNA methylation error pattern in SRS.

Phenotypic differences between ART patients and those conceived naturally

The increased frequency of DNA methylation errors at other loci in the ART cases suggested that the BWS and SRS cases born after ART might exhibit additional phenotypic characteristics. However, when we compared in detail the clinical features from both categories of conception (Supplementary data, Table SV), we found no major differences between ART and naturally conceived patients with BWS and SRS.

Discussion

Our key finding from this study was a possible association between ART and the imprinting disorders, BWS and SRS. We did not find a similar association with PWS and AS but our numbers were quite low in this study and a larger due to the questionnaire return rate and relative rarity of the diseases, international study will be required to reach definitive conclusions. Furthermore, factors such as PCR and/or cloning bias in the bisulfite method and correction for changing rate of ART over time must be considered when analyzing any results.

In addition to the possible association between ART and BWS/SRS, we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART shown by us and others is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable. The process of ART exposes the developing epigenome to many external influences, which have been shown to influence the proper establishment and maintenance of genomic imprints, including hormone stimulation (Sato *et al.*, 2007), *in vitro* culturing (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003), cryopreservation (Emiliani *et al.*, 2000; Honda *et al.*, 2001) and the timing of embryo transfer (Shimizu *et al.*, 2004; Miura and Niikawa, 2005). Furthermore, we and others have also shown that some infertile males, particularly those with oligozoospermia, carry pre-existing imprinting errors in their sperm (Marques *et al.*, 2004; Kobayashi *et al.*, 2007; Marques *et al.*, 2008) which might account for the association between ART and imprinting disorders.

Imprinting syndromes and their association with ART

We report the first Japanese nationwide epidemiological study to examine four well-known imprinting diseases and their possible association with ART. We found that the frequency of ART use in both BWS and SRS was higher than anticipated based on the nationwide frequency of ART use at the time when these patients were born. Several other reports have raised concerns that children conceived by ART have an increased risk of disorders (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Maher *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Lim and Maher, 2009). However, the association is not clear in every study (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007). The studies reporting an association were mainly from case reports or case series whereas the studies where no association was reported were cohort studies. Therefore, the differences in the epidemiological analytical methods might accounts for the disparity in findings.

Owing to the rare nature of the imprinting syndromes, statistical analysis is challenging. In addition, the diagnosis of imprinting diseases is not always clear cut. Many of the syndromes have a broad clinical spectrum, different molecular pathogenesis, and the infant has to have reached a certain age before these diseases become clinically detectable. It is therefore likely that some children with these diseases are not recorded with the specific diagnosis code for these syndromes. Nonetheless, in this study we were examining the relationship between ART and the imprinting syndromes and these confounding factors are likely to apply equally to both groups.

Both BWS and SRS occurred after ART but our numbers for PWS and AS were low, precluding any definitive conclusion for these two disorders. However, while most cases of BWS and SRS are caused by an epimutation, epimutations are very rare in PWS and AS (only 1–4%) and ART would not be expected to increase chromosome 15

Table II Abnormal methylation in patients with SRS and BWS.

Case	ART	Abnormal methylation			
SRS					
SRS-1	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated	PEG10 hypermethylated (mosaic)	GRB10 hypermethylated; ZNF597 hypomethylated
SRS-2	IVF-ET	H19 hypomethylated (mosaic)			
SRS-3	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated (mosaic)		
SRS-4	IVF-ET	H19 hypomethylated	GRB10 hypermethylated		
SRS-5	IVF-ET	H19 hypomethylated (mosaic)	INPP5F hypermethylated		
SRS-6		H19 hypomethylated			
SRS-7		H19 hypomethylated (mosaic)	ZNF597 hypermethylated (mosaic)	ZNF331 hypomethylated (mosaic)	
SRS-8		H19 hypomethylated			
SRS-9		H19 hypomethylated (mosaic)			
SRS-10		H19 hypomethylated			
SRS-11		H19 hypomethylated (mosaic)	PEG1 hypermethylated		
SRS-12		H19 hypomethylated			
SRS-13		H19 hypomethylated (mosaic)	FAM50B hypomethylated		
SRS-14		H19 hypomethylated			
SRS-15		H19 hypomethylated			
BWS					
BWS-1	ICSI	LIT1 hypomethylated	ZDBF2 hypermethylated	PEG1 hypermethylated	NESPAS hypomethylated (mosaic)
BWS-2		LIT1 hypomethylated			
BWS-3		LIT1 hypomethylated			
BWS-4		LIT1 hypomethylated			
BWS-5		LIT1 hypomethylated			
BWS-6		LIT1 hypomethylated	ZDBF2 hypomethylated	ZNF331 hypomethylated (mosaic)	
BWS-7		LIT1 hypomethylated			

ET, embryo transfer. Summary of the abnormal methylation patterns in the ART conceived and naturally conceived patients with Silver-Russell syndrome (SRS) and Beckwith-Wiedemann syndrome (BWS) with epimutations. Numbers in parentheses show the results of the methylation rates obtained using bisulfite-PCR sequencing. The % of DNA methylation of 22 gDMRs in all patients with SRS and BWS examined are presented in Supplementary data, Table SIV. Depictions in red represent DMRs normally exclusively methylated on the maternal allele, while blue represent paternally methylated sites.

deletions or uniparental disomy, consistent with our findings. Prior to this investigation, there was some evidence for an increased prevalence of BWS after ART but less evidence for an increased prevalence of SRS, with five SRS patients reported linked to ART (Svensson *et al.*, 2005; Bliek *et al.*, 2006; Kagami *et al.*, 2007; Galli-Tsinopoulou *et al.*, 2008). Our population-wide study provides evidence to suggest that both BWS and SRS occur more frequently after ART in the Japanese population.

Mechanisms of epimutation in the patients conceived by ART

By performing a comprehensive survey of all the known gDMRs in a number of patients with BWS and SRS, we found that multiple loci were more likely to be affected in ART cases than those conceived naturally. Lim *et al.* (2009) have reported a similarly increased frequency of multiple errors after ART, with 37.5% of patients conceived with ART and 6.4% of naturally conceived patients displaying abnormal methylation at additional imprinted loci. However, while Bliek *et al.* (2009) reported alterations in multiple imprinted loci in 17 patients out of 81 BWS cases with hypomethylation of *KCNQ1OT1(LIT1)* ICR, only 1 of the cases with multiple alterations was born after ART. Similarly, Rossignol *et al.* (2006) reported that 3 of 11 (27%) ART-conceived patients and 7 of 29 (24%) naturally conceived patients displayed abnormal methylation at additional loci. In these four earlier studies, not all gDMRs were assayed and it may be that by doing so, these incongruities will be resolved.

The pattern of cellular mosaicism we observed in some patients suggested that the imprinting defects occurred after fertilization rather than in the gamete as DNA methylation alterations arising in the gamete would be anticipated to be present in every somatic cell. This suggested the possibility that the DNA methylation errors occurred as a consequence of impaired maintenance of the germline imprints rather than a failure to establish these imprints in the germline or a loss of these imprints in the sperm or oocytes *in vitro*. Furthermore, some patients conceived by ART with SRS and BWS showed

alterations at both maternally and paternally methylated gDMRs suggesting that the defects were not limited to one parental germline. The mechanisms controlling the protection of imprinted loci against demethylation early in the development remain unclear. Our data suggested that this protection may fail in ART resulting in the tissue-specific loss of imprints, though it remains unclear if this ever occurs naturally. Potential factors involved could include the culture conditions for the newly fertilized oocyte and the length of exposure to specific media or growth factors, as part of the ART procedure. Some of the naturally conceived patients also had abnormal methylation at both maternally and paternally methylated gDMRs, which were in some cases mosaic. This could indicate that fertility issues arise as a consequence of pre-existing mutations in factors required to protect and maintain imprints early in life and it may therefore be possible to identify genetic mutations in these factors in this group of patients.

Clinical features

In our large-scale epidemiological study, we found differences in the frequency of some classic features of SRS and BWS between patients conceived by ART and those conceived naturally. We found that 7/7 (100%) ART conceived SRS patients showed body asymmetry, whereas only 30/54 (55.5%) who were conceived naturally possessed this feature. Similarly in BWS, earlobe creases were present in 4/7 (57.1%) ART conceived cases and 44/89 (49.4%) naturally conceived, bulging eyes in 3/7 (42.8%) versus 21/89 (23.6%), exomphalos in 6/7 (85.7%) versus 61/89 (68.5%) and nephromegaly in 2/7 (28.6%) versus 18/89 (20.2%), respectively. It is therefore possible that the dysregulation of the additional genes does modify the typical SRS and BWS phenotypes (Azzi *et al.*, 2010). BWS patients with multiple hypermethylation sites have been reported with complex clinical phenotypes (Bliek *et al.*, 2009) and a recently recognized BWS-like syndrome involving overgrowth with severe developmental delay was reported after IVF/ICSI (Shah *et al.*, 2006).

In our study patients with diagnosed imprinting disorders that presented with defects at additional loci (i.e. other than the domain responsible for that disorder) did not display additional phenotypes not normally reported in BWS or SRS. Since we were effectively selecting for classic cases of BWS and SRS in the first instance, it is possible that there are individuals born through ART showing entirely novel or confounding phenotypes that were not identified in our survey. Alternatively, as many of the alterations we observed showed a mosaic pattern, it is possible that mosaic individuals have more subtle phenotypes. In light of this new information on mosaicism, we may be able to use our knowledge of the individual's epigenotype to uncover these subtle changes.

This study, and the work of our colleagues, highlights the pressing need to conduct long-term international studies on ART treatment and the prevalence of imprinting disorders, particularly as the use of ART is increasing worldwide. It remains to be seen if other very rare epigenetic disorders will also have a possible association with the use of ART. Furthermore, it is not yet known what other pathologies might be influenced by ART. For example, in addition to general growth abnormalities, many imprint methylation errors also lead to the occurrence of various cancers (Okamoto *et al.*, 1997; Cui *et al.*, 1998). Further molecular studies will be required to understand the pathogenesis of these associations, and also to identify preventative methods to reduce the risk of occurrence of these syndromes following ART.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

H.H., H.O., N.M., F.S. and A.S. performed the DNA methylation analyses. M.K., K.N. and H.S. collected the samples of the patients. K.N. did the statistical analyses. H.H., M.V.D.P., R.M.J. and T.A. wrote this manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Aberrant Methylation of H19-DMR Acquired After Implantation Was Dissimilar in Soma Versus Placenta of Patients With Beckwith–Wiedemann Syndrome

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Gain of methylation (GOM) at the H19-differentially methylated region (H19-DMR) is one of several causative alterations in Beckwith–Wiedemann syndrome (BWS), an imprinting-related disorder. In most patients with epigenetic changes at H19-DMR, the timing of and mechanism mediating GOM is unknown. To clarify this, we analyzed methylation at the imprinting control regions of somatic tissues and the placenta from two unrelated, naturally conceived patients with sporadic BWS. Maternal H19-DMR was abnormally and variably hypermethylated in both patients, indicating epigenetic mosaicism. Aberrant methylation levels were consistently lower in placenta than in blood and skin. Mosaic and discordant methylation strongly suggested that aberrant hypermethylation occurred after implantation, when genome-wide de novo methylation normally occurs. We expect aberrant de novo hypermethylation of H19-DMR happens to a greater extent in embryos than in placentas, as this is normally the case for de novo methylation. In addition, of 16 primary imprinted DMRs analyzed, only H19-DMR was aberrantly methylated, except for NNAT DMR in the placental chorangioma of Patient 2. To our knowledge, these are the first data suggesting when GOM of H19-DMR occurs. © 2012 Wiley Periodicals, Inc.

Key words: Beckwith–Wiedemann syndrome; H19-DMR; aberrant DNA methylation; after implantation

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INTRODUCTION

Beckwith–Wiedemann syndrome (BWS) is an imprinting-related condition characterized by macrosomia, macroglossia, and abdominal wall defects (OMIM #130650). The relevant imprinted chromosomal region in BWS, 11p15.5, consists of two independent imprinted domains, *IGF2/H19* and *CDKN1C/KCNQ1OT1*. Imprinted genes within each domain are regulated by two imprinting control regions (ICR), the H19-differentially methylated region (H19-DMR) or KvDMR1 [Weksberg et al., 2010]. Several causative alterations have been identified in patients with BWS: loss of methylation (LOM) at KvDMR1, gain of methylation (GOM) at H19-DMR, paternal uniparental disomy (UPD), *CDKN1C* mutations, and chromosomal abnormality involving 11p15 [Sasaki et al., 2007; Weksberg et al., 2010].

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Methylation of H19-DMR is erased in primordial germ cells (PGCs) but becomes reestablished during spermatogenesis [Li, 2002; Sasaki and Matsui, 2008]: this methylation regulates the expression of *IGF2* and *H19* by functioning as a chromatin insulator, restricting access to shared enhancers [Bell and Felsenfeld, 2000; Hark et al., 2000]. GOM on the maternal H19-DMR leads to expression of both *IGF2* alleles and silencing of both *H19* alleles. Dominant maternal transmissions of microdeletions and/or base substitutions within H19-DMR have recently been reported in a few patients of BWS with H19-DMR GOM [Demars et al., 2010]. However, when and how the GOM on the maternal H19-DMR occurs is not clear.

Here, we found epigenetic mosaicism in two BWS patients. We also found that GOM at H19-DMR was discordant in blood and skin versus placenta; specifically, methylation levels were lower in placental samples. These findings strongly suggest that aberrant methylation of H19-DMR occurred after implantation. As a result, we expect aberrant de novo methylation happens to a greater extent in embryos than in placentas.

MATERIALS AND METHODS

Patients

Two unrelated patients with sporadic BWS, Patient 1 (BWS047) and Patient 2 (bwsh21-015), were delivered by cesarean in the third trimester of pregnancy. The mothers of both patients conceived naturally. Patient 1 and Patient 2 met clinical criteria for BWS as described by Elliott et al. [1994] and Weksberg et al. [2001], respectively (Table I). The placenta of Patient 1 was large and weighed 1,065 g, but was without any pathological abnormality. The placenta of Patient 2 was also large, weighing 1,620 g, and had an encapsulated placental chorangioma, as reported previously [Aoki et al., 2011]. The standard G-banding chromosome analysis using peripheral blood samples showed no abnormalities in either patient. This study was approved by the Ethics Committee for Human Genome and Gene Analyses of the Faculty of Medicine, Saga University.

Southern Blot Analysis

Genomic DNA was extracted from embryo-derived somatic tissues and the placentas of the patients (Fig. 1). Methylation-sensitive Southern blots with *Bam*HI and *Not*I were employed for KvDMR1, and blots with *Pst*I and *Mlu*I were employed for H19-DMR, as described previously [Soejima et al., 2004]. Band intensity was measured using the FLA-7000 fluoro-image analyzer (Fujifilm, Tokyo, Japan). The methylation index (MI, %) was then calculated (Fig. 1). Southern blots with *Apa*I were used to identify the microdeletion of H19-DMR as described previously [Sparago et al., 2004].

Bisulfite Sequencing and Combined Bisulfite Restriction Analysis (COBRA)

Bisulfite sequencing covering the sixth CTCF binding site (CTS6) was performed. For COBRA, PCR products of each primary imprinted DMR were digested with the appropriate restriction endonucleases and were then separated using the MultiNA Microchip Electrophoresis System (Shimazdu, Japan). The methylation index was also calculated. All PCR primer sets used in this study have been listed in Supplementary Table SI (See Supporting Information online).

DNA Polymorphism Analyses

For quantitative polymorphism analyses, tetranucleotide repeat markers (*D11S1997* and *HUMTH01*) and a triplet repeat marker (*D11S2362*) from 11p15.4–p15.5 were amplified and separated by electrophoresis on an Applied Biosystems 3130 genetic analyzer (Applied Biosystems, NY); data were quantitatively analyzed with the GeneMapper software. The peak height ratios of paternal allele to maternal allele were calculated. A single nucleotide polymorphism (SNP) for the *Rsa*I recognition site in *H19* exon 5 (rs2839703) was also quantitatively analyzed using hot-stop PCR [Uejima et al., 2000]. Band intensity was measured using the FLA-7000 fluoro-image analyzer (Fujifilm).

Mutation Search of H19-DMR

To search for mutations in the binding sites of CTCF, OCT4, and SOX2, we sequenced a genomic region in and around H19-DMR, which included seven CTCF-binding sites, three OCT4 sites, and one SOX2 site.

TABLE I. Clinical Information of BWS Patients

Patient ID	Conception	Birth weight (gestational age)	Clinical features	Karyotype	Placental weight and pathology	Placental–fetal weight ratio
Patient 1 (BWS047)	Natural	4,506 g (36w2d)	macrosomia macroglossia abdominal wall defect hypoglycemia	46,XY	1,065 g no pathological findings	0.236
Patient 2 (bwsh21-015)	Natural	2,540 g (33w5d)	macrosomia macroglossia hypoglycemia renal malformation hepatosplenomegaly	46,XX	1,620 g placental chorangioma	0.638

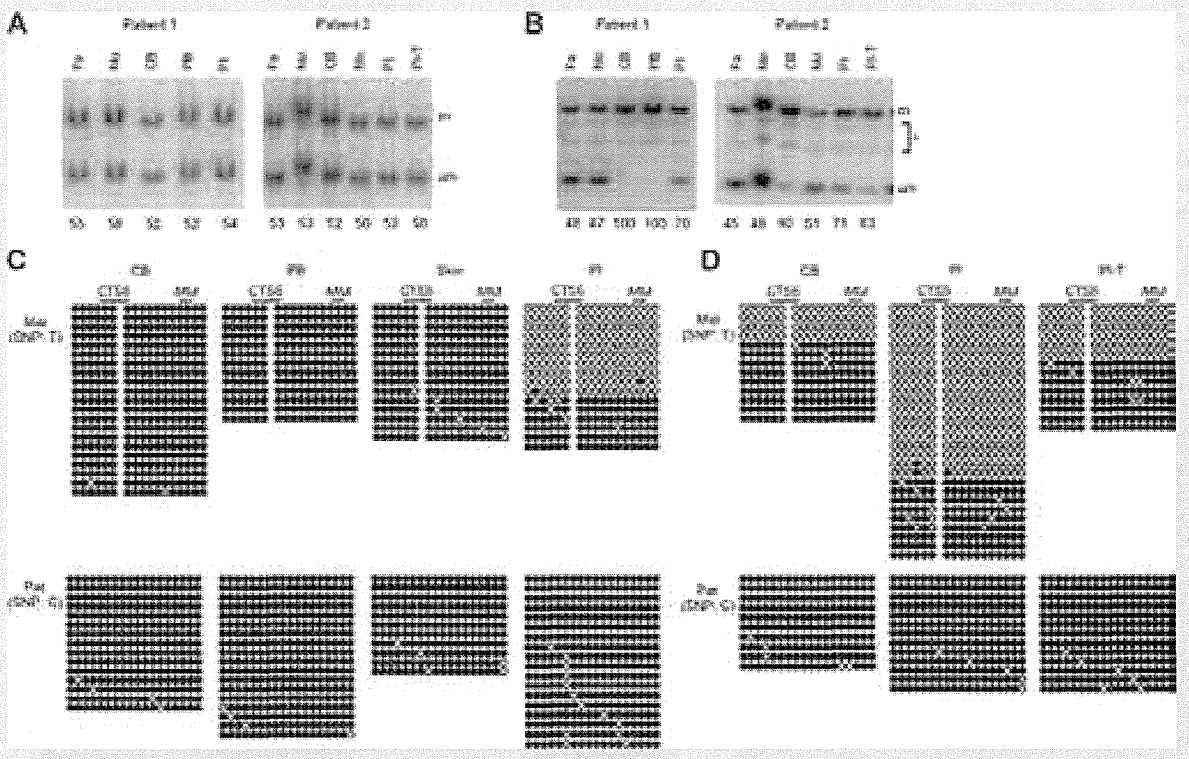

FIG. 1. Methylation analyses of KvDMR1 and H19-DMR. A: Methylation-sensitive Southern blots for KvDMR1. Genomic DNA was extracted from the cord blood, peripheral blood, skin, and placenta of Patient 1 and from the cord blood, placenta, and placental chorangioma of Patient 2. Methylation at KvDMR1 was normal in all samples analyzed. Methylation indices (MI, %) are shown under the figure. B: Methylation-sensitive Southern blots for H19-DMR. The MIs of blood samples were higher than the MIs of placental samples. MI was calculated using the equation $[M/(M+U)] \times 100$, where M is the intensity of the methylated band and U is the intensity of the unmethylated band. C: Bisulfite sequencing of H19-DMR in Patient 1. The two parental alleles were distinguishable by differences in SNPs. Both parental alleles were completely methylated in the cord blood, peripheral blood, and skin samples, and the maternal allele, which is normally unmethylated, was partially methylated in the placenta. D: Bisulfite sequencing of H19-DMR in Patient 2. Methylation of the maternal allele was higher in the cord blood than in the placenta or placental chorangioma. These results were consistent with the results of the Southern blot analysis. We confirmed complete methylation of paternal H19-DMR alleles and complete demethylation of maternal H19-DMR alleles in four normal control placentas that were heterozygous for identifiable SNPs (data not shown). Fa, father; Mo, mother; CB, cord blood; PB, peripheral blood; Pl, placenta; Pl-T, placental chorangioma; m, methylated band; um, unmethylated band; *, nonspecific bands; Mat, maternal allele; Pat, paternal allele; CTS6, sixth CTCF binding site; *MluI*, a restriction site approximately 80 bp downstream of CTS6 assayed by methylation-sensitive Southern blot and COBRA.

RESULTS

We first examined the methylation status of the two ICRs, KvDMR1, and H19-DMR, at 11p15.5 using methylation-sensitive Southern blot analysis. Methylation at KvDMR1 was normal in all samples collected (Fig. 1A); however, methylation at H19-DMR was aberrant (Fig. 1B). In Patient 1, hypermethylation at H19-DMR was complete in cord blood and peripheral blood samples (MI = 100%), and hypermethylation in the placenta was partial (MI = 70%). In Patient 2, H19-DMR was partially hypermethylated in cord blood (MI = 90%) but less so in the placenta and placental chorangioma (MI = 71% and MI = 83%, respectively). For further investigation of differences in methylation between the patients' somatic tissues and placentas, the CTS6 site was subjected to bisulfite sequencing (Fig. 1C and D). We could distinguish the two parental alleles in each patient sample using informative SNPs (rs10732516 and rs2071094). The maternal allele, which is normally unmethylated, was completely methylated in the cord blood, peripheral blood, and skin from Patient 1. However, in placental samples from Patient 1, the maternal allele was only partially methylated: 36% of all CpGs analyzed were methylated. Similar results were observed in Patient 2: the maternal allele in the cord blood was 68% methylated; however, the maternal allele was only 31% and 55% methylated in the placenta and chorangioma samples, respectively. The paternal alleles, which are normally fully methylated, were fully methylated in all samples. These findings supported the results of the Southern blots. Furthermore, we could not find any microdeletions or mutations in or around H19-DMR,

including seven CTCF-binding sites, three OCT4 sites, and one SOX2 site, indicating that there was no genetic cause of the hypermethylation (Fig. 2A and data not shown).

Next, we analyzed polymorphic markers at 11p15.4–p15.5 to determine whether copy number abnormalities or paternal UPD might be involved in these BWS patients. Although smaller PCR products were more easily amplified, paternal–maternal allele ratios in blood samples were between 0.92 and 1.33, indicating that both parental alleles were equally represented in both patients (Fig. 2B). Therefore, we could rule out copy number abnormality and paternal UPD within the patients' blood. We also investigated maternal contamination in the placenta. *D11S1997* and *HUMTH01* for Patient 1 and the *Rsa*I polymorphism in *H19* (rs2839703) for Patient 2 were used for this investigation because the mothers were expected to be homozygous for such polymorphisms. Thus, we investigated contamination of our samples by assessing the homozygosity of the polymorphisms in the mothers. The paternal–maternal ratios in Patient 1 were 0.94 and 1.03, indicating an equal contribution of both parental alleles and suggesting no contamination (Fig. 2B). In Patient 2, the ratios were 0.77 and 0.78 in the placenta and chorangioma, respectively, suggesting a small amount of contamination (Fig. 2C). However, such contamination was too

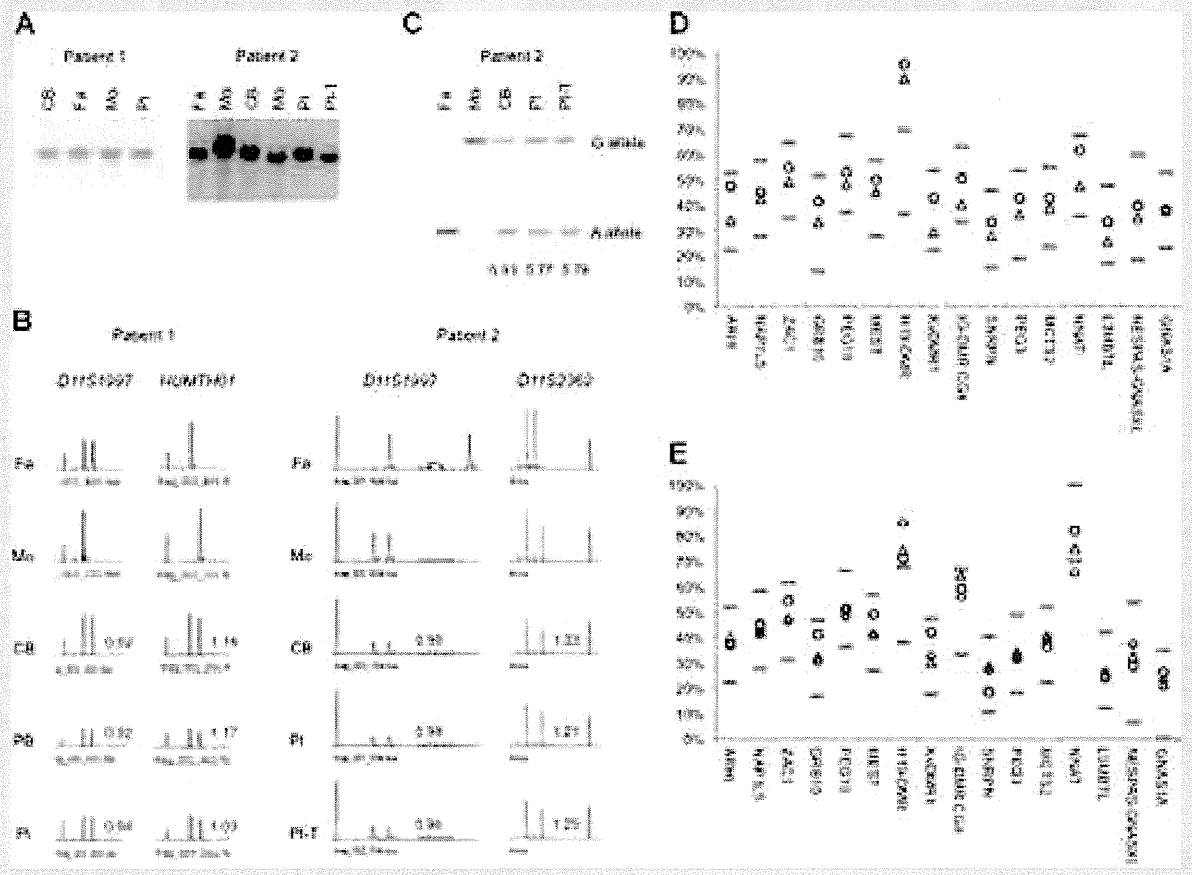

FIG. 2. Microdeletion analysis of H19-DMR, polymorphism analyses, and COBRA of primary imprinted DMRs in embryo-derived and placental samples. A: Southern blots identifying a microdeletion of H19-DMR. A genomic fragment (7.7 kb) generated by *Apa*I digestion, which included the entire H19-DMR, was evident in all samples, indicating that there was no microdeletion in this DMR. B: Microsatellite markers at 11p15.4–p15.5. The peak heights associated with each parental allele in all samples were quantitatively analyzed. The results indicated that both parental alleles were present and equally represented. C: Hot-stop PCR of an *Rsa*I polymorphic site in Patient 2. The ratios of paternal allele to maternal allele are shown under the figure. Although the ratios in the placenta and placental chorangioma are lower than in the cord blood, suggesting a small amount of maternal contamination, this was not enough to affect the results of the methylation analyses. COBRA of cord blood (D) and placentas (E), demonstrating that H19-DMR was hypermethylated. CTS6 is contained within H19-DMR. Methylation at other DMRs was normal in all samples, except for methylation at NNAT, which was aberrant in the placental chorangioma. Cord blood and placentas from 24 normal individuals were used as controls. The upper limit of normal methylation was defined as the higher of these two values: (1) the average of controls + 3 SD, or (2) the average + 15%. Similarly, the lower limit of normal methylation was definite as the lower of these two values: (1) the average of controls − 3 SD, or (2) the average − 15%. The upper and lower limits are indicated by gray bars. ○: Patient 1; △: Patient 2; ◇: placental chorangioma of Patient 2.

small to affect the results of the methylation analyses. In addition, sequence analysis did not show any mutations in *CDKN1C* (data not shown). These findings indicated that H19-DMR was aberrantly hypermethylated in both BWS patients and their associated placentas, but the aberrant methylation was consistently lower in the placenta, and that the H19-DMR GOM was strictly an isolated epimutation.

Finally, we analyzed the methylation status of 16 primary imprinted DMRs scattered throughout the genome-using COBRA (Fig. 2D and E). Only H19-DMR showed aberrant methylation among all primary DMRs in all samples, except for NNAT DMR, which was abnormal only in the placental chorangioma, indicating that the *IGF2/H19* imprinted domain was targeted for aberrant methylation in both somatic tissues and the placenta.

DISCUSSION

Methylation associated with parental imprints are erased in PGC and reestablished during gametogenesis in a sex-specific manner. The paternal pronucleus in the zygote undergoes active demethylation; extensive passive demethylation then ensues on maternal and paternal chromosomes during the pre-implantation period. After implantation, de novo methylation results in a rapid increase in DNA methylation in the inner cell mass (ICM), which gives rise to the entire embryo; in contrast, de novo methylation is either inhibited or not maintained in the trophoblast, which gives rise to the placenta [Li, 2002; Sasaki and Matsui, 2008]. The imprinted DMRs, however, escape these demethylation and de novo methylation events that occur in early embryogenesis. H19-DMR GOM in BWS patients is considered an error in imprint erasure in female PGCs [Horsthemke, 2010]. However, H19-DMR GOM, whether with or without microdeletions within H19-DMR, was partial, indicating a mosaic of normal cells and aberrantly methylated cells [Sparago et al., 2007; Cerrato et al., 2008]. These findings demonstrated that aberrant hypermethylation at H19-DMR was acquired after fertilization, although the precise timing was unknown.

Both participants in this study had isolated GOM at H19-DMR. The partial and variable hypermethylation among samples suggested epigenetic mosaicism. Furthermore, methylation levels in the placentas were lower than those in the blood and skin, suggesting that the aberrant methylation was acquired after implantation—when genome-wide de novo methylation normally occurs. Aberrant de novo methylation at H19-DMR is expected to be more widespread in the embryo than in the placenta, as this is normally the case for de novo methylation [Li, 2002; Sasaki and Matsui, 2008]; this disparity in efficiency could lead to the discordance between hypermethylation in trophoblast-derived placenta and that in embryo-derived blood and skin. This hypothesis is supported by a mouse experiment in which a mutant maternal allele harboring a deletion of four CTCF binding sites was hypomethylated in oocytes and blastocysts, yet was highly methylated after implantation [Engel et al., 2006]. To our knowledge, this is the first evidence demonstrating that aberrant hypermethylation of maternal H19-DMR is acquired after implantation in humans.

We found that of 16 primary imprinted DMRs analyzed, only H19-DMR showed aberrant methylation; even methylation at IG-DMR CG4, another paternally methylated, primary imprinted DMR, was normal in our patients. Although we only studied two patients, this finding indicated that the *IGF2/H19* imprinted domain in both the embryo and placenta was more susceptible than other imprinted domains to aberrant methylation acquired after implantation.

In conclusion, we found that methylation of H19-DMR was discordant in embryo-derived somatic tissue and placenta, strongly suggesting that the aberrant de novo methylation occurred after implantation. However, the precise mechanism of isolated H19-DMR GOM is still unknown. Since no mutations in *CTCF*, an important trans-acting imprinting factor, were found in these patients with isolated GOM at H19-DMR, the potential for mutations in the OCT and SOX transcription factors should be investigated because mutations of OCT-binding sites have previously been found in a few patients with H19-DMR GOM [Cerrato et al., 2008; Demars et al., 2010].

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Methylation screening of reciprocal genome-wide UPDs identifies novel human-specific imprinted genes†

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Nuclear transfer experiments undertaken in the mid-80's revealed that both maternal and paternal genomes are necessary for normal development. This is due to genomic imprinting, an epigenetic mechanism that results in parent-of-origin monoallelic expression of genes regulated by germline-derived allelic methylation. To date, ~100 imprinted transcripts have been identified in mouse, with approximately two-thirds showing conservation in humans. It is currently unknown how many imprinted genes are present in humans, and to what extent these transcripts exhibit human-specific imprinted expression. This is mainly due to the fact that the majority of screens for imprinted genes have been undertaken in mouse, with subsequent analysis of the human orthologues. Utilizing extremely rare reciprocal genome-wide uniparental disomy samples presenting with Beckwith–Wiedemann and Silver–Russell syndrome-like phenotypes, we analyzed ~0.1% of CpG dinculeotides present in the human genome for imprinted differentially methylated regions (DMRs) using the Illumina Infinium methylation27 BeadChip microarray. This approach identified 15 imprinted DMRs associated with characterized imprinted domains, and confirmed the maternal methylation of the *RB1* DMR. In addition, we discovered two novel DMRs, first, one maternally methylated region overlapping the *FAM50B* promoter CpG island, which results in paternal expression of this retrotransposon. Secondly, we found a paternally methylated, bidirectional repressor located between maternally expressed *ZNF597* and *NAT15* genes. These three genes are biallelically expressed in mice due to lack of differential methylation, suggesting that these genes have become imprinted after the divergence of mouse and humans.

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INTRODUCTION

Genomic imprinting is an epigenetic process in which one allele is repressed, resulting in parent-of-origin specific monoallelic expression (1). To date, around 100 imprinted transcripts have been identified in mouse, including protein coding genes, long non-coding RNAs (ncRNA) and microRNAs. Approximately two-thirds show conserved imprinting status between mouse and humans, while some show imprinting restricted to humans (http://igc.otago.ac.nz/home.html).

Genomic imprinting is regulated by epigenetic modifications such as DNA methylation, along with repressive histone modifications that are transmitted through the gametes from the parental germlines (1). Many imprinted regions contain differentially methylated regions (DMRs) that exhibit parent-of-origin-dependent DNA methylation. Of the 21 known DMRs in mouse, a subset have been shown to function as *cis*-acting imprinting control regions (ICRs) orchestrating the monoallelic expression of genes over more than 100 kbp away (2). The establishment of imprinted methylation in both the maternal and paternal germlines requires the *de novo* DNA methyltransferae Dnmt3a and its related protein Dnmt3L (3,4). Maintenance of these DMRs is stable throughout somatic development and is regulated by Dnmt1 and Uhrf1 during DNA replication (5,6).

The identification of novel imprinted genes is important as it is becoming increasing evident that alterations in the fine-tuning of imprinted gene expression can influence a number of complex diseases such as obesity, diabetes, neurological diseases and cancer (7–9), in addition to the well-defined imprinting syndromes associated with severe disruption of imprinted domains.

The identification of imprinted genes has traditionally been performed in mouse owing to the ease of embryo and genetic manipulations, and has utilized gynogenetic and androgenetic embryos, or mice harboring regions of uniparental disomy (UPD), where two copies of an entire chromosome or chromosomal region is inherited from only one parent (reviewed in 10). These embryos have then been used in expression screen-based approaches such as subtractive hybridization, differential display or expression array hybridization (11,12). However, these screens are not deemed comprehensive, as imprinted gene expression can be both tissue- and developmental-stage specific. Previously, sophisticated screens have detected allelic differences in DNA methylation at imprinted DMRs present in all somatic tissues, irrespective of temporal and spatial expression. Techniques such as restriction landmark genomic screening, methylation-sensitive representation difference analysis (Me-RDA) and methylated DNA immunoprecipitation (MeDIP) have identified regions of allelic DNA methylation associated with chromosomal regions controlling several imprinted genes in mice (13–15) and humans (16,17).

In order to identify novel imprinted genes in humans, we have performed a quantitative genome-wide methylation screen comparing the methylomes of three-genome-wide paternal UPD (pUPD) samples identified with Beckwith–Wiedemann-like phenotypes and one genome-wide maternal UPD (mUPD) Silver–Russell-like syndrome case (18–21) with the methylomes of six normal somatic tissues. The genome-wide UPD samples were all mosaic, and we utilized DNA extracted from leukocytes as these presented with lowest level of the biparental cell line. The DNA methylation profiles of these samples only differ at imprinted DMRs, since they are all derived from leukocytes, making them ideal to screen for novel imprinted loci. We utilized the Illumina Infinium Human Methylation27 BeadChip microarray and were able to identify 15 imprinted DMRs associated with known imprinted transcripts, and confirm the allelic methylation within intron 2 of the *RB1* gene (22).

By comparing the methylation profiles of six somatic tissues and the genome-wide UPD cases, we identified a novel paternally methylated DMR which acts as a directional silencer resulting in the maternal expression of *ZNF597* (also known as *FLJ33071*) and *NAT15* on chromosome 16, and a maternally methylated DMRs encompassing the promoter region of the *FAM50B* retrotransposon on chromosome 6, which is paternally expressed in human tissues. Interestingly, the CpG islands of the mouse orthologues of *ZNF597*, *NAT15* and *FAM50B* are all unmethylated, resulting in biallelic expression in mid-gestation embryonic tissues.

RESULTS

Defining a hemimethylated data set

Almost all imprinted domains contain at least one region of allelic DNA methylation which is thought to regulate imprinting *in cis* (1). In order to identify new imprinted genes in humans, we performed a methyation screen of six different normal somatic tissues derived from the three germinal layers (placenta, leukocytes, brain, muscle, fat, buccal cells) and compared the data set with the methylation profiles from reciprocal genome-wide UPD samples. Genomic DNA was modified by sodium bisulfite treatment and hybridized to the Illumina Infinium Human Methylation27 platform. This array covers 27 578 CpG dinucleotides associated with 14 000 human genes. To identify novel imprinted DMRs, we took advantage of the fact that these CpG-rich sequences have a methylation profile of ~50% in all somatic tissues. We identified 78 CpG probes associated with 15 known imprinted DMRs on the array (average methylation 52%, SD 11.7) (Supplementary Material, Fig. S1). To define a range in which novel imprinted DMRs should lie, we used the mean for the known imprinted DMR ± 1.5 SD (range 34.4–69.6). After applying these defined cutoffs, we identified 3212 CpG probes for which the mean methylation value for all normal tissues was within this range. To rule out the possibility that a mean of ~52% was caused by extreme values of hyper- and hypomethylation as a result of tissue-specific methylation, we only assessed those within 1.8 times SD distance from the methylation average. This step ensures that the ~52% methylation value is representative of all tissues. Using these criteria, we reduced the data set to 1836 CpG probes, which were in addition to 72 probes mapping to known imprinted DMRs.

Determining the allelic methylation using genome-wide UPDs

To identify novel imprinted DMRs within the above hemi-methylated data set outlined earlier, we compared the tissue methylation profiles to those obtained for the samples with genome-wide UPD. Of the 1836 CpG probes, only 14 gave methylation profiles consistent with an imprinted profile (Supplementary Material, Fig. S2). We subsequently mapped the exact location of the candidate CpGs using the genomic sequence of the unconverted DNA probes in the BLAT search tool (UCSC Genome Bioinformatics http://genome.ucsc.edu/). These 14 CpG probes were located close to nine autosomal genes, *RB1* (5), *FAM50B* (2), *ZNF597* (1), *TRPC3* (1), *SYCE1* (2), *TSP50* (1), *SORD* (1) and *ZBTB16* (1). We identified five independent probes located throughout CpG 85 (the CpG island identifier on the UCSC genome browser, build GRCh37/hg19) of the recently identified *RB1* imprinted gene on chromosome 13. These probes were unmethylated with average β-values of 0.21, 0.17 and 0.18 in the three genome-wide pUPD samples but hypermethylated, having an average β-value 0.88, in the genome-wide mUPD sample (a complete unmethylated CpG has a β-value of 0, and a fully methylated dinucleotide being 1). Using bisulphite PCR incorporating the single-nucleotide polymorphism (SNP) rs2804094 and sequencing of individual DNA strands, we were able to confirm that this 1.2 kb CpG island is a maternally methylated DMR in placenta, leukocyte and kidney-derived DNA and unmethylated in sperm (Supplementary Material, Fig. S3).

We identified one probe was located close to CpG 55 of the *TRPC3* gene on human chromosome 4 that was suggestive of a maternally methylated DMR. Subsequent allelic bisulphite PCR encompassing the SNP rs13121031 revealed that this region was subject to SNP-associated methylation and not parent-of-origin methylation (data not shown). The CpG islands within the promoters of *ZBTB16*, *TSP50* and *SORD* each had one probe that was suggestive of imprinted methylation, however allele-specific bisulphite PCR analysis revealed that these regions had a mosaic methylated profile (data not shown).

Two probes mapping to CpG 124 of *SYCE1/SPRNP1* on chromosome 10 also had a methylation profile consistent with an imprinted DMR. However, these probes were unable to discriminate *SYCE1* from *SPRN*, a second region that shared 93% homology. Due to the difficulty in designing bisulphite PCR primers that could specifically target *SYCE1*, we were unable to validate our initial observations.

The *ZNF597/NAT15* CpG island is a paternally methylated DMR

To date, only seven paternally methylated DMRs have been identified, the somatic DMRs at the *NESP, IGF2-P0* and *MEG3/GTL2* promoters, the germline *H19* differentially methylated domain (DMD), *Rasgrf1* DMD, IG-DMR and *ZDBF2* DMR (15,23–26). The *RASGRF1* is not imprinted in humans due to lack of the DNA repeat elements that are involved in establishing germline methylation (27). We identify two CpG probes, one mapping to CpG 41 between the promoters of *ZNF597* and *NAT15*, the other 500 bp away, in a region flanking CpG 41. Both probes were hypermethylated in the three genome-wide pUPD samples (β-values of 0.83, 0.42, 0.75) and hypomethylated (β-value of 0.08) in the genome-wide mUPD sample. Using bisulphite PCR and subsequent sequencing of heterozygous DNA samples for the SNP rs2270499, we were able to confirm that the methylation was solely on the paternally derived allele in placenta, leukocyte and kidney (Fig. 1). This is consistent with the previous report that *ZNF597* is maternally expressed in human leukocytes (28). Bisulphite PCR and sequencing of sperm DNA revealed that this region lack methylation, indicating that CpG41 is not a germline DMR. Using allele-specific RT–PCR that incorporated coding SNPs within exon 3, we observed maternal expression in brain ($n = 1$) and placenta ($n = 3$), and confirmed imprinting in leukocytes ($n = 2$).

The gene encoding *N*-acetyltransferase 15, *NAT15*, is encoded by two different transcripts (Fig. 1A). To determine whether *NAT15* is also subject to genomic imprinting, we performed allelic RT–PCR using PCR primers that could discriminate each isoform. We find that *NAT15* isoform 1 is maternally expressed in both placenta ($n = 5$) and leukocytes ($n = 1$), whereas isoform 2 is biallelically expressed ($n = 4$) which is consistent with CpG 101 being unmethylated (Fig. 1, data not shown).

FAM50B DMR shows graduated methylation

We identified two probes mapping to a 1.7 kbp CpG island within the *FAM50B* promoter. These probes were hypermethylated in the genome-wide mUPD (average β-values of 0.86), but hypomethylation in the three pUPD samples mUPD (β-value of 0.23, 0.39, 0.31). Allelic bisulphite sequencing showed that the methylation profile of CpG 143 differs between the 5′ and 3′ ends. The 5′ region flanking the SNP rs2239713, overlapping the *FAM50B* promoter, is a maternally methylated DMR in placenta-, leukocyte- and kidney-derived DNA, while the 3′ region near rs34635612 is fully methylated on both parental alleles. Despite this methylation gradient, the *FAM50B* gene is paternally expressed in placenta ($n = 6$) (Fig. 2).

The absence of allelic methylation at the mouse orthologues *of ZNF597, NAT15* and *FAM50B* is associated with biallelic expression

To determine whether the allelic expression of the novel imprinted transcripts was conserved in mouse, we investigated the allele-specific expression using RT–PCR amplification across transcribed SNPs. Mouse tissues were derived from interspecies crosses at both embryonic day E9.5 and post-natal day 1. The *Fam50b* gene has two isoforms with alternative first exons. We could only detect expression in testis, which was derived from both parental alleles. Exon 2 of Fam50b corresponds to an X-chromosome-derived retrogene and overlaps a methylated CpG island.

The *Nat15* and *Znf597* genes share two different promoter CpG islands, CpG 35 and CpG 87 that are orthologous to the *ZNF597* DMR and the *NAT15* isoform 2 promoters, respectively. In mouse, both of these regions are unmethylated. Both *Nat15* isoforms are predominantly expressed in

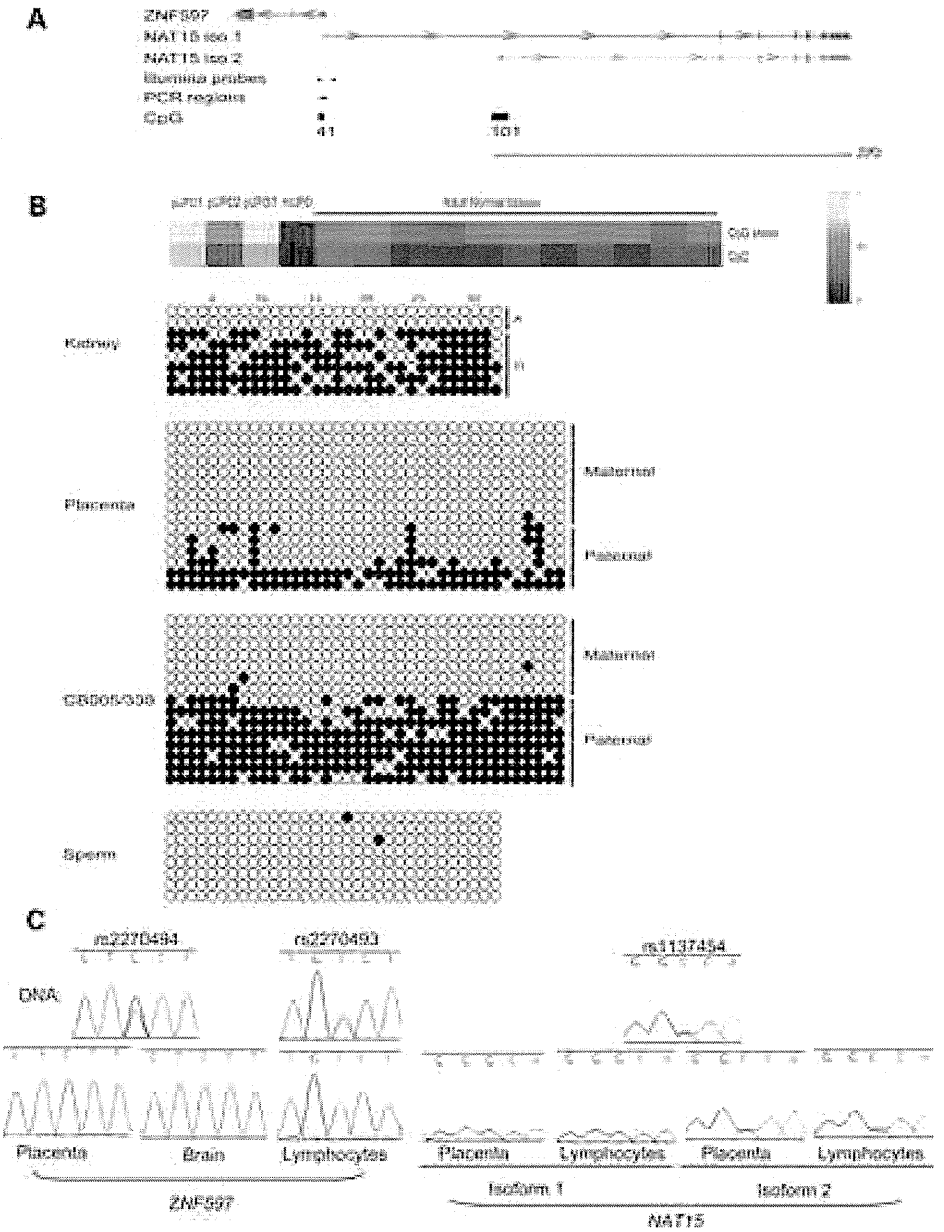

Figure 1. (**A**) Map of the *ZNF597-NAT15* locus on human chromosome 16, showing the location of the various transcripts, CpG islands, Illumina probes and bisulphite PCR regions (red transcripts are maternally expressed, blue paternally expressed and grey are expressed from both parental alleles. Arrows represent the direction of transcription) (not drawn to scale). (**B**) Heat map of the Infinium HumanMethylation27 BeadChIP for the *ZNF597* CpG probes (cg24333473 in CpG island; cg14654875 in CpG shore), with confirmation of allelic methylation in kidney, placenta and cord blood derived DNA. Each circle represents a single CpG dinucleotide and the strand, a methylated cytosine (filled circle) or an unmethylated cytosine (open circle). The same region was analyzed in sperm-derived DNA. (**C**) The sequence traces show allelic expression for the *ZNF597* and *NAT15* genes.

brain and testis, which is equally derived from both parental alleles. The variants of *Znf597* were expressed in E9.5 whole embryo, yolk sac and placenta, and in individual tissues later in development. Allelic expression analysis revealed that these transcripts were not imprinted, with equal expression from both parental chromosomes (Fig. 3).

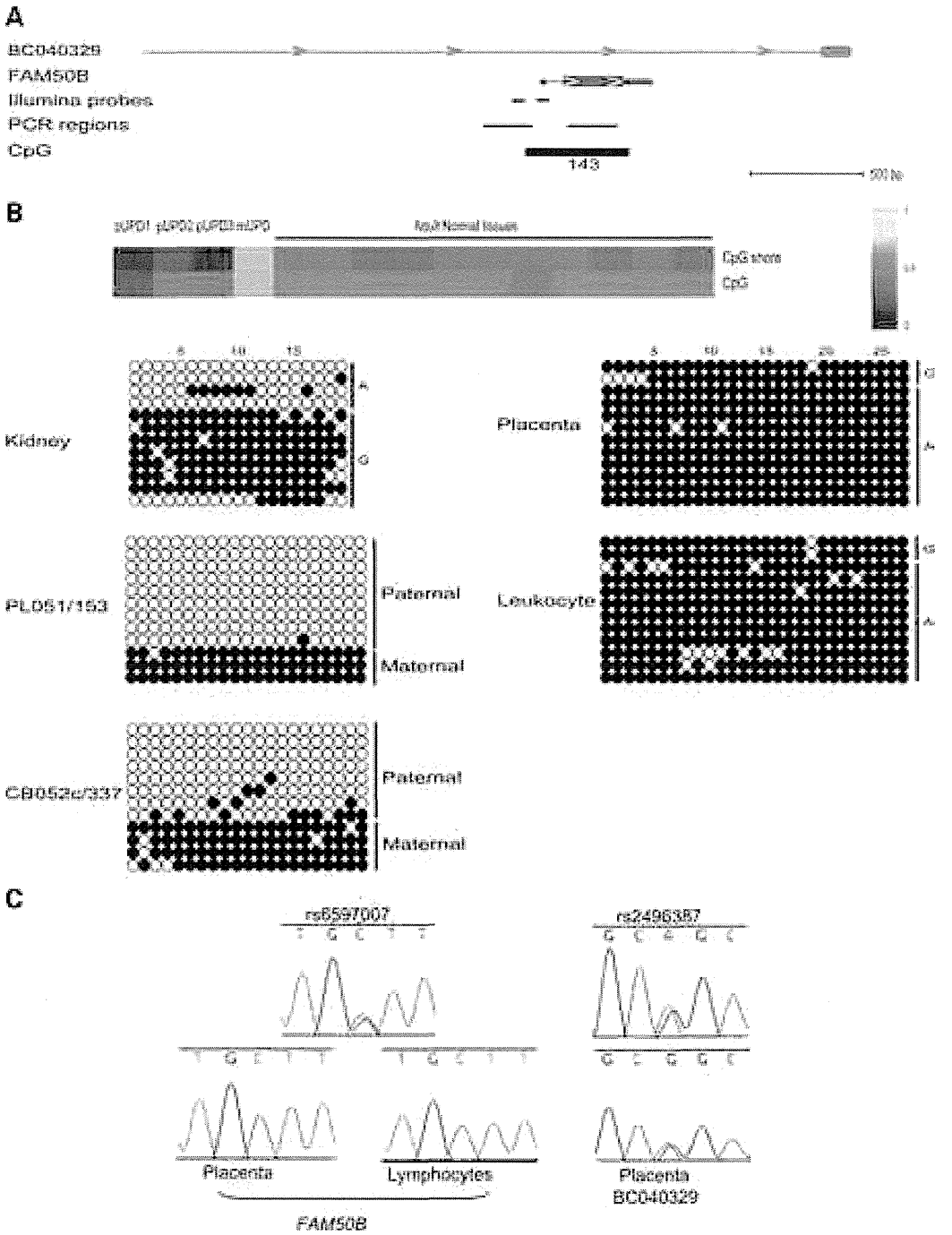

Figure 2. (**A**) A map of the *FAM50B/BC040329* locus, with the location of the of the CpG island (not to scale). (**B**) Heat map for CpG probes mapping to the *FAM50B* promoter (cg01570885; cg03202897) and the subsequent analysis of allelic methylation in various tissues. The methylation profiles on the left are from the 5′ CpG island region, while those on the right are from the 3′ region. (**C**) The allelic expression of *FAM50B* and the host gene in term placenta and leukocytes.

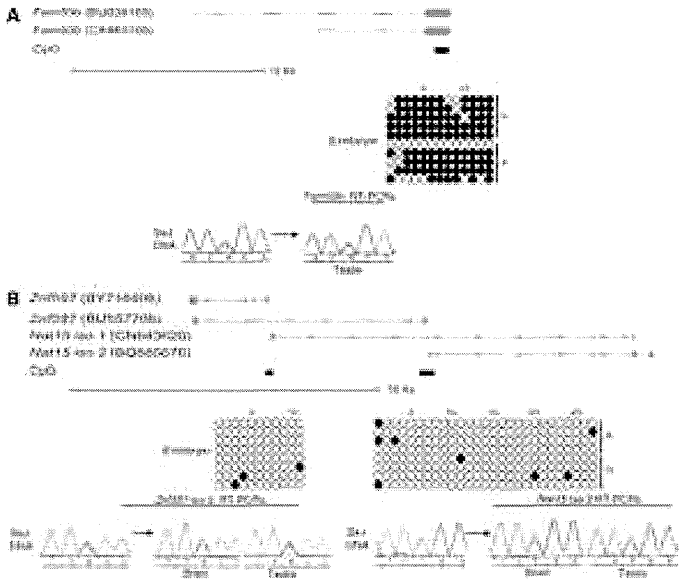

Figure 3. Schematic maps of the *Fam50b* (**A**) and *Znf597/Nat15* genes (**B**), with the location of the alternative promoter regions. The methylation status of the orthologous CpG islands associated with each domain was examined in embryo-derived DNA. The allelic expression of each gene in various mouse tissues from reciprocal mouse crosses. For clarity, only the expression in B6 × JF1 tissues is shown.

DISCUSSION

Identification of new human imprinted genes requires screening human samples

Most screens for new imprinted genes are undertaken in mouse with subsequent confirmation of the imprinting status of the human orthologues. Despite the success, this approach will not identify imprinted loci specifically imprinted in humans. To date, very few imprinted genes are human-specific, however, these rare transcripts do exist as highlighted by the paternally expressed *L3MBTL, C19MC* and *RB1* genes (22,29,30). Using DNA from Beckwith–Wiedemann and Silver–Russell-like phenotypes with reciprocal genome-wide UPDs, we have performed a comprehensive screen of ~0.1% of the human methylome. Despite the extensive coverage of Illumina Infinium Human Methylation27 BeadChip microarray, we identified very few novel imprinted loci. However, it must be noted that paternal germline DMRs are not associated with CpG islands, and therefore maybe remote from gene promoters and promoter CpG islands present on the array.

The predicted number of imprinted genes varies with estimates from 200–2000 transcripts in mouse, with one transcriptome-wide analysis, using the ultra sensitive RNA-seq technology, identifying over 1000 transcripts in brain with parent-of-origin expression bias (31). Recent studies have predicted and experimentally verified imprinted genes based on sequence and epigenetic characteristics. For example, human imprinted regions significantly lack short interspersed transposable elements in comparison with the rest of the genome and are associated with CpG islands (32,33). Using a bioinformatics approach, Luedi *et al.* (34) predicted 156 imprinted genes in humans based on similarity with known imprinted transcripts, confirming the maternal expression of *KCNK9*. In addition, the paternally expressed *MCTS2* gene was identified through a hypothesis-driven search for intronic X-chromosome-derived retrotransposons that are associated with CpG island promoters (35). Interestingly, *FAM50B* is also an imprinted X-chromosome-derived retrogene gene and was correctly identified by Luedi *et al.* (34) during their computational screening and the imprinting status recently confirmed (36).

We wished to identify additional imprinted loci based on data generated in previously published analyses. We have compared our hemimethylated data set against the 156 bio-informatically predicted imprinted genes and the 82 candidates predicted due to unequal representation of alleles in public EST libraries and expression genotype arrays (37,38). We found that fifteen out of one hundred and fifty-six and nine out of eighty-two, respectively, were present in our data set. However, none of these additional genes had a methylation profile consistent with an imprinted DMR, highlighting the high false-positive rates of bioinformatic predictions (Supplementary Material, Fig. S4). From our observations, we predict that the majority of human DMRs overlapping promoters have been identified. Following analysis of more than 14 000 genes, we identified only two new imprinted DMRs. Extrapolating this trend to the 34 702 annotated RefSeq genes, we predict that there will be around five additional unidentified DMRs in the human genome, resulting in a total of ~35.

Parent-of-origin DNA methylation is not the only epigenetic signature associated with imprinted DMRs (reviewed in 9). Recently, a chromatin signature has been shown to mark imprinted DMRs; with trimethylation of lysine 9 of histone

H3 (H3K9me3) and trimethylation of lysine 20 of histone H4 (H4K20me3) associated with the DNA methylated allele (39), while the unmethylated allele is enriched for the transcriptionally permissive Lysine 4 methylation of histone H3 (H3K4me2/3) (40). The combination of differential DNA methylation between sperm and somatic tissues and an overlapping H3K9me3 and H3K4me3 signature has recently been used to identify 11 new candidate DMRs in mouse (41). With the availability of human ChIP-seq derived genome-wide data sets for most histone modifications (42,43), it would be interesting to determine if this histone signature recognized in mouse can be used to identify novel human imprinted DMRs. Interrogation of the NHLBI ChIP-seq data set (http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcell.aspx) revealed that the *RB1*, *ZNF597* and *FAM50B* DMRs are enriched for both H3K4me3 and H3K9me3, with the later two regions harboring functional CTCF binding sites (data not shown).

The regulation of imprinted domains on human chromosomes 13 and 16

The *RB1* DMR has previously been proposed to contain the promoter of the paternally expressed *E2B-RB1* isoform (22). We were unable to identify coding SNPs within the *RB1* gene that would allow us to determine the allelic expression in our cohort of tissues. However, we were able to show that the *LPAR6* gene, encoding lysophosphatidic acid receptor 6 located in intron 16 of *RB1* is biallelically expressed, suggesting that the *RB1* DMR does not influence the expression of this gene (Supplementary Material, Fig. S3).

The maternal expression of *ZNF597* has previously been shown in leukocytes (28). Here, we show that the *ZNF597* DMR acts as a bidirectional silencer, which orchestrates the paternal silencing of *ZNF597* and *NAT15*. This organization is reminiscent of *PEG10-SGCE* domain on human 7p22 (44). We did not observe methylation in DNA isolated from mature sperm, which suggests that this region acquires methylation during early somatic development (Fig. 1). All known somatic DMR are associated with nearby germline DMRs, which regulate the methylation in a hierarchical fashion (23,45,46), implying a yet to be identified germline DMR is situated within the vicinity of the *ZNF597* gene.

The maternally expressed *NAT15* is a highly conserved protein coding gene with two alternative first exons, with only isoform I subject to imprinting. In addition, there is evidence from EST libraries of an ncRNA (genbank: DA387972) that originates from the *NAT15* isoform 1 promoter and continues past the exon–intron splice site to produce a ~550 bp transcript. Unfortunately, we were unable to detect expression of this transcript in our tissue set, so we could not determine if this ncRNA is imprinted.

FAM50B is an imprinted retrogene

Sequence analysis revealed that the *FAM50B* transcript (previously named *X5L*) is a retrotransposon that originated from *FAM50A/XAP5* within Xq28. Unlike other classical retrogenes, this gene has an intron in the 5′ UTR in both humans and mouse, which has no counterpart in its parental gene. It is likely that the intron was inserted after retroposition, possibly during recruitment of a functional promoter region (47). Interestingly, several other imprinted genes have been shown to originate from retrotransposition from the X-chromosome genes (35,48). *FAM50B* is ubiquitously expressed, and is inserted within the intron of a host transcript *BC040329*, which is predominantly expressed in testis with low detection in brain and placenta (data not shown). This host gene is biallelically expressed in placenta ($n = 7$) (Fig. 2), of which two samples exhibited imprinted expression of *FAM50B*.

Discrepancy between imprinted DMR methylation screens

The quantitative methylation values obtained using the Illumina Infinium platform makes it suitable for comparing reference and test samples. This approach has previously been used to screen for imprinted DMRs using paternally derived androgenetic complete hydatidiform moles versus maternally derived mature cystic ovarian teratomas and in patients with maternal hypomethylation syndrome (24,49). In both cases, the genetic material analyzed is not ideally suited for comprehensive screening for novel imprinted loci. This is because it is currently unknown to what extent the DNA methylation profile is altered in ovarian teratomas, and any differences maybe due to the uniparental nature of the sample or tumorgenic changes, and candidates obtained from comparisons with complete hydatidiform moles may simply reflect tissue-specific differences. This is highlighted by the fact that of the 95 candidate probes identified by Choufani *et al.* (49), 68 overlapped with our hemimethylated data set (Supplementary Material, Fig. S4) with only *ZNF597* DMR being identified in both screens. These authors also suggest that *AXL*-promoter region is a DMR, but this was not identified using our genome-wide UPDs, and bisulphite PCR and sequencing of our samples revealed a non-allelic mosaic methylation profile (Supplementary Material, Fig. S5). In addition, the methylation profiles obtained from comparing normal and maternal hypomethylation samples will only facilitate the identification of a subset of imprinted DMRs, since *ZFP57* mutations do not effect the maintenance of all maternally methylated imprinted DMRs equally (50,51).

Functional relevance of the new imprinted domains

Very little is known about the role of *FAM50B*, *ZNF597* and *NAT15*, with no previous publications describing functional studies. The three new imprinted regions we identify all map to chromosomes for which recurrent chromosomal UPDs have been reported. With the exception of pUPD and the overexpression of *PLAGL1/HYMAI* in Transient Neonatal Diabetes Mellitus, the UPDs for these chromosomes are not associated with obvious developmental phenotypes and most cases were identified because of the unmasking of mutant recessive alleles (reviewed in 52,53).

CONCLUSIONS

Our study has assisted in defining a comprehensive catalog of human imprinted genes. The use of extremely rare reciprocal