born after ART.

After analysing the second questionnaire, 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), correct the frequencies of DNA methylation errors (epimutations) 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 Figure 3, Table Ⅱ, Supplementary Table IV**). In 4 out of the 5 ART cases, DNA methylation errors were not restricted to the *H19* gDMR, and were present at both maternally and paternally methylated gDMRs. These 4 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 Table IV**), and no significant differences were found in methylation ratios between patients conceived by ART and natural conception.

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 7 BWS samplessamples with DNA methylation errors of the *LIT1* gDMR, one of which was derived from ART patient and 6 from natural conception (**Supplementary Figure 3, Table II, Supplementary Table IV**). In the one ART (ICSI) case, we identified 4 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 naturally conceived.

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 Table V**) 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 analysis 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). 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, 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 deletions or uniparental disomy (UPD), 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 of multiple imprinted loci in 17 patients out of 81 BWS cases with hypomethylation of *KCNQ1OT1*(*LIT1*) ICR, only one 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 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 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 epi-genotype 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.

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AUTHORS' ROLES

HH, HO, NM, FS and AS performed the DNA methylation analyses. MK, KN, HS collected the samples of the patients. KN did the statistical analyses. HH, MVDP, RMJ and TA wrote this manuscript. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Table Ⅰ. The 2009 frequency of four imprinting diseases in Japan in relation to use of assisted reproduction techniques (ART). 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 the frequencies of these diseases and the epimutation rates. BWS: Beckwith-Wiedemann syndrome, AS: Angelman syndrome, PWS: Prader-Willi syndrome and SRS: Silver-Russell syndrome.

Table Ⅱ. Abnormal methylation in patients with SRS and BWS. 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 for the methylation rates obtained using bisulfite-PCR sequencing. The % of DNA methylation of 22 gametic differentially methylated regions (gDMRs) in all patients with SRS and BWS examined are presented in Supplementary Table IV. Depictions in red represent DMRs normally exclusively methylated on the maternal allele, while blue represent paternally methylated sites.

Supplementary Figure 1. Genomic structures of 22 human gDMRs. (Upper part) Genomic maps of the paternally methylated gDMRs: *ZDBF2* (a), *H19* (b) and *GTL2(IG-DMR)* (c), the maternally methylated gDMRs: *DIRAS3* (d), *NAP1L5* (e), *FAM50B* (f), *ZAC* (g), *GRB10* (h), *PEG10* (i), *PEG1* (j), *INPP5Fv2* (k), *LIT1* (l), *RB1* (m), *SNRPN* (n), *ZNF597* (o), *ZNF331* (p), *PEG3* (q), *PSIMCT-1* (r), *NNAT* (s),

L3MBTL (t), *NESPAS* (u) and *GNAS1A* (v). Non-imprinted regions: *LINE-1* (w) and *Alu* (x). The extent of the regions analyzed in this study and the Genbank accession numbers are shown in parentheses. Filled boxes and horizontal arrows indicate the genes and orientations. Open boxes represent the gDMRs. The horizontal arrows indicate the primers. Vertical arrows indicate unique polymorphisms. (Lower part) DNA methylation analyses by bisulfite-PCR sequencing within the SNPs of genomic DNA prepared from normal blood and sperm. Each row represents a unique methylation profile with an average of 20 clones sequenced for each sample. Samples were heterozygous for polymorphism allowing differentiation between the two parental alleles. Closed and open circles represent methylated and unmethylated CpGs, respectively. In *LINE-1* and *Alu* cases, 'T' represents TpG sites that were either unmethylated or mutated. 'A' represents TpA sites that were mutated. 'X' represents other mutations.

Supplementary Figure 2. The numbers and age distributions of patients with the four imprinting diseases. We conducted a nationwide investigation during 2009 to determine the frequency of the four imprinting diseases in Japan. Age distributions of 97 patients with BWS (A), 137 patients with AS (B), 279 patients with PWS (C) and 53 patients with SRS (D).

Supplementary Figure 3. DNA methylation analyses of the gDMRs in patients with SRS and BWS conceived by ART. Bisulfite-PCR sequencing results for gDMR regions spanning DNA polymorphisms from blood genomic DNA of patients with SRS (1-5) and BWS (1). Methylation profiles of all patients with SRS and BWS originating from ART are summarized in Table II and Supplementary Table IV. Multiple abnormal DNA methylation patterns are shown for patients SRS-1,3,4,5 and BWS-1.Mosaic methylation patterns are shown for patients SRS-1,2,3,5 and BWS-1. Dotted lines highlight mosaic pattern, where expected methylation was not observed.

Supplementary Table Ⅰ. Sequences of PCR primers and conditions used for bisulfite-PCR methylation analysis with a SNP.

Supplementary Table II. Results of the first postal survey and the number of four imprinting disorders.

Supplementary Table III. Results of the second postal survey and the number of four imprinting disorders.

Supplementary Table IV. Bisulfite PCR sequence methylation profiles of the 22 gDMRs and 2 non-imprinted regions in the DNA of 15 SRS and 7 BWS patients.
% values indicate average methylation values at the gDMRs obtained using bisulfite PCR sequencing methylation analysis.

Supplementary Table V. Clinical characteristics of patients with SRS and BWS. Differences in the methylation patterns between patients who were conceived after ART and naturally were not significantly reflected in their clinical features and severity. Circle (○) confirms positive diagnosis of symptom, while dash (-) confirms negative. (X) is used where no diagnosis was made. Clinical features were as follows, 1. Body asymmetry 2. Failure to thrive 3. Sweating 4. Short stature 5. Mental retardation 6. Hydrocephalus 7. Unusual dermatoglyphics 8. Ptosis 9. Clinodactyly of the fifth fingers 10. Difficulty in hearing 11. Renal hypoplasia 12. Triangular shaped face 13. Hypoglycemia 14. Diabetes 15. Heart malformation 16. Gastrointestinal injury 17. Hypertension 18. Earlobe creases 19. Ocular hypertelorism 20. Occlusal interference 21. Macroglossia 22. Microcephaly 23. Bulging eyes 24. Exophalos 25. Umbilical hernia 26. Hemihypertrophy 27. Splenomegaly 28. Malrotation of intestine 29. Nephromegaly 30. Diaphragmatic hernia 31. Adrenomegaly 32. Cardiomegaly 33. Urinary malformation 34. Hepatomegaly 35. Cryptorchism.