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DNA methylation errors at imprinted loci after assisted conception originate in the parental sperm

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There is an increased prevalence of imprinting disorders, such as Beckwith–Wiedemann syndrome, associated with human assisted reproductive technologies (ART). Work on animal models suggests that *in vitro* **culture may be the source of these imprinting errors. However, in this study we report that, in some cases, the errors are inherited from the father. We analyzed DNA methylation at seven autosomal imprinted loci and the** *XIST* **locus in 78 paired DNA samples. In seven out of seventeen cases where there was abnormal DNA methylation in the ART sample (41%), the identical alterations were present in the parental sperm. Furthermore, we also identified DNA sequence variations in the gene encoding** *DNMT3L*, **which were associated with the abnormal paternal DNA methylation. Both the imprinting errors and the DNA sequence variants were more prevalent in patients with oligospermia. Our data suggest that the increase in the incidence of imprinting disorders in individuals born by ART may be due, in some cases, to the use of sperm with intrinsic imprinting mutations.**
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Introduction

Recent studies have identified an increased incidence of the normally rare imprinting disorders, Beckwith–Wiedemann syndrome (BWS; NIM130650) and Angelman syndrome (AS; NIM105830), in babies conceived after assisted conceptions.[1–3] Epigenetic marks acquired in the germ line drive the allele-specific expression of specific 'imprinted' genes in the embryo.[4–6] One of the best studied epigenetic marks is DNA methylation which is found associated with only one parental allele within discrete locations

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known as differentially methylated regions (DMRs).[4] The identification of epigenetic changes at imprinted loci in assisted reproductive technology (ART) infants has led to the suggestion that the technique itself may predispose embryos to acquire imprinting errors.[1–3] Both *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are associated with the increased risk of imprinting disorders and it is not clear at what point these imprinting errors arise.[7,8]

Major epigenetic events take place during both germ cell development and the preimplantation stages of embryonic development.[9] *In vitro* culture may expose the genome to environmental factors that prevent the proper establishment of the imprint. However, the risks cannot easily be evaluated for ART treatment because patients who receive ART treatment may differ both demographically and genetically from the general population.

In mice, the *de novo* DNA methyltransferase *Dnmt3a* and the accessory protein DNA methyltransferase 3-like protein (Dnmt3L) are key regulators of DNA methylation co-operating to *de novo* methylate DNA in the germ line with *Dnmt3L* functioning to activate the DNA methyltransferase and recognize the target sequence based on nucleosome modification and CpG spacing.[10–17] Female mice that lack either *Dnmt3a* or *Dnmt3L* are fertile but their heterozygous progeny lacks the maternal imprint and the mice die before mid-gestation. Male mice that lack these methylases are infertile and oligospermic.[12]

We, and others, previously demonstrated that methylation errors are present at imprinted loci in the sperm of infertile men, particularly those with oligospermia.[18,19] In this new study, we examined the methylation profile of seven autosomal imprinted genes (*H19*, *GTL2*, *PEG1*, *KCNQ1OT1*, *ZAC*, *PEG3* and *SNRPN*) and the X-linked *XIST* gene in DNA obtained from ART conceptuses and matched parental sperm to determine whether these alterations were inherited.

Materials and methods
Conceptus sample collection
A total of 78 aborted samples, ranging from 6 to 9 weeks gestation (mean 7 weeks and 3 days) were obtained after treatment by ART (IVF and ICSI) after ethics committee approval and written informed consent. Trophoblastic villi were dissected away from decidual tissues and washed repeatedly with the saline solution to avoid maternal contamination. A total of 38 non-ART aborted conceptus samples, ranging from 6 to 8 weeks gestation (mean 7 weeks and 5 days) were used as controls. Parental sperm and genomic DNA were prepared as described previously.[19]

Bisulfite treatment PCR
Methylation assays were performed using combined bisulfite PCR restriction analysis (COBRA) and bisulfite sequencing.[19] Seven autosomal imprinted genes (*H19*, *GTL2*, *PEG1*, *KCNQ1OT1*, *ZAC*, *PEG3* and *SNRPN*), the *XIST* gene and the repetitive sequences (long interspersed nucleotide elements (*LINE1*) and *Alu*) were analyzed as described previously.[19,20] Each DNA sample was treated with sodium bisulfite using the EZ methylation kit (Zymo Research, Orange, CA, USA) and amplified by PCR. Restriction enzymes were as follows: *H19*, *Taq*I; *GTL2*, *Taq*I; *PEG1*, *Sau*3AI; *KCNQ1OT1*, *Rsa*I; *ZAC*, *Rsa*I; *PEG3*, *Hin*fI; *SNRPN*, *Bsi*EI; *XIST*, *Tai*I; *Alu*, *Mbo*I and *LINE1*, *Hin*fI. Digested samples were resolved on 10% polyacrylamide gels and/or 3% agarose gels. Ethidium bromide-stained bands were quantified with a VersaDoc 5000 and Quantity One software (Bio-Rad, Hercules, CA, USA) and percentage of methylated restriction enzyme sites in each genomic sample was calculated from the ratio between the enzyme-cleaved PCR product and the total amount of PCR product.

PCR products were purified, cloned into pGEM-T (Promega, Madison, WI, USA) and an average of 20 clones per individual sequenced using M13 reverse primer and an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster city, CA, USA). Sodium modification treatments were carried out in duplicate for each DNA sample and at least three independent amplification experiments were performed for each individual examined.

Karyotyping of the chromosomes
Chromosomal compliment was determined by FISH using multicolored and chromosome-specific probes. Cases of chromosomal number abnormalities, deletion and duplication were omitted. XY karyotypes were determined by the DNA amplification for X- (*DMD* gene) and Y- (*SRY* gene) chromosome-specific sequences as described.[21]

Histological examinations
Histological examination (hematoxylin and eosin) was performed to confirm the diagnosis of the abortion except in the case of trophoblastic neoplasia.

Mutation analyses
Mutation analyses of *DNMT3A* and *DNMT3L* were performed using the DNA obtained from the male patient's blood. Differences in migration were initially identified by PCR and single-strand conformation polymorphism (SSCP) analyses.[22] Primers used for the amplification and sequencing of *DNMT3A* and *DNMT3L* are shown in Supplementary Table. Sequence alterations were identified by direct cycle sequencing using BigDye fluorescent terminators and the ABI sequencer (Applied Biosystems).

Results
Aberrant paternal imprint methylation in the ART-treated conceptus
We first examined the methylation status of seven imprinted loci and the *XIST* DMR in DNA samples prepared from 78 XY karyotyped ART conceptuses and 38 non-ART conceptuses using COBRA (Figure 1a and b). In women, the *XIST* DMR is DNA methylated on one X chromosome whereas the other chromosome is unmethylated. In men, who have only one X chromosome, the single locus is normally methylated. Abnormal methylation was taken as being over the range of ± 2SD (standard deviations) of the mean of the methylation in the normal fertilization products. Among the 78 ART-treated cases, we found 8 cases of aberrant paternal methylation (*H19*, 6 cases; *GTL2*, 2 cases; Table 1). We confirmed the results of the COBRA assay by sequencing (Figure 2). We examined global DNA methylation of the non-imprinted repetitive elements *Alu* and *LINE1*. There were no detectable differences between ART and normal samples (Table 1; Figure 1c). Loss of DNA methylation was specific to the imprinted loci.
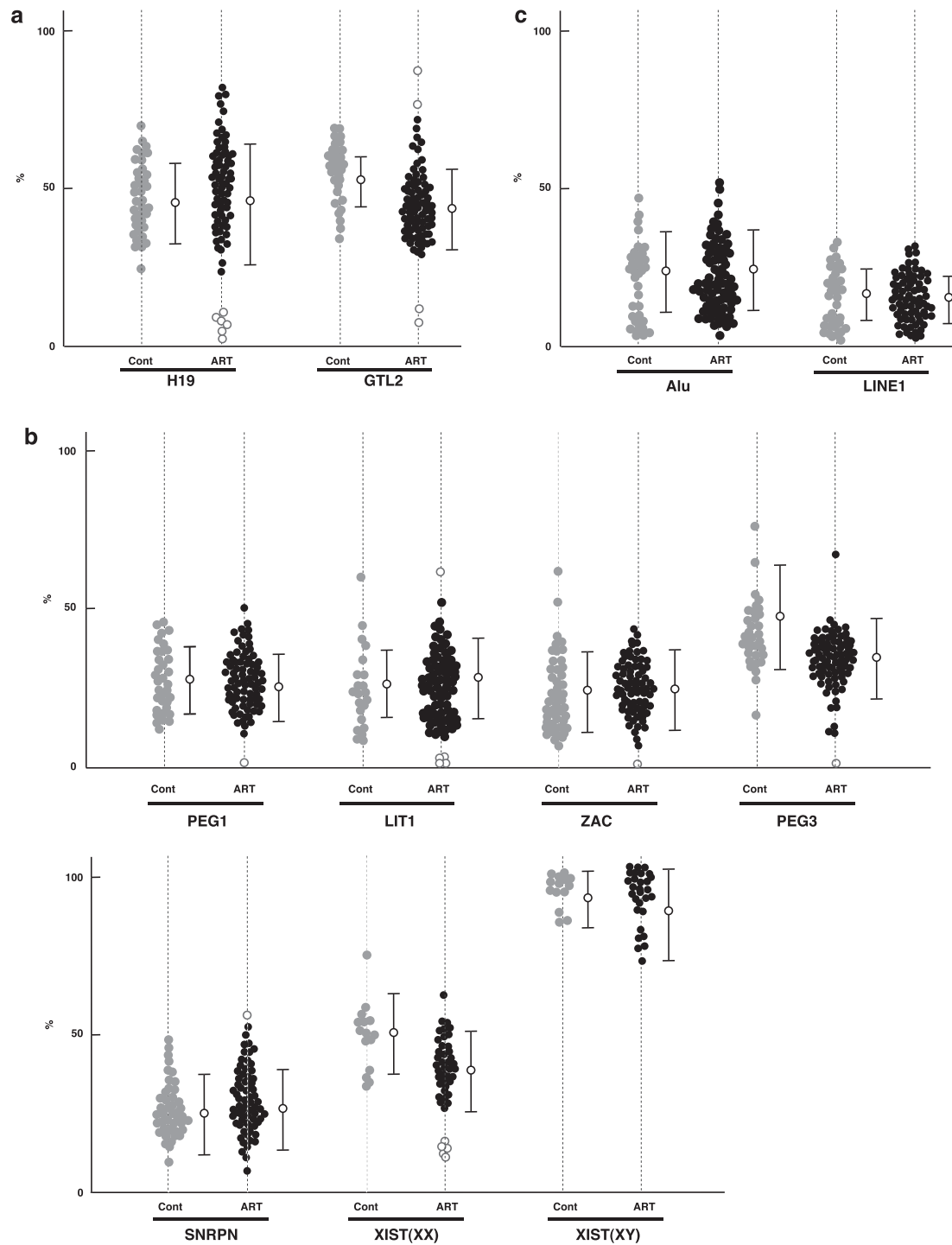
Figure 1 Methylation profile of natural and ART conceptus samples at imprinted and non-imprinted loci. Aberrant methylation over the range of ± 2SD (standard deviations) of the mean of the normal fertilization methylation profile is indicated by red circles. White circles and bar indicate the mean and SD. Paternally methylated loci, *H19* and *GTL2* (**a**); maternally methylated loci, *PEG1*, *KCNQ1OT1(LIT1)*, *ZAC*, *PEG3*, *SNRPN*, *XIST* of XX karyotype (non-ART conceptus samples, *n* = 19; ART, *n* = 46) and *XIST* of XY karyotype (non-ART conceptus samples, *n* = 19; ART, *n* = 32) (**b**), non-imprinted repetitive elements, *Alu* and *LINE1* (**c**).

Table 1 Abnormal methylation profiles detected by bisulfite PCR sequence in sperm and ART conceptus DNA

Case (age)	ART treatment	Paternally methylated DMR				Maternally methylated DMR					Repetitive sequences	
		H19	GTL2	PEG1	LIT1	ZAC	PEG3	SNRPN	XIST(XX)	XIST(XY)	Alu	LINE1
13 (28)	IVF (Cryo)	39.4	34.6	16.7	29.3	24.3	34.7	34.7	**19.6**		45.1	29.0
		(83.1)	(98.6)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)				(23.7)
23 (33)[a]	ICSI	**4.0**	55.8	**1.1**	28.1	32.4	30.6	30.6		82.8	24.1	14.5
		(9.8)	(99.2)	(0.0)	(0.0)	(2.0)	(0.0)	(0.0)			(22.8)	(24.4)
28 (39)[a,b]	ICSI	**6.5**	**18.3**	19.4	22.6	25.4	36.8	24.5	43.6		26.2	11.2
		(16.7)	(13.5)	(23.0)	(0.0)	(0.0)	(0.0)	(0.0)			(23.1)	(18.9)
44 (36)[c]	ICSI (Cryo)	**12.8**	48.9	31.4	35.4	33.6	37.8	27.8		82.4	28.5	22.6
		(9.8)	(99.8)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)			(18.9)	(19.2)
50 (43)[b,c]	ICSI	**19.7**	37.2	26.0	28.7	29.7	38.4	28.4		78.9	12.9	21.0
		(33.3)	(93.1)	(0.0)	**(12.0)**	(0.0)	(0.0)	(0.0)			(20.1)	(25.4)
52 (41)[c]	ICSI	**23.5**	37.3	23.0	25.1	41.3	46.2	41.3		82.0	14.7	12.0
		(17.6)	(96.6)	(1.2)	(0.0)	(0.0)	(0.0)	(0.0)			(30.3)	(22.4)
53 (34)[c]	ICSI	57.6	33.5	29.8	24.9	**1.0**	42.6	32.6	51.3		19.5	21.8
		(76.6)	(95.8)	0.0	0.0	0.0	0.0	(5.6)			(10.4)	(24.4)
57 (42)[a]	ICSI	41.4	**16.9**	31.8	28.1	34.9	**1.1**	25.4	39.4		17.2	18.8
		(95.5)	(21.6)	0.0	(3.8)	0.0	0.0	0.0			(19.2)	(25.4)
61(33)	IVF	30.5	41.4	22.8	20.9	24.0	34.5	34.5	**18.3**		28.4	11.1
		(47.6)	(99.0)	0.0	0.0	0.0	0.0	0.0			(23.1)	(25.1)
67 (36)[c]	IVF	**10.3**	37.7	31.2	26.7	31.1	41.5	28.4		84.8	19.3	16.6
		(11.8)	(93.5)	0.0	0.0	0.0	0.0	0.0			(30.3)	(21.4)
70 (36)[c]	IVF	27.6	37.2	28.7	**3.0**	21.8	35.2	51.5	51.3		13.4	11.7
		(98.6)	**(81.6)**	0.0	0.0	0.0	0.0	0.0			(30.6)	(29.2)
30 (32)[d]	Spontaneous	68.9	48.9	25.3	**6.5**	29.5	33.9	26.9	45.9		19.5	22.8
46 (34)[d]	Spontaneous	44.1	34.4	26.1	**1.9**	28.1	41.3	41.3		83.8	14.7	21.8
62 (30)[d]	AIH	56.7	50.4	27.6	**2.3**	24.1	43.2	23.4		81.7	19.3	25.1
32 (27)[d]	Spontaneous	45.7	38.7	25.4	20.9	24.0	34.5	25.5	**17.7**		20.6	21.2
40 (33)[a,d]	AIH	68.2	34.7	25.1	26.6	22.8	41.6	21.9	**19.0**		26.2	21.2
63 (34)[c,d]	AIH	56.4	36.5	28.8	24.9	20.3	44.6	34.6	**20.6**		19.5	22.3

Abbreviations: AIH, artificial insemination by husband; ART, assisted reproductive technology; DMR, differentially methylated region; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization.
Each value is the percentage methylation determined by bisulfite PCR sequencing analyses. Bold text highlights the ART-treated conceptuses where mean values are abnormal. The percentage methylation in the parental sperm DNA is shown in parentheses below the value for the conceptus.
[a]Moderate oligospermia (sperm count ⩾5 to 20 × 10^6 per ml).
[b]In case 28, the nucleotide at position 518 (AAF05812) in exon 2 of *DNMT3L* was changed from a G to an A on both alleles of the gene. This is predicted to result in a substitution of Glu to Lys. In case 50, nucleotide 787 of exon 5 of *DNMT3L* was changed from a C to a T (Table 2).
[c]Severe oligospermia (sperm count < 5 × 10^6 per ml).
[d]Not tested in the sperm DNA.

Aberrant maternal imprint methylation in the ART-treated conceptus

We found 12 cases of aberrant maternal methylation in the ART samples (*PEG1*, 1 case; *KCNQ1OT1*, 4 cases; *ZAC*, 1 case; *PEG3*, 1 case; *XIST*, 5 cases; Table 1). The methylation errors were predominantly found at the *H19* and *KCNQ1OT1* DMRs, which commonly show alterations in DNA methylation in BWS patients. Five cases with an XX female karyotype were found with a methylation defect at the *XIST* locus but not at the autosomal DMRs. No samples showed complete imprint erasure.

In total, we identified 17 fetal samples with imprint methylation errors. Among them, a completely unmethylated pattern at a single locus was found in 14 cases and a mosaic pattern of aberrant methylation was identified at more than one loci in 3 cases.

Seven cases (21.8%) were the products of fertilization by ICSI, four cases (26.6%) were the products of IVF, two cases (11.8%) were involved a cryopreserved embryo and three cases (13.0%) were involved artificial insemination with husband's sperm (AIH). We also identified errors in three samples from our control group. We identified methylation errors in both the ICSI/IVF ($n = 39$) and non-ICSI/IVF ($n = 39$) groups indicating that ICSI was not responsible for these changes. There was no significant difference in the maternal ages between the ICSI/IVF and non-ICSI/IVF groups; 34.3 ± 4.3 and 35.0 ± 3.7 years old, respectively.

Relationship between aberrant methylation in sperm and conceptus after ART

We compared the quality of the parental sperm sample in the 17 cases where we identified imprinting errors. Seven samples were severely oligospermic (50.0%), four showed moderate oligospermia (25.0%) and six appeared normal (8.6%). There was an increased frequency in visible sperm abnormalities in the father associated with imprint errors in the conceptuses in line with our previous findings.[19]
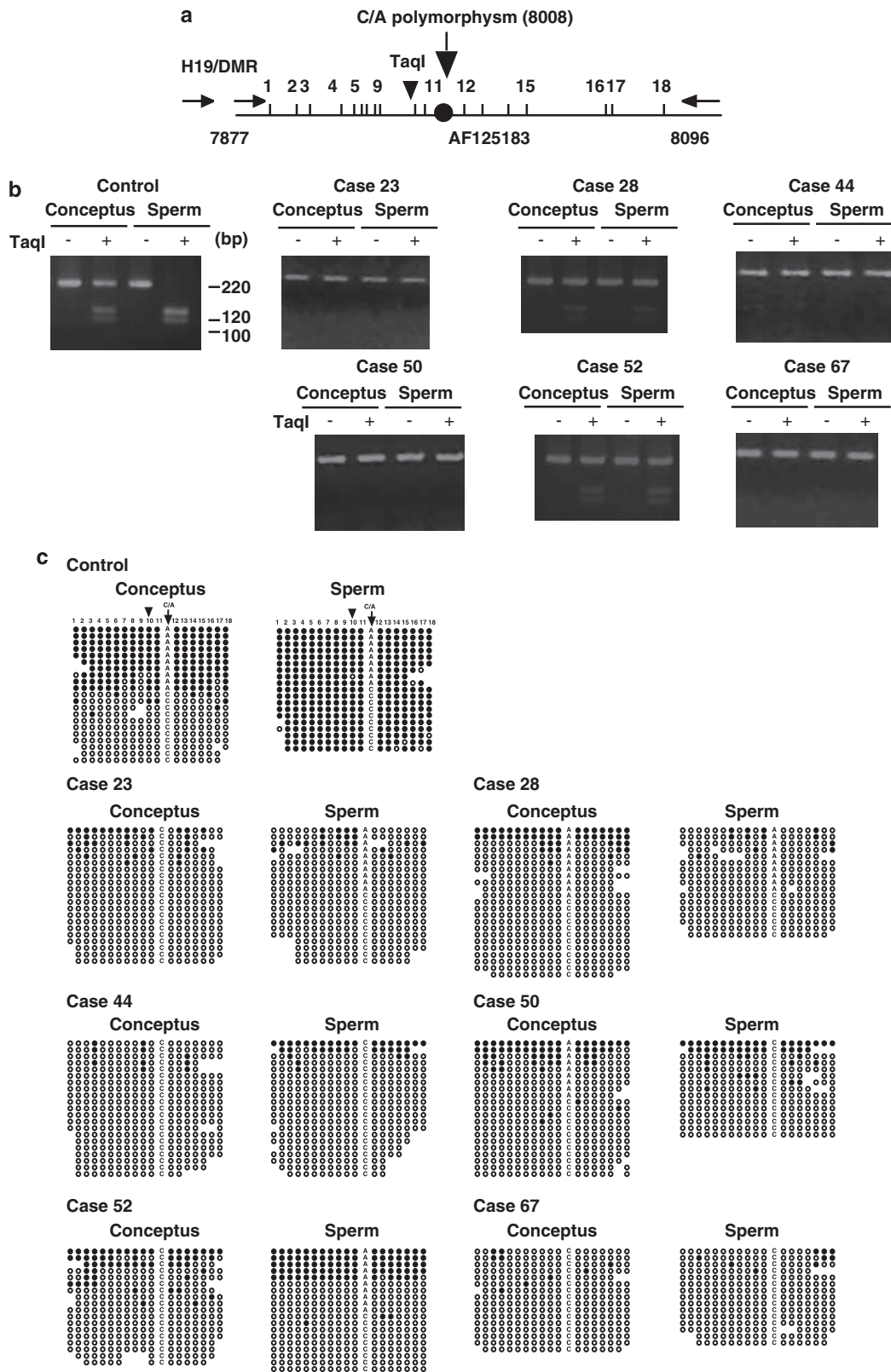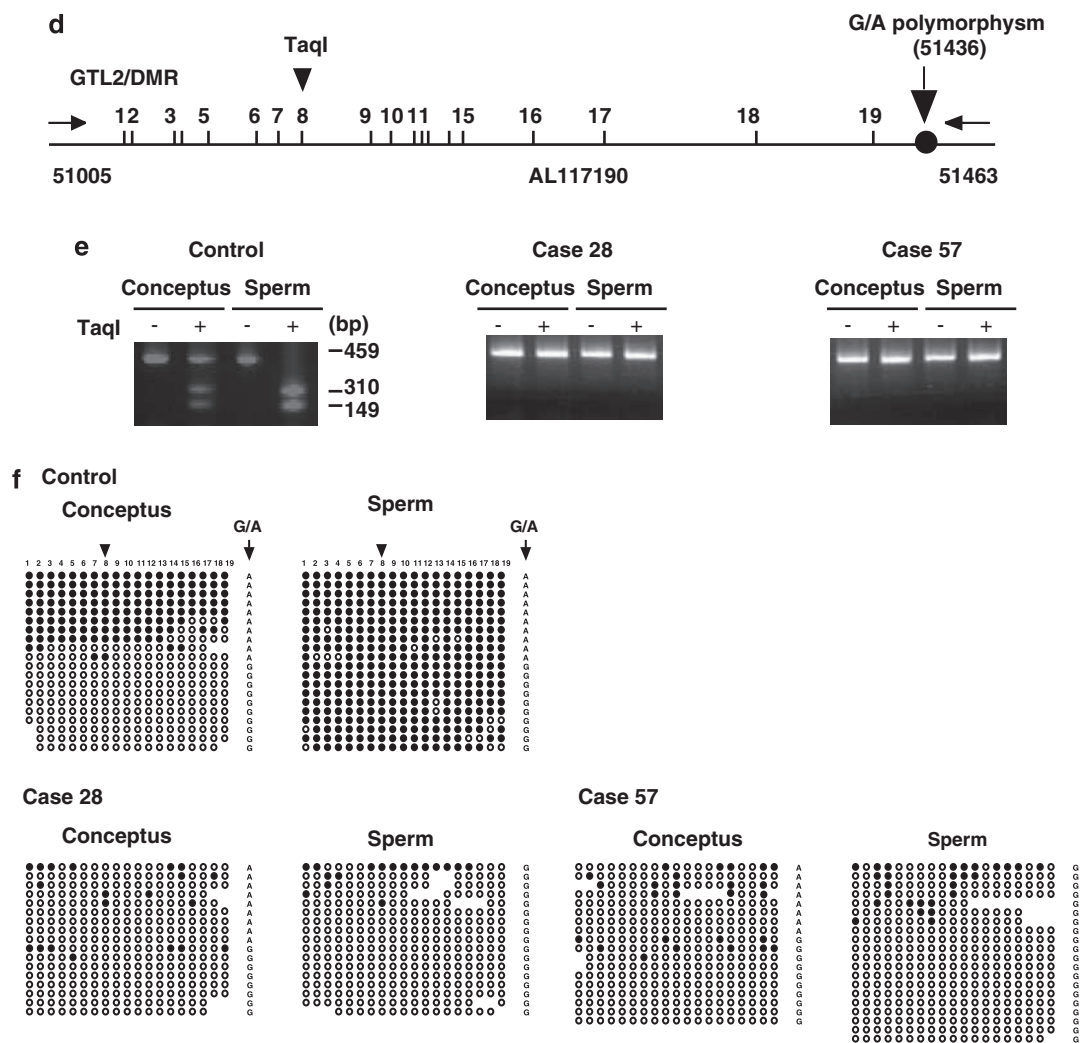
Figure 2 Continued.

Figure 2 DNA methylation analyses in sperm and ART-treated conceptuses revealed abnormal methylation at *H19* and *GTL2* DMRs. Genomic structure of the human DMRs of *H19* (**a**). The extent of the region analyzed in this study and GenBank accession number are shown under the line. The **horizontal arrows** represent primer positions. **Vertical arrowhead** indicates the unique bisulfite PCR restriction enzyme site analyzed by *Taq*I. Arrow shows the unique C/A polymorphism at nucleotide 8008 in the amplified region of the *H19* DMR. The vertical bars represent CpG sites. DNA methylation analyses by COBRA (**b**) and bisulfite-PCR sequencing (**c**) of genomic DNA prepared from the sperm and ART conceptus samples fertilized from the same sperm shown to have an abnormal pattern of DNA methylation at the human DMR of *H19*. Conceptus sample, DNA obtained from ART conceptus; sperm, sperm DNA from the male patient. In the COBRA assay, bisulfite-treated DNA was amplified by PCR and digested with restriction enzymes *Taq*I only cuts the PCR product if the site was originally methylated in the genome. In the bisulfite-PCR sequencing of ART conceptus and sperm DNA at *H19* (cases 23, 28, 44, 50, 52 and 67), each row represents a unique methylation profile with an average of 20 clones sequenced for each sample. Closed and open circles represent methylated and unmethylated CpGs, respectively. Samples were heterozygous for a C/A polymorphism allowing differentiation between the two parental alleles. The results are summarized in Table 1. Genomic structure of human DMR of *GTL2* (**d**). Vertical arrowhead indicates the unique bisulfite PCR restriction enzyme site analyzed by *Taq*I and arrow shows the unique G/A polymorphism at nucleotide 51436 in the amplified region of the *GTL2* DMR. Like *H19*, DNA methylation analysis was performed for the human DMR of *GTL2* by COBRA (**e**) and bisulfite-PCR sequencing (**f**) of genomic DNA (cases 28 and 57) was prepared from the ART conceptus shown to have an abnormal pattern and the matched parental sperm.

Using DNA polymorphisms at the *H19* and *GTL2* loci, we found seven cases where the precise DNA methylation error was present in the father sperm and the conceptuses (Table 1; Figure 2), suggesting that the methylation errors were preexisting and not a consequence of the ART. We also identified four sperm samples (cases 13, 50, 53 and 70)

that showed abnormal DNA methylation that was not present in the conceptus.

Mutation analyses of *DNMT3A* and *DNMT3L*
The consequences of *Dnmt3a* and *Dnmt3L* deficiency in mice are loss of imprints and oligospermia, so we next

Table 2 Summary of the *DNMT3L* and *DNMT3A* sequence alterations identified in infertile men with abnormal imprint methylation in the sperm

DNMT3L alterations	Genotype	Case	Abnormal imprinted methylation	Sperm analyses
Exon 2 $G^{518} \rightarrow A$ ($Glu^{12} \rightarrow Lys$)	A/A	28[a]	H19, GTL2	Moderate
	G/A	P 48	—	Severe
		P 78	—	Normal
		P 80	—	Normal
Exon 10 $A^{1316} \rightarrow G$ ($Arg^{278} \rightarrow Gly$)	G/G	P 62	GTL2, PEG1, PEG3,	Severe
		P 63	H19, GTL2, PEG1, LIT1	Severe
	G/A	P 49	GTL2	Normal
		P 52	LIT1	Normal
		P 85	PEG1	Moderate
		P 90	SNRPN	Normal
		P 12	—	Normal
		P 33	—	Moderate
		P 45	—	Moderate
		P 61	—	Normal
		P 78	—	Normal
		P 93	—	Normal
		P 97	—	Normal
		P 98	—	Normal
Exon 5 $C^{787} \rightarrow T$	T/T	*50	H19	Severe
		P 30	GTL2, PEG1	Normal
		P 59	PEG1, LIT1	Normal
		P 62	GTL2, PEG1, PEG3	Severe
		P 67	H19, PEG1	Severe
		P 82	GTL2, ZAC	Severe
	C/T	P 46	—	Normal
		P 74	—	Normal
		P 92	—	Normal
Exon 6 $C^{937} \rightarrow T$	T/T	P 79	PEG3	Normal
	C/T	P 52	LIT1	Normal
		P 54	GTL2, PEG1, PEG3	Normal
		P 90	SNRPN	Normal
		P 46	—	Normal
		P 55	—	Moderate
		P 65	—	Normal
		P 76	—	Normal
		P 78	—	Normal
		P 91	—	Normal
		P 97	—	Moderate
		P 83	—	Severe
DNMT3A alterations				
Exon 7 $A^{971} \rightarrow G$ ($Asp^{212} \rightarrow Gly$)	A/G	P 20	PEG1	Normal
Exon 17 $G^{2193} \rightarrow T$ ($Arg^{659} \rightarrow Leu$)	T/T	P 8	PEG1	Normal
	A/A	P 83	GTL2, ZAC, SNRPN	Severe
		P 87	PEG3	Normal
Exon 10 $G^{1483} \rightarrow A$		P 9	—	Normal
		P 18	—	Normal
		P 74	—	Normal

Nucleotide numbering refers to *DNMT3L* and *DNMT3A* cDNA sequences AF194032 and AF331856, respectively.
[a]Male patients from this paper with an abnormal imprint in both the conceptus and the sperm. Others samples are previously reported male patients (14).

examined 136 infertile men for gene mutations using SSCP and DNA sequencing. This group included the 7 male patients with an abnormal imprint in both the conceptuses and the sperm, 24 patients for which we previously reported an abnormal sperm imprint[19] and an additional 105 patients who had undergone some fertility treatment where we had not identified a difference in DNA methylation. Of these, 27 had severe oligospermia, 30 had moderate oligospermia and 79 appeared normal.

We identified two sequence differences in *DNMT3L* that were predicted to alter the amino-acid sequence, one within exon 2 and one within the methylation enzyme activation motif IV in exon 10 (Table 2). The individual homozygous for the exon 2 polymorphism (^{518}G/G) had DNA methylation errors at *H19* and *GTL2* and moderate oligospermia. Three heterozygous individuals (^{518}G/A) showed normal methylation although one had severe oligospermia. Two individuals homozygous for the exon

10 alteration (^{1316}G/G) had aberrant methylation of paternal and maternal imprinted loci and severe oligospermia. Of the twelve individuals that were heterozygous for this polymorphism (^{1316}G/A), four showed abnormal methylation whereas eight appeared normal. They all had normal sperm or only moderate oligospermia.

We also identified sequence differences that were predicted to result in nonsense mutations. One was a ^{787}C to T conversion in exon 5 located in the PHD domain. Six individuals homozygous for this alteration had abnormal methylation at some paternal and maternal loci and four also had severe oligospermia. The three heterozygous individuals had normal-appearing sperm and no detectable methylation errors. One individual homozygous for a nonsense variant (^{937}C–T) in exon 6, also located in a PHD domain, had abnormal methylation at PEG3 in his sperm and three of the eleven individuals heterozygous for this sequence alteration had abnormally methylated sperm but none had oligospermia.

Of the seven male patients with an abnormal imprint in both the ART conceptuses and the sperm, two cases had variations in DNMT3L (case 50, ^{787}C/C in exon 5 and case 28, ^{518}G/G in exon 2), which could suggest that these variants affect the function of DNMT3L.

We identified three types of DNMT3A sequence alterations but these did not correlate with either an abnormal imprint or abnormalities in the number of sperm (Table 2).

In conclusion, we report seven cases where imprinting mutations in ART conceptuses match those present in the parental sperm and in two of these cases, the father also carried an alteration in the sequence of the DNMT3L gene. These findings suggest that the increased prevalence of imprinting disorders linked to ART does not originate solely through the process of ART but, in a significant number of cases, these alterations in DNA methylation preexist in the father's germ line. We may be selecting these cases because they are also linked with a low sperm count.

Discussion

Although it is clear that the *in vitro* culture of gametes can result in the altered regulation of imprinted genes, in this study we have identified another source of imprinting errors in ART, direct inheritance from the father's sperm. We found 17 of 78 ART samples (21.8%) that showed abnormal DNA methylation at one or more than one imprinted loci. Although some of the imprinting errors we identified may have occurred during the process of ART, we identified seven cases where the same imprinting errors were present in the parental sperm and the matched ART sample, suggesting that abnormal hypomethylation at H19 and GTL2 was transmitted directly from the father's sperm. Loss of a paternal methylation imprint cannot account for

an increased prevalence of BWS or AS in ART children as these arise from loss of the maternal imprint but could account for the increased frequency of syndromes such as SRS.[23–25] Given the known role of imprinted genes in regulating embryonic and extraembryonic growth (MRC Mammalian Genetics Unit, Harwell UK, URL: http://www.mgu.har.mrc.ac.uk/research/imprinting/function.html), the increased frequency of low birth weight in ART infants may also be due to alterations at imprinted loci although such changes have not, as yet, been reported.[26] Other obstetric complications are frequently linked to ART such as miscarriage, placental dysfunction, premature labor, placenta previa and maternal hypertension.[27–31] These may also reflect accumulated small changes in DNA methylation at imprinted loci.

We identified some samples that showed both paternal and maternal methylation errors. This could suggest a more wide-ranging defect in recognizing or maintaining appropriate methylation in the early conceptus. In this respect, our observation that there are alterations in the DNMT3L gene linked to infertility and alteration in DNA methylation is intriguing. The existence of heterozygous cases and the fact that we only observe the partial loss of imprints could suggest haploinsufficiency or a dominant-negative function either in the developing sperm and/or in the early embryo. Alternatively, the sequence alterations in DNMT3L result in an altered specificity rather than loss of function, which could account for the hypomethylation of two paternal DMRs and gain of a maternal DMR methylation that we observed in sperm DNA of patient 28. In addition, two ART fetuses (cases 23 and 57) showed hypomethylation of both a paternal and a maternal DMR, which might also suggest a problem with the identification of the target sequence. It will clearly be important to test this hypothesis by asking whether the sequence alterations we have identified result in aberrant function *in vitro*.

Imprint-specific methylation errors and their plasticity

In all cases, the global methylation status of our samples was not detectably altered suggesting that the DNA methylation marks at imprinted loci are more readily altered. There also appeared to be differences in the vulnerability of different DMRs to alteration. It has been shown that the short tandem repeat elements within the DMRs of some imprinted genes are important for imprinting.[32] The degree to which imprinted genes are repeat-like may be a factor in determining their methylation status and we are extending our current work to other DNA methylated loci to further investigate this possibility.

In a few cases, the methylation abnormalities in the sperm were not present in the ART conceptus suggesting that either the parental sperm is mosaic for the methylation errors or that remodeling of DNA methylation can

occur after fertilization. Nutrients, including methyl substrates such as vitamin B6 and folic acid, influence DNA methylation and histone modification at gene promoters and this approach has already been applied to treat autism.[33] Imprinting errors in sperm at the paternally methylated DMRs may be reversed using a similar approach.

Standard ART treatment

As a result of our studies and the work of others in this area, we recommend that imprint methylation analyses be added to the routine sperm examination to identify preexisting imprint mutations. The relative ease of COBRA would make this approach feasible within a clinical setting and substantially reduce the likelihood of abnormal samples being used in ART. Altered expression and methylation of imprinted genes is a frequent event in cancer[34] and it will be important to also determine the frequency of cancer occurrence in ART offspring. A retrospective examination of imprinted loci and the constitution of children born after each ART method will reveal the safest and most ethical approach to use, which will be invaluable for the future development of standard ART treatments.

Conflict of interest statement

None declared.

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