TABLE 2

Characterization of the methylation errors of the imprinted genes in the sperm DNA.

Sperm property	Abnormal methylation	Paternal	Maternal	Paternal/ Maternal
Normal	Single locus	1	8	—
	Two loci	0	0	2
Moderate	Single locus	5	6	—
	Two loci	0	1	1
	Three loci	0	0	1
Severe	Single locus	9	3	—
	Two loci	1	0	3
	Three loci	2	0	4
Total		18	18	11

Note: Paternal = case number of the aberrant methylation of paternally methylated differentially methylated regions (DMRs). Maternal = case number of the aberrant methylation of maternally methylated DMRs. Paternal/Maternal = case number of the aberrant methylation of both paternally and maternally methylated DMRs.

Sato. Imprint methylation loss in oligozoospermia. Fertil Steril 2011.

PEG1, LIT1, ZAC, PEG3, and *SNRPN,* the cutoff value was less than 15%, agreement rates were high, and the cases of the agreement were examined in 98.2%, 97.9%, 100%, 96.4%, and 100%, respectively. Thus, we defined less than 90% of the paternally methylated DMRs and more than 15% of the maternally methylated DMRs in the BPL assay as abnormal sperm DNA methylation (Supplementary Fig. 2, available online).

Analyses of the Methylation Status of Paternally Methylated DMRs in Sperm

We performed BPL analysis on three paternally methylated DMRs in 337 sperm DNA samples from the male partner of couples presenting with fertility problems. The BPL assay for the DMR of *ZDBF2* showed it to be fully methylated in all but three samples (1.32%) that showed a reduction in methylation (Fig. 1B, C; Table 1). We also performed COBRA and bisulfite-PCR sequencing of the CpG sites of the DMR in the *ZDBF2*. This confirmed the relative hypomethylation of the *ZDBF2* DMR in the three samples. Ten samples (3.01%) showed reduced methylation of the *H19* DMR, and 23 samples (7.37%) showed reduced methylation of the *GTL2* DMR (Table 1).

We next investigated whether the sperm carrying abnormal DNA methylation shared any properties (Table 1; Supplementary Table 2 [online]). Only three cases with an abnormal methylation pattern were phenotypically normal. Overall, the occurrence of abnormal methylation at the paternally methylated loci was statistically significantly increased in oligozoospermic patients when compared with normozoospermic patients (Table 1; Fig. 2).

Analyses of the Methylation Status of Maternally Methylated DMRs in Sperm

In 29 of 337 samples, an abnormal methylation pattern was found at one or more of the maternally methylated regions associated with *PEG1, LIT1, ZAC, PEG3,* and *SNRPN* (Table 1), which confirmed the usefulness of the bisulfite-based sequencing method (data not shown). Similar to the paternal DMRs, altered maternal DMRs were associated with abnormal appearing sperm, except in the case of the *SNRPN* DMR. Also, it was often the case that at least two DMRs were affected. There were only two samples with abnormal imprints that appeared microscopically normal (normozoospermia) (Table 2). In addition, of the 22 most severe oligozoospermia cases with imprinting errors, seven cases carried abnormal methylation patterns at both maternal and paternal loci.

DISCUSSION

In humans, ejaculated and mature sperm should be fully methylated at paternal DMRs and completely unmethylated at maternal DMRs. We have developed a novel BPL method for analyzing DNA methylation and have demonstrated the effectiveness of this technique on a large number of sperm samples. This method is a rapid, high-throughput, and quantitative methylation analysis technique employing PCR-SSOP protocols and the xMAP technology developed by Luminex Corporation (22, 23). This technique has been used to recognize genetic polymorphisms in human disease and HLA typing, but our study is the first to use this approach to detect DNA methylation status.

The most frequent error in males with fertility problems was at the PEG1 DMR (8.84%), similar to previous findings (16, 24). This suggests that this DMR is particularly prone to errors. In the mouse model, Peg1-deficient mice are growth restricted (25). In humans, ART-treatment infants generally are characterized by low-weight birth (26), and low-birth weight in non-ART babies has been linked to the altered expression of several imprinted genes, including *PEG1* (27, 28). Imprinting errors inherited from abnormal sperm may underlie some cases of low birth weight in ART.

Further, our study demonstrates that human sperm from subfertile men contains abnormal paternal and maternal imprinting marks, suggesting a link between genomic imprinting defects and infertility. The DNA methyltransferase (Dnmt) 3a- and 3l-deficient male mice are oligozoospermic (29, 30). We previously reported DNA sequence variations in the gene encoding *DNMT3L* in oligozoospermic men with abnormal paternal DNA methylation (31), which could suggest that altered DNMT3L function underlies both the oligozoospermia and the defects in methylation. Arnaud et al. (32) showed that a maternal imprint could be acquired in the absence of Dnmt3L in female germ cells at some loci and in some embryos. Variable penetrance of loss of imprinting as a consequence of DNMT3L deficiency shares some similarity with our findings, where not all loci are affected consistently.

We reported several miscarriage cases where imprinting mutations in ART conceptuses matched those present in the parental sperm (31). Use of ICSI may bypass the natural elimination process of abnormal sperm and may allow the inheritance of imprinting mutations. This may be linked to the increased frequency of miscarriage, placental dysfunction, premature labor, intrauterine growth retardation, placenta previa and maternal hypertension, and obstetric and neonatal complications associated with ART (33–37).

To our knowledge, our is the first study to report on using Luminex analyses for the examination of DNA methylation at imprinted loci. The relative ease of the BPL method would make this approach feasible within a clinical setting and could be applied to reduce the likelihood of abnormal samples being used in ART. There are also many other applications for this protocol, including a retrospective examination of infants born after each ART method with a focus on imprinted genes and their DMRs.

Acknowledgments: The authors thank Takuya Koshizaka, Ph.D., and all of the members of our laboratory for their technical assistance, support, and valuable suggestions; and, in particular, Dr. Rosalind M. John for comments on the manuscript.

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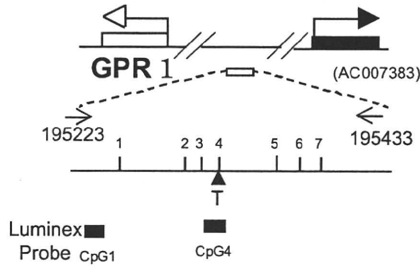
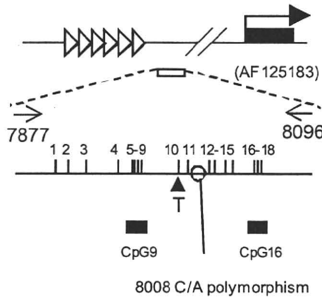
SUPPLEMENTARY FIGURE 1

Genomic structures of human differentially methylated regions (DMRs). (**A**) *ZDBF2,* (**B**) *H19,* (**C**) *GTL2,* (**D**) *PEG1,* (**E**) *LIT1,* (**F**) *ZAC,* (**G**) *PEG3,* and (**H**) *SNRPN.* Extent of the regions analyzed in this study and GenBank accession numbers are shown under the line. Filled boxes and horizontal arrows indicate genes and their orientation. Open boxes represent the DMRs associated with the genes. Arrowheads above the CpGs indicate which of these sites are contained within a repeat sequence. The horizontal arrows represent position of primers. Vertical arrows indicate the unique bisulfite PCR restriction enzyme sites analyzed. Restriction enzymes are shown: T, *TaqI*; and H, *HhaI*. The vertical bars represent CpG sites. Black boxes indicate the probes used in BPL.

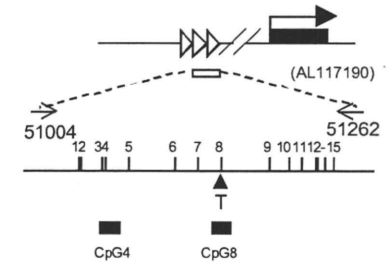
A ZDBF2/DMR



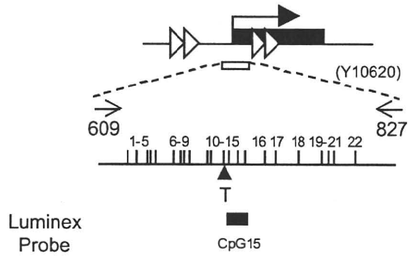
B H19/DMR



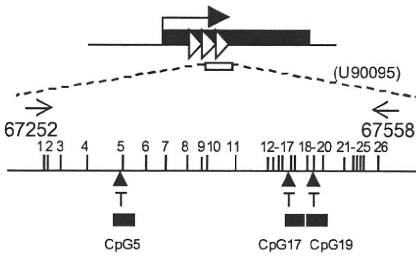
C GTL2/DMR



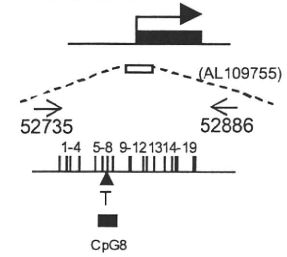
D PEG1/DMR



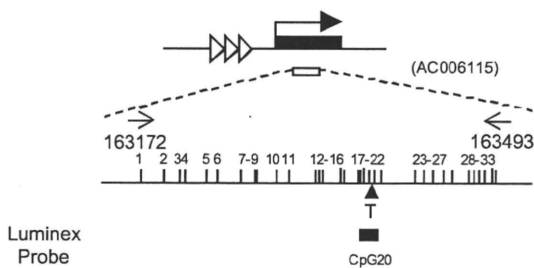
E LIT1/DMR



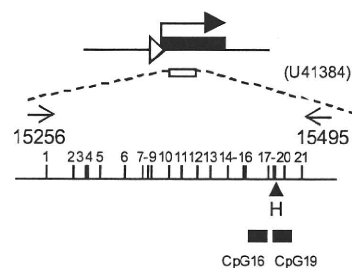
F ZAC/DMR



G PEG3/DMR



H SNRPN/DMR



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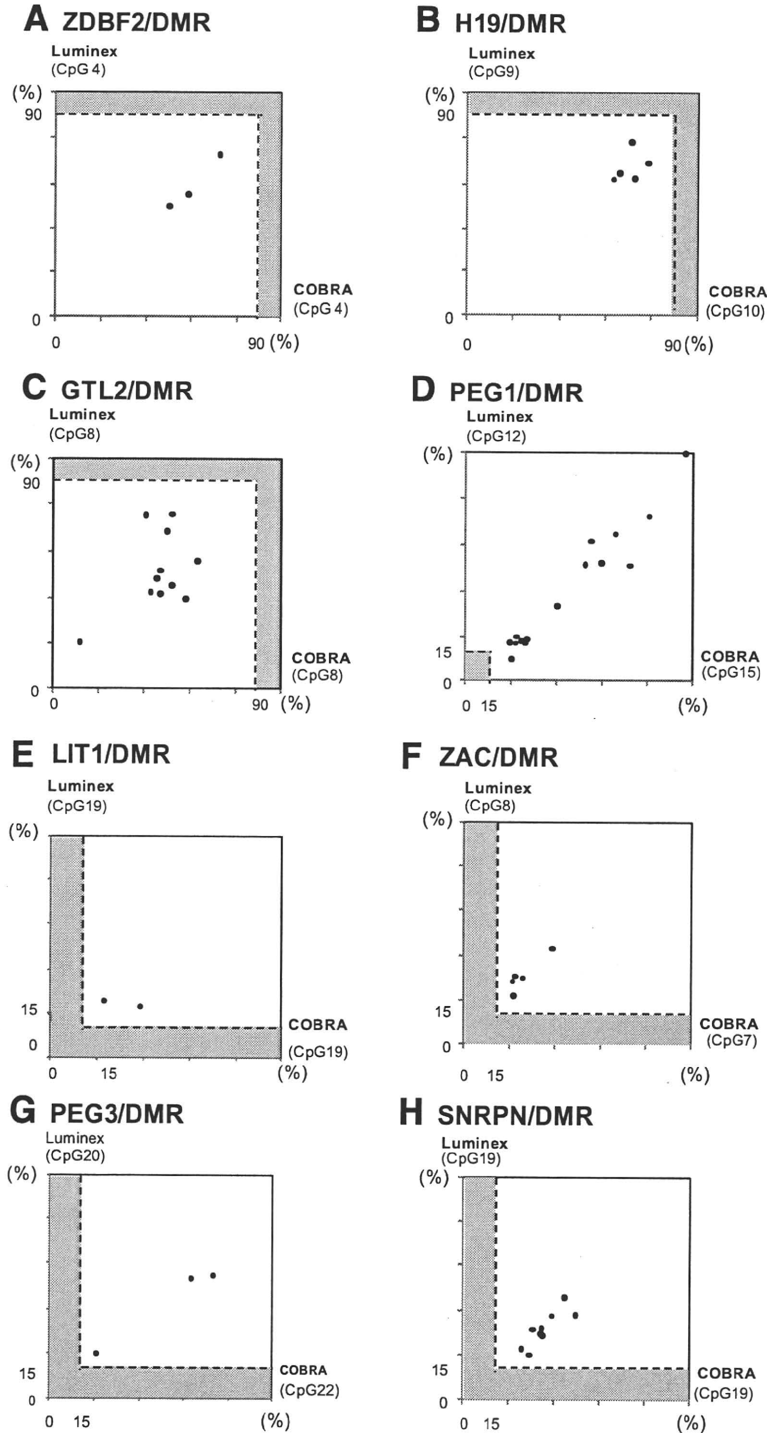
SUPPLEMENTARY FIGURE 2

Validation of bisulfite polymerase chain reaction Luminex (BPL) analyses by comparison with the combined bisulfite polymerase chain reaction restriction analysis (COBRA) assay. Eight imprinted human differentially methylated regions (DMRs) with aberrant methylation in sperm DNA samples are compared. The number was calculated by Spearman's rank method. **(A)** *ZDBF2*, **(B)** *H19*, **(C)** *GTL2*, **(D)** *PEG1*, **(E)** *LIT1*, **(F)** *ZAC*, **(G)** *PEG3*, and **(H)** *SNRPN*. We defined the cutoff values, less than 90% of the paternally methylated DMRs and more than 15% of the maternally methylated DMRs, in the BPL assay as abnormality of the sperm DNA methylation (gray zone).



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SUPPLEMENTARY TABLE 1

Primer sets and oligonucleotide probes of the bisulfite polymerase chain reaction Luminex (BPL) analysis for eight imprinted genes.

Target genes			Sequence(5'–3')
ZDBF2	Primer F		GTTTTGTTAGTTAGATTGGAAAATA
	Primer R		AAAATAATAATTACCTAAAAATAAAAAC
	probe(CpG 1)	Me	ATTCAAAACCGCAATAAACT
		Un	ATTCAAAACCACAATAAACTA
	probe(CpG 4)	Me	CAACTACTCGAATAACTAAA
		Un	CAACTACTCAAATAACTAAA
H19	Primer F		TATATGGGTATTTTTGGAGGTTTTT
	Primer R		ATAAATATCCTATTCCCAAATAACCCC
	probe(CpG 9)	Me	TTATAGTTCGAGTTCGTTT
		Un	ATTATAGTTTGAGTTTGTTT
	probe(CpG 16)	Me	AGTTACGCGTCGTAGG
		Un	AGTTATGTGTTGTAGGG
GTL2	Primer F		GGGTTGGGTTTTGTTAGTTGTT
	Primer R		CCAATTACAATACCACAAAATTAC
	probe(CpG 4)	Me	CCTAATAAATCGCGAACAA
		Un	CCTAATAAATCACAAACAA
	probe(CpG 8)	Me	GTTGTTCGAGGTTTATAG
		Un	CTATAAACCTCAAACAACT
PEG1	Primer F		TYGTTGTTGGTTAGTTTTGTAYGGTT
	Primer R		ACCACCAACCACACCCCCTC
	probe(CpG 15)	Me	TTATGGTGCGTCGAGAT
		Un	GGTTATGGTGTGTTGAGAT
LIT1	Primer F		TTTTGGTAGGATTTTGTTGAGGAGT
	Primer R		CCTCACACCCAACCAATACCTC
	probe(CpG 5)	Me	GTTATTGGTCGAAAGAGTT
		Un	GTTATTGGTTGAAAGAGTT
	probe(CpG 17)	Me	TGTTTTCGTCGTTGTCGAT
		Un	TTGTTTTTGTTGTTGTTGTTGAT
	probe(CpG 19)	Me	TGCGGTAGCGTTTCGAT
		Un	ATTGTGGTAGTGTTTTGATT
ZAC	Primer F		GGGGTAGTYGTGTTTATAGTTTAGTA
	Primer R		CRAACACCCAAACACCTACCCTA
	probe(CpG 8)	Me	GGTACGTTCGAGCGGT
		Un	GGTATGTTTGAGTGGTT
PEG3	Primer F		GTAAGAYGGTTATTTTGGTTTAGAG
	Primer R		AAAAATATCCACCCTAAACTAATAA
	probe(CpG 20)	Me	GCGGTCGAAGGCGTATTTA
		Un	GTGGTTGAAGGTGTATTTA
SNRPN	Primer F		AGGGAGTTGGGATTTTTGTATTG
	Primer R		ACTAACCACTCCTCAAACAAATAC
	probe(CpG 16)	Me	AGGTTGGCGCGTATGTT
		Un	AGGTTGGCGCGTATGTT
	probe(CpG 19)	Me	AGGTTGGCGCGTATGTT
		Un	AGGTTGGCGCGTATGTT

Note: The number of CpG sites are represented at the position in Figure 1A. Me = methylated probe; Un = unmethylated probe; F = forward primer; R = reverse primer.

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SUPPLEMENTARY TABLE 2

Comparison of imprinting errors by sperm property.

Property	H19	GTL2	ZDBF2	PEG1	ZAC	PEG3	SNRPN	LIT1
Motility								
<40%	3.5% (4/114)	13.2% (15/114)[a]	0.9% (1/114)	5.2% (6/114)	1.8% (2/114)	1.8% (2/114)	2.6% (3/114)	0.9% (1/114)
40–60%	5.7% (4/70)	7.1% (5/70)	2.9% (2/70)	7.1% (5/70)	2.9% (2/70)	1.4% (1/70)	2.9% (2/70)	0.0 (0/70)
>60%	1.3% (2/153)	2.0% (3/153)[a]	0.0 (0/153)	3.3% (5/153)	0.7% (1/153)	0.7% (1/153)	0.7% (1/153)	0.7% (1/153)
Morphologic abnormality								
>70%	3.2% (3/93)	7.5% (7/93)	3.2% (3/93)	4.3% (4/93)	1.1% (1/93)	1.1% (1/93)	3.2% (3/93)	1.1% (1/93)
50–70%	4.0% (5/125)	10.4% (13/125)[b]	0.0 (0/125)	4.0% (5/125)	3.2% (4/125)	1.6% (2/125)	0.8% (1/125)	0.0 (0/125)
<50%	1.7% (2/119)	2.5% (3/119)[b]	0.0 (0/119)	5.9% (7/119)	0.0 (0/119)	0.8% (1/119)	1.7% (2/119)	0.8% (1/119)

Note: There were no differences among groups for the volume and abstinence period parameters.

[a] Statistically significant difference between the two groups: $P < .01$.

[b] Statistically significant difference between the two groups: $P < .05$.

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