In contrast, methylation is initiated at paternal DMRs prenatally during embryonic germ cell development and completed by the pachytene phase of postnatal spermatogenesis (9–12). The gametic imprints are maintained stably after fertilization despite overall epigenetic reprogramming, and persist during development and into adulthood.

Methyl-substrates and DNA methyltransferases (Dnmts) are required for both the acquisition and the maintenance of DNA methylation. In mice, Dnmt3a and the accessory protein, Dnmt3l, establish imprinted DNA methylation in the germ line (13–15). Dnmt3a has a central role in the *de novo* methylation process at the paternally methylated *H19*, *Gtl2* (intergenic DMR; IG-DMR) and *Rasgrf1* loci, while the role of Dnmt3b appears to be specific to the *Rasgrf1* locus (15,16). Dnmt3l has a plant homeodomain (PHD)-like motif but lacks DNA methylation activity (14,17). Instead, Dnmt3l cooperates with Dnmt3a to *de novo* methylate DNA (18,19). It may serve to activate the functional Dnmts and/or play a role in recognizing the target sequence (20,21). Germ line conditional knockout mice that lack either Dnmt3a or Dnmt3l do not acquire the maternal or paternal methylation imprints (15,16).

To date, DNA methylation is acquired on the paternal allele at 4 DMRs and on the maternal allele at 18 DMRs (22–26). There are additional DMRs where allele-specific methylation is acquired after fertilization. Disruption of the methylating machinery in the germ line primarily results in global loss of imprinting (14,27,28), while loss of the maintenance DNA methylase can affect the expression of a subset of imprinted genes within a domain (29–31).

The number of known imprinted genes is ~100 but the total number is unknown. A number of approaches have been used to identify new candidates (32). A drawback of expression-based approaches is in the identification of genes expressed at different stages of development or ones that are imprinted only in a subset of tissues. In contrast, approaches based on detecting regions of allele-specific epigenetic marks between the maternal and paternal genomes are applicable to all tissue types at all time points. Tiling array technology and chromatin immunoprecipitation (ChIP-on-chip) has been successfully applied to decipher chromatin structure (33–35). In this study, we applied this technology in combination with the methylated DNA binding column technique (36,37) using the antibody against 5-methyl-cytosine (methylated-DNA immunoprecipitation; meDIP) to determine how effectively we could identify known and novel DMRs.

MATERIALS AND METHODS

Mouse strains and the preparations of DNA and RNA

Derivation of PG-, AG-derived stem and TS cells was described previously in detail (38). C57BL/6 (B6) females were mated with JF1 (39) males to generate B6/JF1 mice and reciprocally crossed to generate JF1/B6 mice. The mature sperm and MII oocytes were obtained from B6 and ICR mice, respectively. Blastocysts were obtained from B6/JF1 mice. Genomic DNAs from mature sperm, MII oocytes, blastocysts and TS cells was prepared as previously described (6,40). Genomic DNA and total RNA were obtained from various organs from B6/JF1 and JF1/B6 mice at embryonic day (E) 13.5, E18.5 and adult stages. For human polymorphic analysis, DNA and RNA were prepared from umbilical cord blood after delivery and from their mothers' peripheral blood using standard protocols. Total RNA was prepared using ISOGEN (Nippon Gene, Tokyo, Japan), treated with DNase I (Promega, WI, USA) to remove genomic DNA. The absence of genomic DNA contamination was confirmed by the lack of genomic DNA amplification of *Gapdh*/*GAPDH* by polymerase chain reaction (PCR).

The isolation of *Dnmt3a*-deficient and wild-type prospermatogonia

To obtain *Dnmt3a*-deficient and wild-type prospermatogonia, male germ cells were isolated from E14.5, E16.5, E18.5 and Postnatal day (P) 7 testes from B6 mice and from P7 testes of the conditional *Dnmt3a* knockout mice by fluorescence activated cell sorting (FACS) as previously described in detail (16).

MeDIP-on-chip analysis

DNA extracted from PG- and AG-derived cells and mature sperm was fragmented to ~200–1000 bp by sonication (Sonics & Materials, Connecticut, USA). Fragment size was checked on 1% agarose gels. Immunoprecipitation was carried out using a specific antibody for 5-methyl-cytosine (AbD Serotec, Oxfordshire, UK). Input and bound DNA was amplified by GenomePlex Complete Whole Genome Amplification kit (Sigma–Aldrich, Missouri, USA). The relative enrichment of DMRs was determined by sequence-specific real-time PCR analyses using a 7500 Real Time PCR System (Applied Biosystems Japan, Tokyo, Japan) and SYBR *Premix Ex Taq* II (Perfect Real Time) (Takara Bio, Kyoto, Japan). Primers and PCR conditions are described in Supplementary Table S1.

For the tiling arrays, input DNA was labeled with a cyan-3 dye and bound DNA was labeled with cyan-5. DNAs were hybridized to the mouse whole genome tiling array (Agilent Technologies Japan, Tokyo, Japan). The methylated sequences were compared between PG- and AG-derived cells and sperm DNA using ChIP Analytics 1.3 software (Agilent Technologies Japan, Tokyo, Japan).

Bisufhite-PCR methylation assay

The methylation assay was performed at the DMRs of *H19*, IG-DMR (*Gtl2*), *Rasgrf1*, *Zdbf2*, *Nespas*, *Gnas1A*, *Peg10*, *Peg1*, *Peg3*, *Snrpn*, *Lit1*, *Zac1*, *U2af1-rs1*, *Igf2r* (DMR2) and *Impact*. The *Zdbf2* methylated regions were analyzed by both combined bisulfite-PCR restriction analysis (COBRA) and bisulfite-PCR sequencing (11). Each DNA sample (MII oocytes, sperm and several organs tissues) was treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) and amplified by PCR as follows: a PCR

reaction mix containing 0.5 μM of each of the primer sets, 200 μM dNTPs, 1 × PCR buffer, 1.25 U of *Ex Taq* Hot Start DNA Polymerase (Takara Bio, Kyoto, Japan) in a total volume of 20 μl. Primers used and PCR conditions are listed in Supplementary Table S1.

COBRA was carried out on bisulfite-treated PCR samples with the following enzymes: *Taq*I for the DMR of *H19*, IG-DMR (*Gtl2*), *Nespas*, *Zac1*, *Igf2r* (DMR2) and *Zdbf2*; *HpyCH4*IV for the DMR of *Gnas1A*, *Peg10*, *Peg1*, *Peg3*, *Snrpn*, *Lit1*, *U2af1-rs1*, *Impact* and *Zdbf2*. Samples were electrophoresed on 2% agarose gels. The PCR products were purified and cloned into the pGEM-T Easy vector (Promega, WI, USA) and individual clones were sequenced using T7 or SP6 primer and an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan). An average of 20 clones for each individual was sequenced. At least two separate sodium modification treatments were carried out for each DNA sample, and at least three independent amplification experiments were performed for each individual.

Reverse transcription PCR analysis

Monoallelic expression of *Gpr1*/*GPR1* was investigated by RT-PCR. DNA-free total RNA (1 μg) from mouse and human tissues was reverse-transcribed into cDNA using AMV reverse transcriptase (Roche Diagnostics, Basel, Switzerland) with either a sense or antisense primer in order to determine the direction of transcription. RT products were then amplified using the specified PCR primers.

In situ hybridization analysis

cDNA probes for mouse *Zdbf2* and *Gpr1* were generated by PCR (Supplementary Table S1) and used to prepare sense and antisense riboprobes by *in vitro* transcription using the DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Sagittal sections of 8 μm from paraffin embedding mouse embryos and placentas at E13.5 were used for *in situ* hybridization as described previously (41). Sections were counterstained with eosin.

Sequence analysis

Nucleotide similarities between mouse DMR1 and human DMRh1 were calculated using the GENETYX software version 11.0 (GENETYX, Tokyo, Japan). Dot-matrix analysis was performed on mouse DMR1 and human DMRh1 to detect homologous regions using Harrplot Ver. 2.0 as part of the computer software GENETYX package.

RESULTS

MeDIP-on-chip screen for the DMRs

To identify novel DMRs, we applied the meDIP-on-chip method to DNA extracted from parthenogenetic (PG)-derived stem cells (two copies of the maternal genome), androgenetic (AG)-derived stem cells (two copies of the paternal genome) and genomic DNA prepared from mature sperm. We first confirmed that the stem cell genomic DNA had the characteristic epigenetic profile of PG- and AG-genomes by analyzing the methylation status at the DMRs of the imprinted genes *H19*, IG-DMR (*Gtl2*), *Rasgrf1*, *Nespas*, *Gnas1A*, *Peg10*, *Peg1*/*Mest*, *Peg3*, *Snrpn*, *Lit1*/*Kcnq1ot1*, *Zac1*/*Plagl1*, *U2af1-rs1*/*Zrsr1*, *Igf2r* (DMR2) and *Impact*. Representative results for one paternal DMR, IG-DMR (*Gtl2*), and one maternal DMR, *Lit1*, are shown (Supplementary Figure S1A). Both stem cells maintained the correct DNA methylation marks all the DMRs except the *H19* DMR, which was hypermethylated in both genomes.

Next, we used antibodies specific for 5-methyl-cytosine to isolate methylated DNA from mouse PG- and AG-derived cells and also from sperm. We used quantitative real-time-polymerase chain reaction to assay for the presence of the known DMRs within the immunoprecipitated material using input DNA as a control. The paternal DMRs of *H19*, IG-DMR (*Gtl2*) and *Rasgrf1* were amplified by real-time-PCR from both AG-derived cell and sperm meDIP samples (Supplementary Figure S1B). The *H19* DMR was amplified from both the AG- and the PG-derived cell samples. The maternal DMRs of *Nespas*, *Peg10*, *Peg1*, *Peg3*, *Lit1*, *U2af1-rs1* and *Igf2r* (DMR2) were amplified from the materials of the meDIP PG-derived cells. We additionally examined sequences where both maternal and paternal alleles were methylated, including *Nanog*, *Rest*, *Aicda*, *Tdrd12*, *Gdf3* and *Slc2a3* (*Aicda* and *Tdrd12* were unmethylated in sperm) and where both alleles were unmethylated, *Utf1* (42). In total, the monoparental stem cells maintained the correct parental methylation pattern at over 94% (16/17) of the loci examined. These data indicated that meDIP was effective at isolating known DMRs.

We next performed meDIP-on-chip by applying the meDIP samples to mouse whole-genome tiling arrays. The fixed quantity value that had been obtained from this array analysis corrected the reference value. We looked for the regions under the following conditions: (i) at least three adjoined methylated probes (using neighborhood model supplied by Agilent Technologies Japan, p (Xbar) < 0.07) and (ii) a similar methylation pattern between AG-derived cells and sperm but dissimilar to PG-derived cells (normalized log ratio of the PG-derived cells probe < 0.5). We identified 458 candidate DMRs in the mouse genome. 141 were paternally methylated DMRs and 317 were maternally methylated DMRs (Figure 1, Tables 1 and 2). Of these, 20 were known DMRs. We correctly identified the IG-DMR (*Gtl2*) and *Lit1* DMRs using the tiling arrays for mouse chromosome 7 and 12 (Figure 1B, upper panel). Using the tiling array for chromosome 1, we found the evidence of three closely linked paternally methylated DMRs (Figure 1B, lower panel) that lay within a 60 kb region between the imprinted *Zdbf2* (zinc finger, DBF-type containing 2) gene and the uncharacterized gene, *Gpr1* (G-protein-coupled receptor 1) (GenBank accession number NM146250) (Figure 2A). We had previously identified *Zdbf2* as an imprinted gene linked to a DMR in a parallel study isolating imprinted genes based on their expression status (26). Not all the known DMRs were identified.

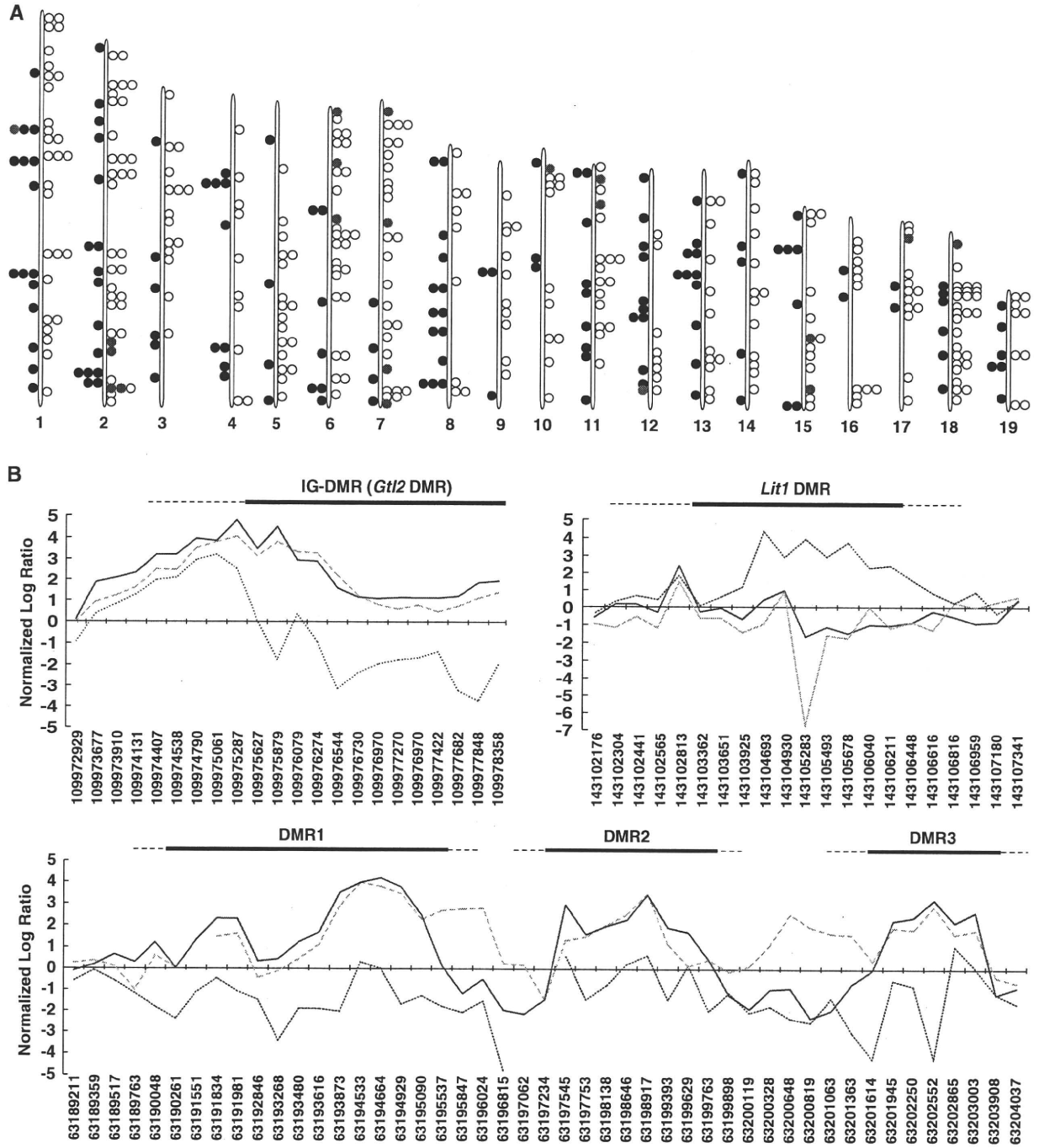

Figure 1. Whole mouse genome meDIP-on-chip and genome tiling array screen for DMRs using PG- and AG-derived stem cells and sperm. (**A**) Chromosome map shows the position of all paternally methylated and maternally methylated DMRs. Red circles to the left-hand side of each chromosome indicate known maternal DMRs and blue circles to the right-hand side of each chromosome indicate known paternal DMRs. Open circles indicate novel maternally methylated DMRs and closed circles indicate novel paternally methylated DMRs. (**B**) The methylation pattern of the meDIP-on-chip assay. (Upper panel) IG-DMR (*Gtl2*) paternal DMR (left) and *Lit1* maternal DMR (right). (Lower panel) Three paternally methylated DMRs between *Gpr1* and *Zdbf2*: DMR1, DMR2 and DMR3. The longitudinal axes indicate normalized log ratio (log2 Cy5-labeling meDIP DNA fragments/Cy3-labeling whole genome DNA fragments DNA), which represents the methylation degree. The numbers of horizontal axes indicate 5′-flanking base position of the tiling array probe in mouse genome browser mm8 assembly which were obtained from the build 36 'essentially complete' assembly by National Center for Biotechnology Information and the Mouse Genome Sequencing Consortium. Blue solid, aqua broken and red dotted lines represent meDIP-on-chip data of sperm, AG- and PG-derived cells samples, respectively. Black lines indicate the position of IG-DMR (*Gtl2*) and *Lit1* DMR.

Table 1. Paternal-allele methylated DMR candidates

Candidate No.	Position (mm8)	Size (kb)	P (Xbar)	Candidate No.	Position (mm8)	Size (kb)	P (Xbar)
1	chr1:033685996-033687689	1.7	0.027230699	72	chr8:095022687-095024090	1.4	0.05286681
2	chr1:063193268-063195587	2.3	0.0218758	73	chr8:111610219-111613561	3.3	0.057668444
3	chr1:063197753-063199822	2.1	0.042529337	74	chr8:123394925-123395984	1.1	0.03046366
4	chr1:063201945-063203062 (A)	1.1	0.038459964	75	chr8:123960459-123965990	5.5	0.035588805
5	chr1:064666213-064668088	1.9	0.042587895	76	chr8:124005188-124009612	4.4	0.038051125
6	chr1:075329727-075332982	3.3	0.022909729	77	chr9:061121907-061124402	2.5	0.036127605
7	chr1:078022275-078024393	2.1	0.027050465	78	chr9:061182468-061184945	2.5	0.05035278
8	chr1:090499895-090501007	1.1	0.028617026	79	chr9:119419980-119420779	0.8	0.025072549
9	chr1:133799192-133800261	1.1	0.02696383	80	chr10:011096967-011097931	1.0	0.03095065
10	chr1:134023790-134025706	1.9	0.030393751	81	chr10:056062305-056063184	0.9	0.02961503
11	chr1:134149907-134150736	0.8	0.03685552	82	chr10:060206859-060208255	1.4	0.04117605
12	chr1:138490891-138491572	0.7	0.04051165	83	chr11:003237473-003238310	0.8	0.027867135
13	chr1:154855403-154856207	0.8	0.032803357	84	chr11:007322890-007324637	1.7	0.03812329
14	chr1:169125711-169127401	1.7	0.021216722	85	chr11:032858949-032860475	1.5	0.02232835
15	chr1:182770196-182772623	2.4	0.03033735	86	chr11:063713890-063716353	2.5	0.068963565
16	chr1:186798464-186800148	1.7	0.025820382	87	chr11:069415271-069416977	1.7	0.030176075
17	chr2:010252113-010252960	0.8	0.034892585	88	chr11:084344242-084346009	1.8	0.06344608
18	chr2:032446637-032448262	1.6	0.055148385	89	chr11:095079200-095080564	1.4	0.029354626
19	chr2:044359572-044360465	0.9	0.04610098	90	chr11:098780556-098782087	1.5	0.048574876
20	chr2:052779932-052781936	2.0	0.0356707	91	chr11:120056577-120057507	0.9	0.03450692
21	chr2:071545340-071547095	1.8	0.027983196	92	chr12:009601787-009602759	1.0	0.04254318
22	chr2:101555546-101557244	1.7	0.03754241	93	chr12:029403118-029403923	0.8	0.029047519
23	chr2:105485539-105487500	2.0	0.033533122	94	chr12:040508958-040510065	1.1	0.04656238
24	chr2:115764618-115765843	1.2	0.023765821	95	chr12:045433938-045434465	0.5	0.018067254
25	chr2:118589797-118591157	1.4	0.044992935	96	chr12:070676298-070678203	1.9	0.035833355
26	chr2:143855825-143857111	1.3	0.043339927	97	chr12:073969064-073971035	2.0	0.01724685
27	chr2:157666102-157666938	0.8	0.025987396	98	chr12:076990926-076992593	1.7	0.038976375
28	chr2:164902535-164903153	0.6	0.024327435	99	chr12:076999983-077001402	1.4	0.010916126
29	chr2:165569294-165572201	2.9	0.014741534	100	chr12:104876896-104879970	3.1	0.061366655
30	chr2:165589044-165591976	2.9	0.019366147	101	chr12:108768086-108769476	1.4	0.04249084
31	chr2:168529651-168530604	1.0	0.021744832	102	chr12:109975627-109980439 (B)	4.8	0.020096103
32	chr2:172816442-172817394	1.0	0.027761048	103	chr13:019379713-019380663	1.0	0.04339324
33	chr3:032093257-032094758	1.5	0.0346579	104	chr13:040724758-040725660	0.9	0.04800732
34	chr3:088058689-088060627	2.0	0.053832173	105	chr13:044715633-044717473	1.8	0.028829992
35	chr3:102212197-102213932	1.7	0.053714477	106	chr13:044593235-044595857	2.6	0.039124887
36	chr3:127453428-127458227	4.8	0.04294138	107	chr13:053240493-053244189	3.7	0.04368776
37	chr3:131091297-131092327	1.0	0.03170784	108	chr13:053424451-053429206	4.8	0.06249129
38	chr3:147900692-147901491	0.8	0.037227307	109	chr13:053443843-053444926	1.1	0.033136632
39	chr4:044273005-044274686	1.7	0.0342338	110	chr13:060625134-060626135	1.0	0.041249607
40	chr4:045675124-045675950	0.8	0.02793303	111	chr13:077617432-077618221	0.8	0.041074943
41	chr4:045785001-045787233	2.2	0.016951019	112	chr13:098168444-098169681	1.2	0.038340382
42	chr4:045790862-045793054	2.2	0.05020369	113	chr13:115171670-115172595	09	0.020752199
43	chr4:064738195-064739382	1.2	0.027920863	114	chr14:010460554-010462803	2.2	0.049559623
44	chr4:128217514-128218988	1.5	0.04806063	115	chr14:047675151-047676493	1.3	0.021633925
45	chr4:129251321-129253174	1.8	0.04603652	116	chr14:054031699-054033685	2.0	0.034764
46	chr4:134438654-134439691	1.0	0.036609355	117	chr14:098222686-098223268	0.6	0.035441127
47	chr4:139097297-139098542	1.2	0.031863578	118	chr14:121390692-121394556	3.9	0.025694156
48	chr5:023937045-023939114	2.1	0.06268672	119	chr15:007427165-007427914	0.7	0.025462002
49	chr5:093884506-093886995	2.5	0.048833344	120	chr15:025697417-025700122	2.7	0.016209736
50	chr5:131881178-131882657	1.5	0.04750135	121	chr15:025706271-025707476	1.2	0.01838927
51	chr5:147464697-147466931	2.2	0.022693422	122	chr15:025718408-025718933	0.5	0.027751224
52	chr6:052109421-052111561	2.1	0.030017477	123	chr15:053006538-053007526	1.0	0.027212601
53	chr6:054026130-054027411	1.3	0.035516605	124	chr15:102070682-102072718	2.0	0.06376759
54	chr6:097066893-097068534	1.6	0.034522403	125	chr15:102830007-102831360	1.4	0.031679183
55	chr6:122672646-122673998	1.4	0.03949519	126	chr16:030125324-030126481	1.2	0.04029876
56	chr6:140298497-140300873	2.4	0.025882334	127	chr16:043719336-043720459	1.1	0.024739949
57	chr6:144207949-144209008	1.1	0.032060467	128	chr17:035110303-035112872	2.6	0.04365454
58	chr6:145073228-145074452	1.2	0.043128457	129	chr17:046081814-046082662	0.8	0.032995045
59	chr7:099433113-099434110	1.0	0.021295292	130	chr18:034674166-034677415	3.2	0.023786515
60	chr7:118306647-118310770	4.1	0.05059889	131	chr18:036410061-036411625	1.6	0.04972436
61	chr7:132646656-132648366	1.7	0.03798946	132	chr18:038504756-038506451	1.7	0.028145561
62	chr7:140825063-140826533	1.5	0.033876684	133	chr18:053592000-053594251	2.3	0.048128698
63	chr8:008487401-008490250	2.8	0.035363488	134	chr18:064469013-064469974	1.0	0.048328057
64	chr8:012503705-012505149	1.4	0.041198887	135	chr18:081142034-081143948	1.9	0.038805358
65	chr8:046469489-046470833	1.3	0.04832795	136	chr19:010297511-010299422	1.9	0.04160669
66	chr8:060216840-60219015	2.2	0.054875467	137	chr19:025669277-025670970	1.7	0.045235418
67	chr8:074455057-074456587	1.5	0.03862939	138	chr19:037879058-037880854	1.8	0.057981245
68	chr8:074616339-074617293	1.0	0.04343615	139	chr19:041064820-041068690	3.9	0.034352854
69	chr8:087664692-087665701	1.0	0.051280405	140	chr19:042462156-042463219	1.1	0.02959308
70	chr8:087667382-087671399	4.0	0.046456236	141	chr19:057171672-057172217	0.5	0.03625847
71	chr8:094959358-094962722	3.4	0.044347655				

P (Xbar) indicates p (Xbar) value of the most significant probe in its region. (A) *Zdbf2* DMR and (B) IG-DMR (*Gtl2*).

Table 2. Maternal-allele methylated DMR candidates

Candidate No.	Position (mm8)	Size (kb)	P (Xbar)	Candidate No.	Position (mm8)	Size (kb)	P (Xbar)
1	chr1:004677405-004680223	2.8	0.0237986	81	chr4:056833933-056836223	2.3	0.022042342
2	chr1:005905383-005908212	2.8	0.018068576	82	chr4:060007834-060010504	2.7	0.027354108
3	chr1:013109934-013114495	4.6	0.019053403	83	chr4:082007129-082010469	3.3	0.022314303
4	chr1:014902821-014905844	3.0	0.022682365	84	chr4:103116841-103119256	2.4	0.031934537
5	chr1:024538601-024542424	3.8	0.020918498	85	chr4:107903819-107903877	1.6	0.026745262
6	chr1:032116554-032116613	1.8	0.026415968	86	chr4:125827000-125827059	1.6	0.033597253
7	chr1:036626972-036628483	1.5	0.022979708	87	chr4:153741937-153743571	1.6	0.029771574
8	chr1:036715035-036716800	1.8	0.021054856	88	chr4:154495756-154498309	2.6	0.022843158
9	chr1:040269834-040271457	1.6	0.021841165	89	chr5:037624391-037627129	2.7	0.030124942
10	chr1:058656604-058658693	2.1	0.021467961	90	chr5:064090611-064092787	2.2	0.032423064
11	chr1:063133112-063135558	2.4	0.018698972	91	chr5:071978637-071980584	1.9	0.03554431
12	chr1:069309424-069312191	2.8	0.019993572	92	chr5:077929980-077934343	4.4	0.027385928
13	chr1:069759227-069760723	1.5	0.024084114	93	chr5:078188911-078192010	3.1	0.045278415
14	chr1:074749647-074750852	1.2	0.02189668	94	chr5:082095599-082097977	2.4	0.027626283
15	chr1:074883540-074885912	2.4	0.023577677	95	chr5:101886786-101889296	2.5	0.028704671
16	chr1:082165204-082169216	4.0	0.021599824	96	chr5:106800163-106801479	1.3	0.027590053
17	chr1:091763096-091766857	3.8	0.0240105	97	chr5:107927323-107929481	2.2	0.033987135
18	chr1:093630290-093634089	3.8	0.02066035	98	chr5:110582368-110584177	1.8	0.029948255
19	chr1:122427617-122432992	5.4	0.01444563	99	chr5:116343153-116344638	1.5	0.02797624
20	chr1:122440765-122442941	2.2	0.018751323	100	chr5:119921542-119924672	3.1	0.03576451
21	chr1:123354835-123356653	1.8	0.023314446	101	chr5:120063522-120065663	2.1	0.028085513
22	chr1:136329040-136330902	1.9	0.018591803	102	chr5:127531574-127533303	1.7	0.028648317
23	chr1:155966454-155968446	2.0	0.015548464	103	chr5:135534407-135535387	1.0	0.020641953
24	chr1:156487452-156488820	1.4	0.01905871	104	chr5:136172390-136173572	1.2	0.022459375
25	chr1:161880204-161881805	1.6	0.038172938	105	chr5:139072890-139074587	1.7	0.016678514
26	chr1:166956508-166959120	2.6	0.016881404	106	chr5:146893529-146896066	2.5	0.02011744
27	chr1:174212164-174214785	2.6	0.022220261	107	chr6:004695854-004698573	2.7	0.019778943
28	chr1:189305059-189311596	6.5	0.017962102	108	chr6:007503950-007507696	3.8	0.023743302
29	chr2:009813042-009815047	2.0	0.022884484	109	chr6:017229969-017232226	2.3	0.025952324
30	chr2:011207696-011209104	1.4	0.01718148	110	chr6:017979125-017983000	3.9	0.017993594
31	chr2:024916269-024918291	2.0	0.022236267	111	chr6:021165838-021167511	1.7	0.030279916
32	chr2:025016418-025018399	2.0	0.019263456	112	chr6:021935187-021937334	2.1	0.02010118
33	chr2:025397984-025402571	4.6	0.014632969	113	chr6:030682464-030689996	7.5	0.01629422
34	chr2:029573050-029574474	1.4	0.026143976	114	chr6:034850411-034851986	1.6	0.017477673
35	chr2:033788267-033789456	1.2	0.019592382	115	chr6:036317254-036319844	2.6	0.016221117
36	chr2:035104192-035104243	1.1	0.028701099	116	chr6:043237998-043240078 (E)	2.1	0.03237432
37	chr2:051793296-051796677	3.4	0.018992666	117	chr6:058835661-058837219 (F)	1.6	0.027194222
38	chr2:061604035-061608674	4.6	0.020744983	118	chr6:065310430-065311666 (G)	1.2	0.021420863
39	chr2:062211510-062213411	1.9	0.023512473	119	chr6:067304657-067306131	1.5	0.03576346
40	chr2:062446005-062447593	1.6	0.020700894	120	chr6:067460849-067466104	5.3	0.026164446
41	chr2:069685551-069687058	1.5	0.030491233	121	chr6:067463404-067466104	2.7	0.015576691
42	chr2:070367063-070369555	2.5	0.018729487	122	chr6:071303159-071304733	1.6	0.020830877
43	chr2:070919022-070920942	1.9	0.01798238	123	chr6:073398337-073400658	2.3	0.041336037
44	chr2:076138655-076140328	1.7	0.01826188	124	chr6:079950693-079953626	2.9	0.025820898
45	chr2:105024797-105026056	1.3	0.018905096	125	chr6:082367687-082369045	1.4	0.023050921
46	chr2:105460287-105463427	3.1	0.016755583	126	chr6:083983935-083985438	1.5	0.023546984
47	chr2:110162692-110165055	2.4	0.02837651	127	chr6:085090127-085092702	2.6	0.022739487
48	chr2:113164875-113167167	2.3	0.023015061	128	chr6:096129315-096132554	3.2	0.025416961
49	chr2:122328928-122330175	1.2	0.02046392	129	chr6:097024399-097026153	1.8	0.029560687
50	chr2:125363369-125365775	2.4	0.032801084	130	chr6:125286598-125287941	1.3	0.01435962
51	chr2:127813988-127816711	2.7	0.012370981	131	chr6:126128327-126130786	2.5	0.025953725
52	chr2:130267142-130269115	2.0	0.022524204	132	chr6:136482254-136483949	1.7	0.018379996
53	chr2:132635416-132638005	2.6	0.013028574	133	chr6:136770656-136774801	4.1	0.031868864
54	chr2:146527611-146531637	4.0	0.018155145	134	chr6:142658106-142660237	2.1	0.016816275
55	chr2:148097997-148100499	2.5	0.009921394	135	chr7:006332204-006336197 (H)	4.0	0.013280352
56	chr2:152377240-152379677 (A)	2.4	0.010118501	136	chr7:016103614-016106029	2.4	0.014163033
57	chr2:157250325-157252581 (B)	2.3	0.008028563	137	chr7:016150455-016153988	3.5	0.014222109
58	chr2:170218228-170220655	2.4	0.0194618	138	chr7:018211612-018213729	2.1	0.017967263
59	chr2:173928712-173931558	2.8	0.009727121	139	chr7:024238192-024242555	4.4	0.016059337
60	chr2:173937217-173941051 (C)	3.8	0.009931667	140	chr7:024293881-024297190	3.3	0.019919932
61	chr2:173968974-173971383 (D)	2.4	0.007812512	141	chr7:029830009-029831950	1.9	0.012292666
62	chr2:178336086-178338064	2.0	0.019697197	142	chr7:035245768-035246777	1.0	0.021291312
63	chr2:181599626-181601824	2.2	0.013717825	143	chr7:043474646-043476273	1.6	0.012648921
64	chr3:007594593-007598083	3.5	0.016771285	144	chr7:046243531-046246986	3.4	0.01994469
65	chr3:034831468-034834969	3.5	0.031780172	145	chr7:049626297-049628167	1.9	0.02512963
66	chr3:036862151-036862198	1.8	0.024218604	146	chr7:059882077-059882135 (I)	2.5	0.014637309
67	chr3:046541933-046543604	1.7	0.02516645	147	chr7:067677277-067678725	1.4	0.01958729
68	chr3:054719962-054723493	3.5	0.023525374	148	chr7:068137247-068139281	2.0	0.034075223
69	chr3:055269547-055271782	2.2	0.020089474	149	chr7:075181242-075183250	2.0	0.014702246
70	chr3:055867538-055872400	4.9	0.02141071	150	chr7:089507231-089508988	1.8	0.03743501
71	chr3:065753115-065756368	3.3	0.033186894	151	chr7:100051451-100053814	2.4	0.014619963
72	chr3:067551215-067551270	1.5	0.02275754	152	chr7:110417089-110421405	4.3	0.019979531
73	chr3:079927589-079929496	1.9	0.02613796	153	chr7:111948575-111950637	2.1	0.015058612
74	chr3:079971586-079973377	1.8	0.026952958	154	chr7:114353321-114354903	1.6	0.023320151
75	chr3:084389767-084391322	1.6	0.02515726	155	chr7:122461720-122463398	1.7	0.026265722
76	chr3:087881593-087883247	1.7	0.027132021	156	chr7:128478998-128481285 (J)	2.3	0.020451799
77	chr3:108023194-108024627	1.4	0.024553038	157	chr7:126327181-126328933	1.8	0.018874127
78	chr3:126354778-126357616	2.8	0.02315601	158	chr7:139434397-139437287	2.9	0.016238498
79	chr4:021856794-021857823	1.0	0.030982286	159	chr7:139829080-139831032	2.0	0.027838653
80	chr4:044730990-044732806	1.8	0.022573303	160	chr7:140772576-140774926	2.4	0.019183591

(continued)

Table 2. Continued

Candidate No.	Position (mm8)	Size (kb)	P (Xbar)	Candidate No.	Position (mm8)	Size (kb)	P (Xbar)
161	chr7:141142723-141144797	2.1	0.02639694	240	chr14:083252269-083257004	4.7	0.011833122
162	chr7:143103651-143106675 (K)	3.0	0.016338563	241	chr14:102728834-102730676	1.8	0.015235411
163	chr8:004204874-004207016	2.1	0.022798432	242	chr14:104990563-104993500	2.9	0.019385036
164	chr8:026413587-026415169	1.6	0.03148531	243	chr14:107793504-107797494	4.0	0.016853297
165	chr8:027459206-027460587	1.4	0.032607652	244	chr14:116969080-116973383	4.3	0.013358023
166	chr8:038199721-038202173	2.5	0.026130743	245	chr15:006526293-006528390	2.1	0.020619694
167	chr8:044803368-044806622	3.3	0.043445475	246	chr15:006821080-006822833	1.8	0.012574255
168	chr8:071204755-071207122	2.4	0.021933837	247	chr15:010121536-010124016	2.5	0.016022267
169	chr8:122877933-122878642	0.7	0.030691648	248	chr15:044616875-044618897	2.0	0.014585087
170	chr8:124158092-124159752	1.7	0.027747734	249	chr15:061866956-061868107	1.2	0.015759477
171	chr8:124353331-124355356	2.0	0.027783262	250	chr15:072372513-072373586	1.1	0.019629903
172	chr9:021087872-021089270	1.4	0.03632289	251	chr15:072636322-072638800 (O)	2.5	0.017643908
173	chr9:037116789-037118411	1.6	0.020918619	252	chr15:075807678-075810850	3.2	0.023505678
174	chr9:037176612-037180135	3.5	0.027288798	253	chr15:079431697-079433748	2.1	0.030031314
175	chr9:039941858-039944124	2.3	0.036523227	254	chr15:085473004-085473063	1.6	0.020536428
176	chr9:050502657-050504704	2.0	0.03374793	255	chr15:096882333-096884933 (P)	2.6	0.017182048
177	chr9:057300197-057303815	3.6	0.018607844	256	chr15:101240038-101241546	1.5	0.03318241
178	chr9:076107910-076110030	2.1	0.026395189	257	chr15:101971141-101973609	2.5	0.020543724
179	chr9:078180813-078182487	1.7	0.025889887	258	chr16:017489943-017489987	0.9	0.01920948
180	chr9:082941778-082943974	2.2	0.025293902	259	chr16:021246116-021248115	2.0	0.026626676
181	chr9:102361832-102363332	1.5	0.020740993	260	chr16:029128734-029130726	2.0	0.027809
182	chr9:108197365-108199028	1.7	0.025672207	261	chr16:030429910-030429969	1.2	0.0295966
183	chr10:012779656-012782167 (L)	2.5	0.019184785	262	chr16:031853152-031855686	2.5	0.023718907
184	chr10:020413715-020416568	2.9	0.01953244	263	chr16:036479910-036481593	1.7	0.020951904
185	chr10:021379027-021380617	1.6	0.0238754	264	chr16:091108135-091109901	1.8	0.037090495
186	chr10:022333858-022335802	1.9	0.017312726	265	chr16:091112310-091115789	3.5	0.030529456
187	chr10:022505019-022510288	5.3	0.017806638	266	chr16:091947817-091948515	0.7	0.039174825
188	chr10:024283881-024285477	1.6	0.016753925	267	chr16:092582773-092585646	2.9	0.028216336
189	chr10:073216320-073218166	1.8	0.047033958	268	chr16:097764922-097766838	1.9	0.024059681
190	chr10:082295933-082297899	2.0	0.03255051	269	chr17:005374631-005376776	2.1	0.03132879
191	chr10:097122222-097124647	2.4	0.025589569	270	chr17:009511762-009513773	2.0	0.024981726
192	chr10:099187071-099189870	2.8	0.02582734	271	chr17:012584437-012586532 (Q)	2.1	0.02412774
193	chr10:106887829-106890671	2.8	0.024832647	272	chr17:031917288-031918966	1.7	0.036958188
194	chr10:126580305-126584057	3.8	0.0229678	273	chr17:034564671-034568817	4.1	0.036491122
195	chr11:003794275-003795370	1.1	0.03017051	274	chr17:036596043-036597688	1.6	0.029813139
196	chr11:011925467-011927317 (M)	1.9	0.026753291	275	chr17:036804142-036806133	2.0	0.032254983
197	chr11:016407175-016408969 (N)	1.8	0.028730938	276	chr17:044937669-044938910	1.2	0.02387914
198	chr11:022872000-022874562	2.6	0.0290943	277	chr17:046191541-046193261	1.7	0.03355552
199	chr11:031268290-031272176	3.9	0.02519847	278	chr17:047558119-047560713	2.6	0.03126954
200	chr11:053300081-053301551	1.5	0.015159988	279	chr17:049759295-049760847	1.6	0.022615742
201	chr11:053305814-053310528	4.7	0.011801407	280	chr17:079521685-079524587	2.9	0.036173925
202	chr11:053870758-053872961	2.2	0.024789684	281	chr17:093502830-093507935	5.1	0.020683607
203	chr11:057642798-057648684	5.9	0.01782747	282	chr18:013114801-013118408 (R)	3.6	0.03690424
204	chr11:062391207-062393390	2.2	0.022354733	283	chr18:020143702-020145888	2.2	0.020389104
205	chr11:062604615-062606649	2.0	0.037107296	284	chr18:034372092-034373598	1.5	0.0217988
206	chr11:069619339-069621611	2.3	0.015448221	285	chr18:034703079-034704303	1.2	0.045119096
207	chr11:072177596-072178833	1.2	0.011860856	286	chr18:035787290-035788030	0.7	0.037418738
208	chr11:084772811-084774719	1.9	0.017397704	287	chr18:037071868-037074704	2.8	0.019358814
209	chr11:085702850-085705929	3.1	0.019243628	288	chr18:037085878-037089320	3.4	0.018705504
210	chr11:088814584-088816685	2.1	0.03279886	289	chr18:037117819-037121107	3.3	0.028756998
211	chr11:101961201-101964545	3.3	0.017420538	290	chr18:037426316-037429037	2.7	0.02729652
212	chr12:037616718-037620453	3.7	0.00981845	291	chr18:037931616-037934633	3.0	0.018353485
213	chr12:040643123-040643182	2.7	0.014325851	292	chr18:039899306-039901593	2.3	0.017372293
214	chr12:084898561-084900520	2.0	0.016897557	293	chr18:042076444-042078040	1.6	0.022621712
215	chr12:096086762-096090337	3.6	0.009172818	294	chr18:046336482-046338773	2.3	0.01798738
216	chr12:099134675-099136350	1.7	0.014613676	295	chr18:046480672-046483104	2.4	0.02476409
217	chr12:102219226-102221216	2.0	0.01636928	296	chr18:046589690-046591475	1.8	0.019116558
218	chr12:105623102-105624458	1.4	0.014140618	297	chr18:046851351-046854314	3.0	0.03013274
219	chr12:111719113-111720871	1.8	0.008191496	298	chr18:054120897-054122418	1.5	0.026858354
220	chr13:019400293-019403773	3.5	0.040705923	299	chr18:062303490-062306672	3.2	0.02011947
221	chr13:019629918-019631961	2.0	0.022918101	300	chr18:066582220-066586790	4.6	0.017490398
222	chr13:031621242-031624357	3.1	0.01656243	301	chr18:066984440-066986686	2.2	0.02194144
223	chr13:047204189-047206166	2.0	0.012271536	302	chr18:069320834-069324481	3.6	0.022667281
224	chr13:065271696-065274388	2.7	0.017073411	303	chr18:070654510-070657602	3.1	0.038828205
225	chr13:075554829-075557522	2.7	0.009975607	304	chr18:072474614-072477050	2.4	0.028672088
226	chr13:092214744-092216480	1.7	0.012622342	305	chr18:077060298-077063598	3.3	0.0280942
227	chr13:096349727-096351467	1.7	0.012113783	306	chr18:080524705-080527164	2.5	0.02553843
228	chr13:096543879-096546003	2.1	0.025604498	307	chr18:082539721-082542232	2.5	0.02821235
229	chr13:099736482-099737805	1.3	0.022977684	308	chr18:086846350-086848475	2.1	0.032687873
230	chr13:113332691-113335190	2.5	0.023172792	309	chr19:006991711-006994911	3.2	0.026918497
231	chr13:116052081-116054730	2.6	0.017533854	310	chr19:007678835-007680562	1.7	0.032486826
232	chr14:011077128-011078632	1.5	0.01759021	311	chr19:009201186-009203180	2.0	0.031132337
233	chr14:015035019-015037201	2.2	0.033699445	312	chr19:016501850-016504566	2.7	0.030156422
234	chr14:028837272-028839252	2.0	0.027515797	313	chr19:018816356-018817936	1.6	0.027075935
235	chr14:039801943-039804245	2.3	0.031648647	314	chr19:037760893-037765203	4.3	0.025636315
236	chr14:054047482-054049683	2.2	0.03008235	315	chr19:038589068-038591035	2.0	0.034372117
237	chr14:068123705-068125678	2.0	0.023587078	316	chr19:059520244-059523338	3.1	0.028666519
238	chr14:068289885-068291193	1.3	0.023119137	317	chr19:060521572-060524423	2.9	0.029403668
239	chr14:073375085-073376336	1.3	0.03023008				

P (Xbar) indicates p (Xbar) value of the most significant probe in its region. (A) *Mcts2* DMR, (B) *Nnat* DMR, (C) *Nespas* DMR, (D) *Gnas1A* DMR, (E) *Peg10* DMR, (F) *Peg1* DMR, (G) *Napl5* DMR, (H) *Peg3* DMR, (I) *Snrpn* DMR, (J) *Inpp5f_v2* DMR, (K) *Lit1* DMR, (L) *Zac1* DMR, (M) *Meg1* DMR, (N) *U2af1-rs1* DMR, (O) *Peg13* DMR, (P) *Slc38a4* DMR, (Q) *Igf2r* DMR2 and (R) *Impact* DMR.

The *Ras/Grf1* DMR could not be identified because the sequence for this region had been excluded from the mouse tiling array due to its highly repetitive sequence. The *H19* DMR was also not identified in the screen and this was most likely because the *H19* DMR was methylated in both ADS and PDS material, as determined by COBRA, and therefore amplified from both meDIP samples. Nonetheless, these data on known DMRs indicated that meDIP would be an effective technique for identifying novel DMRs.

Paternally methylated DMRs in the *Gpr1-Zdbf2* imprinted domain

Within our tiling array, there were three separate regions of differential methylation on chromosome 1 in close proximity. In our very recent study on *Zdbf2*, we identified one paternally methylated region on chromosome 1 in this vicinity (26). We chose to characterize these three DMRs in greater detail in order to determine their relationship to the *Zdbf2* DMR. Using the combined bisulfite-PCR restriction analysis (COBRA) and bisulfite-PCR sequencing, we confirmed that these three DMRs were methylated in genomic DNA from mature sperm and unmethylated in metaphase II (MII) oocytes DNA and differentially methylated in blastocysts DNA from B6/JF1 mice (Figure 2B). Genomic DNA from somatic tissues from B6/JF1 and JF1/B6 embryos at E13.5 and adult mice was assayed by the same methylation protocol. All of the tissues of both adult and embryo, including the liver, lung, heart, kidney, spleen and brain, were differentially methylated and the methylation was reprogrammed in the next generation and stably maintained after tissue differentiation (Supplementary Figure S2A).

We called these paternal DMRs DMR1, DMR2 and DMR3. They were 5.0, 3.0 and 4.2 kb, respectively. None of the DMRs would be defined as CpG islands using the following standard criteria: minimum length 100 bp; GC content > 50%; Obs/Exp CpG > 0.6. Instead, they exhibited a low G+C content (43.5%, 46.6% and 42.4%, respectively) and a low frequency of CpG dinucleotides (CpG observed/expected = 0.22, 0.34 and 0.19, respectively). Analysis of the primary sequence of the DMR1 region revealed five repeats of the 37 bp repetitive sequence. Many imprinted DMRs are characterized by repeat sequence elements. DMR3 contained the 341 bp sequence of the *Zdbf2* DMR that we reported previously (26). Further analysis demonstrated that the three DMRs were closely linked within a 16 kb region separated by regions the lacked allele-specific methylation (Supplementary Figures S2B-1, 8, 14 and 20).

The *de novo* methylation of the DMRs linked to *Gpr1-Zdbf2* is dependent on methyltransferase Dnmt3a

To investigate the developmental changes in methylation at the paternally methylated DMRs in the *Gpr1-Zdbf2* domain, we carried out bisulfite-PCR methylation analysis in genomic DNA isolated from male germ cells at E14.5, E16.5 and E18.5. The paternally methylated *H19* and the maternally methylated *Lit1* DMRs were included as controls. The regions we analyzed and the CpG sites in this study are shown in Figure 2A.

In E14.5 prospermatogonia, DMR2 was ~15% methylated while DMR1 and DMR3 were unmethylated (Figure 3A). In contrast, the paternally methylated *H19* DMR was unmethylated in E14.5 prospermatogonia. This was different to the Kato's *et al.* (16) paper that reported that the *H19* DMR was hypomethylated (5–15%) in E14.5 prospermatogonia. The maternally methylated *Lit1* DMR was almost unmethylated. In E16.5 prospermatogonia,

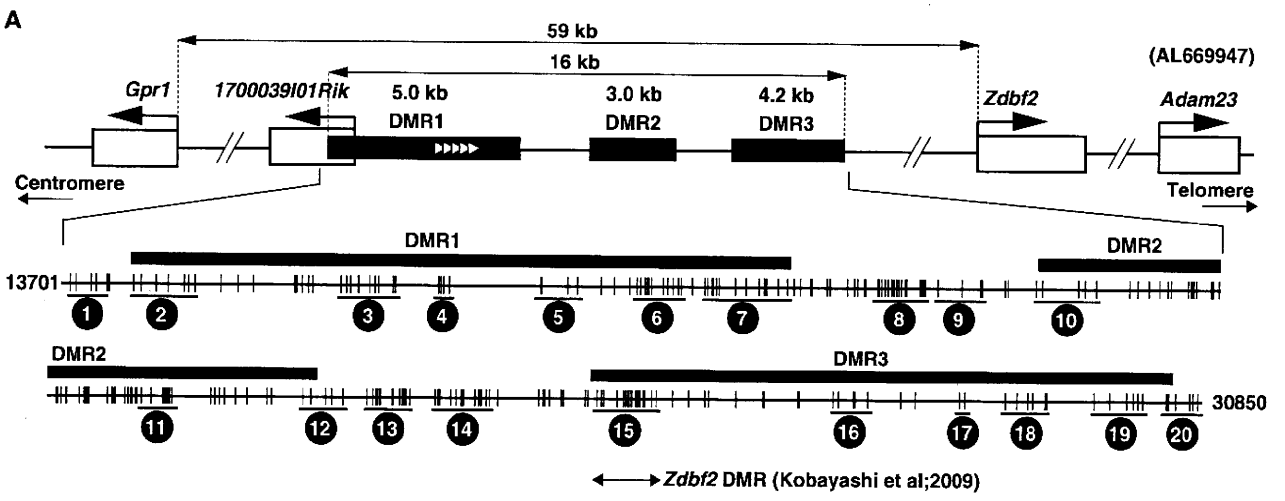

Figure 2. Three paternally methylated DMRs on the *Gpr1-Zdbf2* imprinted domain. (**A**) Physical map of the mouse *Gpr1-Zdbf2* locus (upper panel). Black boxes represent the position of three paternally methylated DMRs, called DMR1 (5.0 kb), DMR2 (3.0 kb) and DMR3 (4.2 kb). Arrows above genes (white boxes) show the direction of transcription. White arrowheads indicate five times repeats of the 37 bp repetitive sequence. Close-up of the three paternally methylated DMRs (lower panel). The vertical bar represents a CpG site. The regions we analyzed bisulfite-PCR methylation sequencings were indicated 1–20. (**B**) Bisulfite-PCR sequencing results for four regions (regions 3, 7, 11 and 15) on genomic DNA prepared from sperm, MII oocytes, blastocysts from B6/JF1 mice and the kidney from B6/JF1 and reciprocal JF1/B6 mice. Each row represents a unique methylation profile within the pool of 20 clones sequenced. Closed and open circles represent methylated and unmethylated CpGs, respectively.

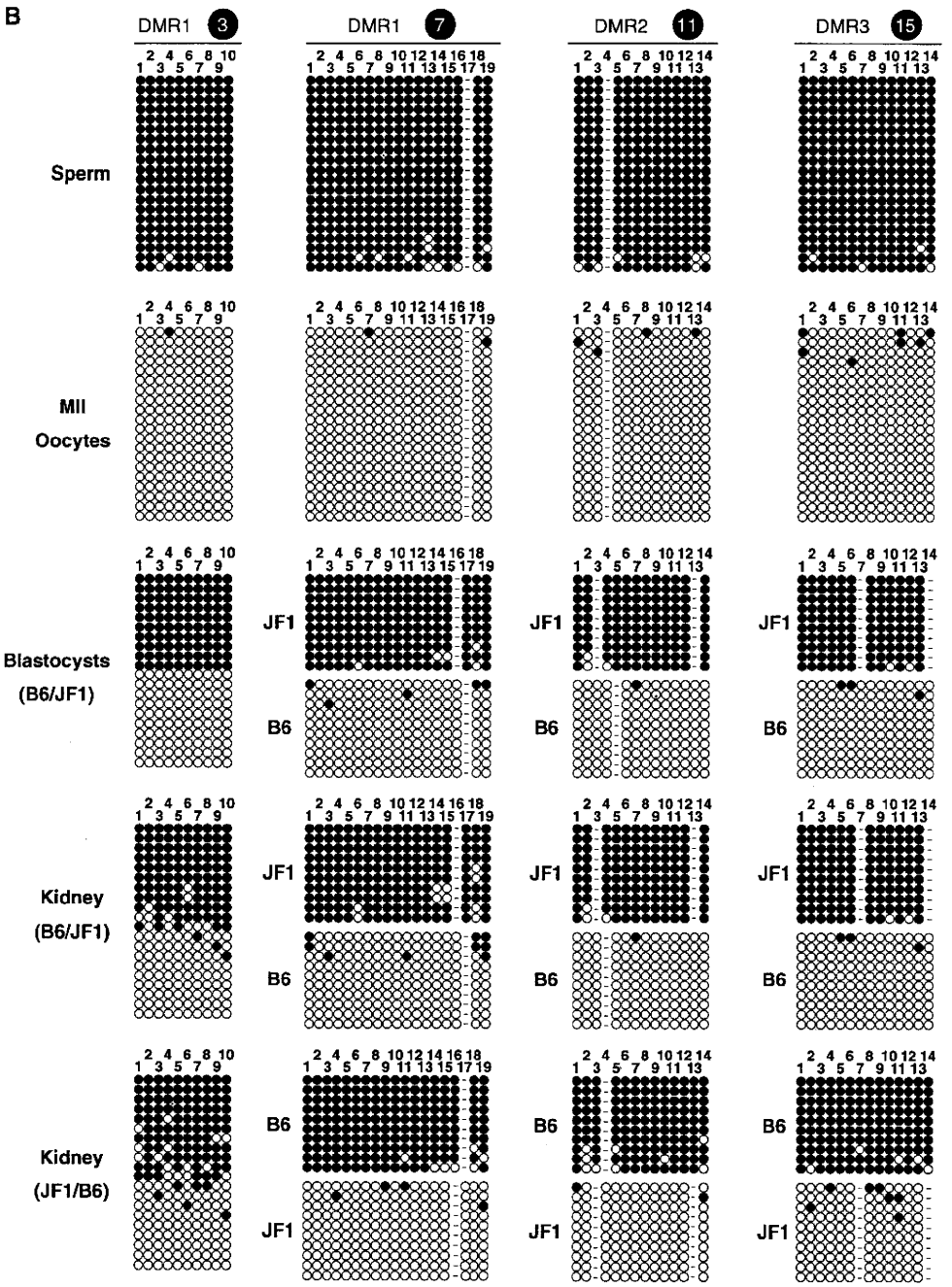

Figure 2. Continued.

methylation at DMR2 had increased, methylation was observed at DMR1 but methylation at DMR3 was mosaic. In E18.5, methylation of DMR2 and DMR3 further increased but DMR1 methylation was still mosaic. These data suggested that the DMR2 region was the first to acquire DNA methylation followed by DMR3 and then DMR1.

The *de novo* methylation of *H19* DMR and IG-DMR (*Gtl2*) is mediated by the *de novo* methyltransferase Dnmt3a (16). We asked whether the *Zdbf2* DMRs were also dependent on Dnmt3a by examining normal and *Dnmt3a*-deficient prospermatogonia. Male germ cells at P7 were isolated from the testes of the conditional *Dnmt3a* knockout mice by FACS as previously described (16). We performed the bisulfite-PCR based assays for the paternally methylated DMRs on this material. The degree of methylation in *Dnmt3a*-deficient prospermatogonia was decreased compared to wild type prospermatogonia

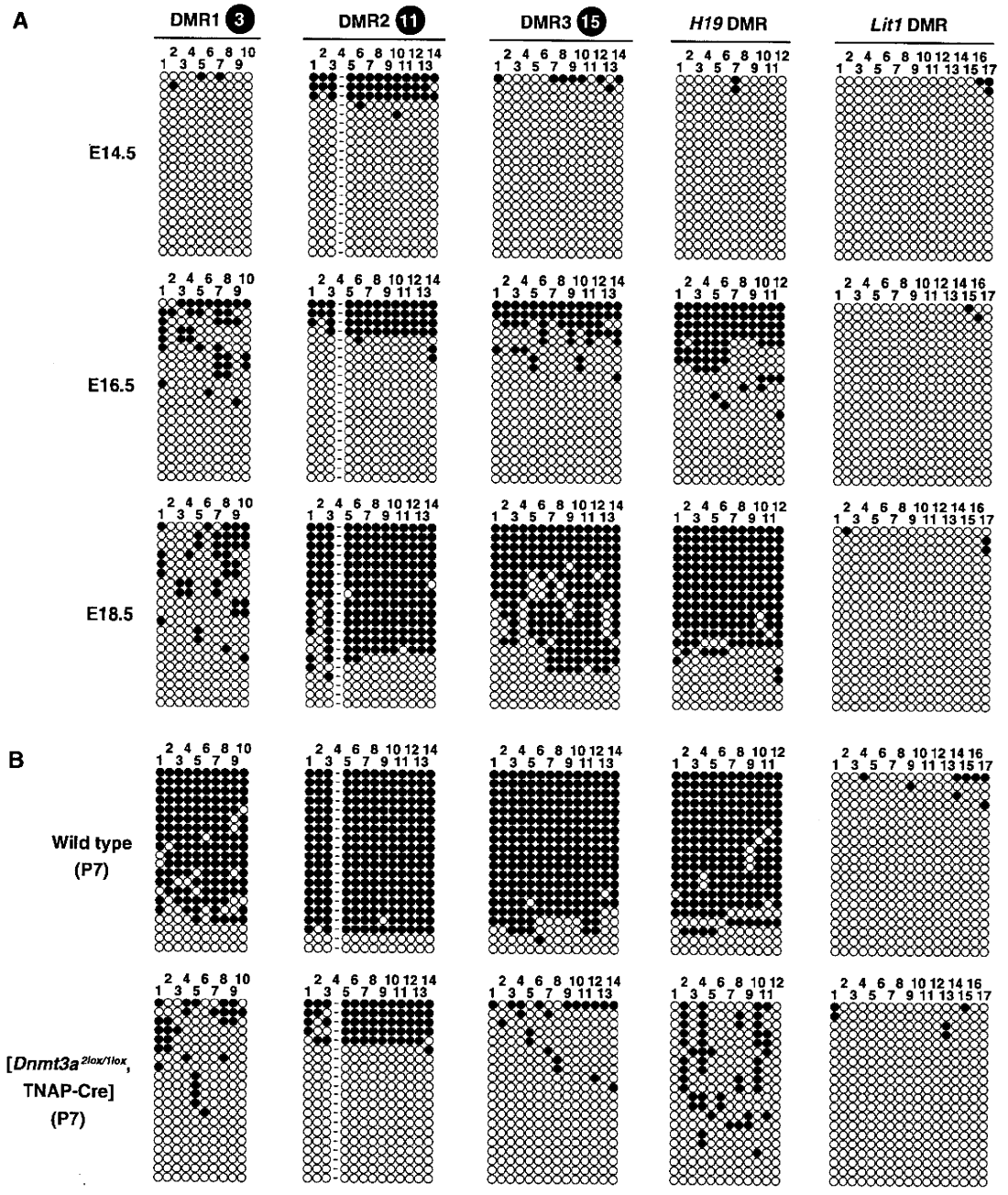

Figure 3. Methylation acquisition during spermatogenesis and absence of methylation imprints in *Dnmt3a*-difecient spermatogonia. (**A**) Methylation status of three *Gpr1-Zdbf2* DMRs in E14.5, E16.5 and E18.5 prospermatogonia. As a control, the paternally methylated *H19* DMR and the maternally methylated *Lit1* DMR were included. The regions we analyzed and the CpG sites in this study are shown in Figure 2A. (**B**) Methylation profile of DMRs in normal and *Dnmt3a*-deficient prospermatogonia. Male germ cells at P7 were isolated from testes of normal and the conditional *Dnmt3a* knockout mice by FACS. The bisulfite-PCR-based assays for the three paternally methylated *Gpr1-Zdbf2* and the *H19* DMRs and maternally methylated *Lit1* DMR.

(Figure 3B). Similar to *H19* DMR and IG-DMR (*Gtl2*), establishment of the *Zdbf2* DMR was dependent on Dnmt3a.

Imprinted genes near *Zdbf2*

Imprinted genes are commonly clustered within the genome. We therefore sought to determine the imprinting status of the nearby *Gpr1* gene. We identified a single nucleotide polymorphism (SNP) in exon 3 of *Gpr1* between the B6 and JF1 strains of mice (Figure 4A). We performed allele-specific reverse transcription-PCR (RT-PCR) sequencing analysis using E18.5 tissues obtained from reciprocal crosses between these strains and also adult material. The transcriptional

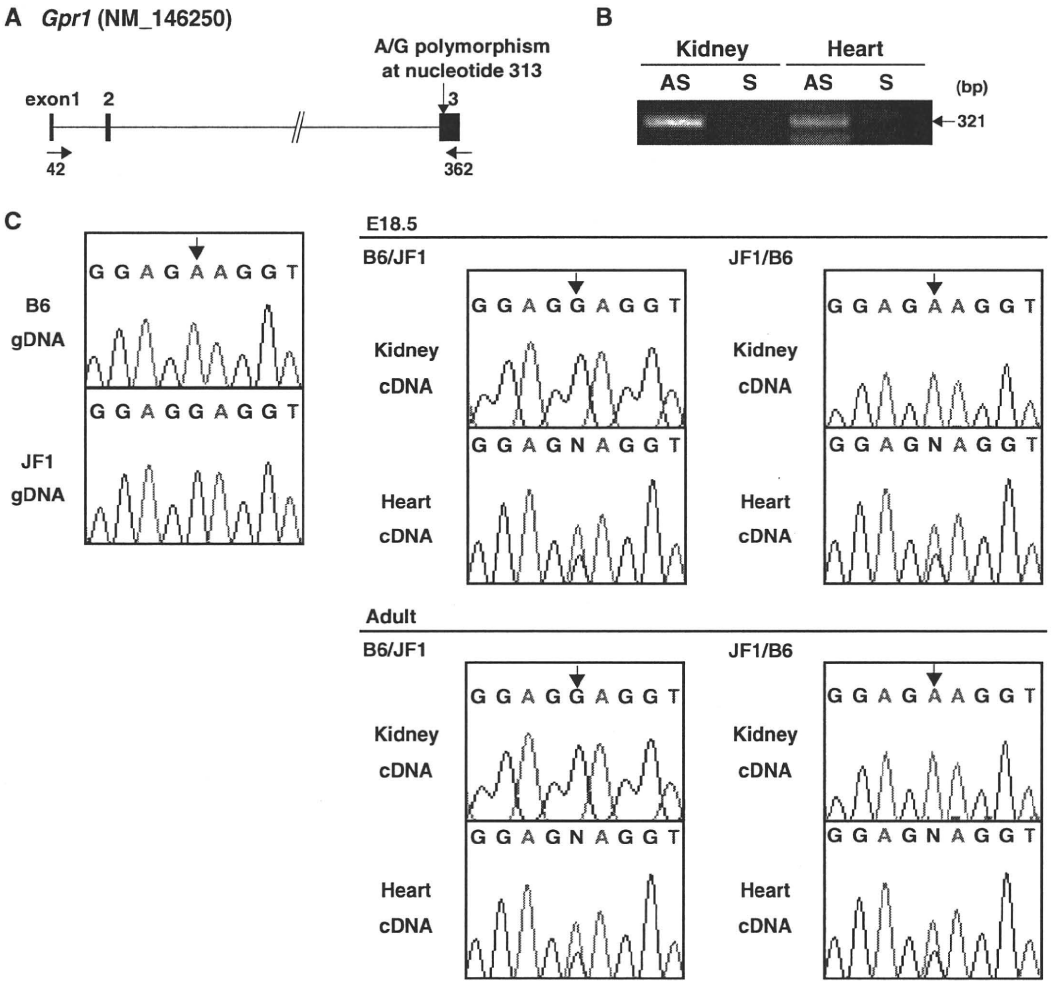

Figure 4. Tissue-specific imprinted expression of mouse *Gpr1*. (**A**) Structure of the mouse *Gpr1* gene. Exons are shown as filled boxes and primers are indicated by arrows. The DNA polymorphism between B6 and JF1 is indicated by the vertical arrow. (**B**) Direction expression analysis of mouse *Gpr1* gene. The first cDNA strands syntheses were performed using either the sense (S) or the antisense (AS) primer of the mouse *Gpr1* gene. Arrow indicates the cDNA product of *Gpr1* gene amplified by RT-PCR on right. (**C**) Allele-specific expression analysis of mouse *Gpr1* gene. cDNA and genomic PCR products were amplified and sequenced directly from E18.5 embryos and adult material obtained from a B6/JF1 and reciprocal crossed JF1/B6 mice.

direction of the RT-PCR products was determined by using either sense or antisense primers as primers for cDNA synthesis (Figure 4B, Supplementary Figure S3A). Only the paternal *Gpr1* allele was detected in kidney cDNA but in brain, lung, liver, heart, spleen, testis and the placenta *Gpr1* was biallelically expressed (Figure 4C and Supplementary Figure S3). We also examined the expressed sequence tag (EST), 1700039l01Rik (GenBank accession number XM 001478509), located ~40 kb upstream of *Gpr1* and overlapping DMR1. This EST consisted of three exons. Using a similar SNP-based assay, we found that the transcript was biallelically expressed in the testis (data not shown). *Adam23* (a disintegrin and metallopeptidase domain 23) (GenBank accession number NM 011780), a gene located ~140 kb downstream of *Zdbf2*, showed biallelic expression in the all tissues which we examined (data not shown).

Expression of mouse *Zdbf2* and *Gpr1*

In order to determine whether *Zdbf2* and *Gpr1* were co-expressed in the same tissues, we examined their expression pattern in E13.5 mouse embryos by *in situ* hybridization. *Zdbf2* was strongly expressed in the mesencephalon, pituitary gland, nasal epithelium, thymus, intestinal epithelium, the mesonephrum in the mouse and in the spongiotrophoblast layer of the placenta (Figure 5A–H). Despite the tissue-specific monoallelic expression of *Gpr1* gene, the gene was widely expressed with the strongest expression being in the diencephalon, dorsal root ganglion, tongue, liver in the mouse embryo and in the spongiotrophoblast layer of the placenta (Figure 5I–M).

Characterizing the *GPR1-ZDBF2* human imprinted domain

We applied the meDIP-on-chip method to human sperm DNA to isolate paternally methylated human DMRs.

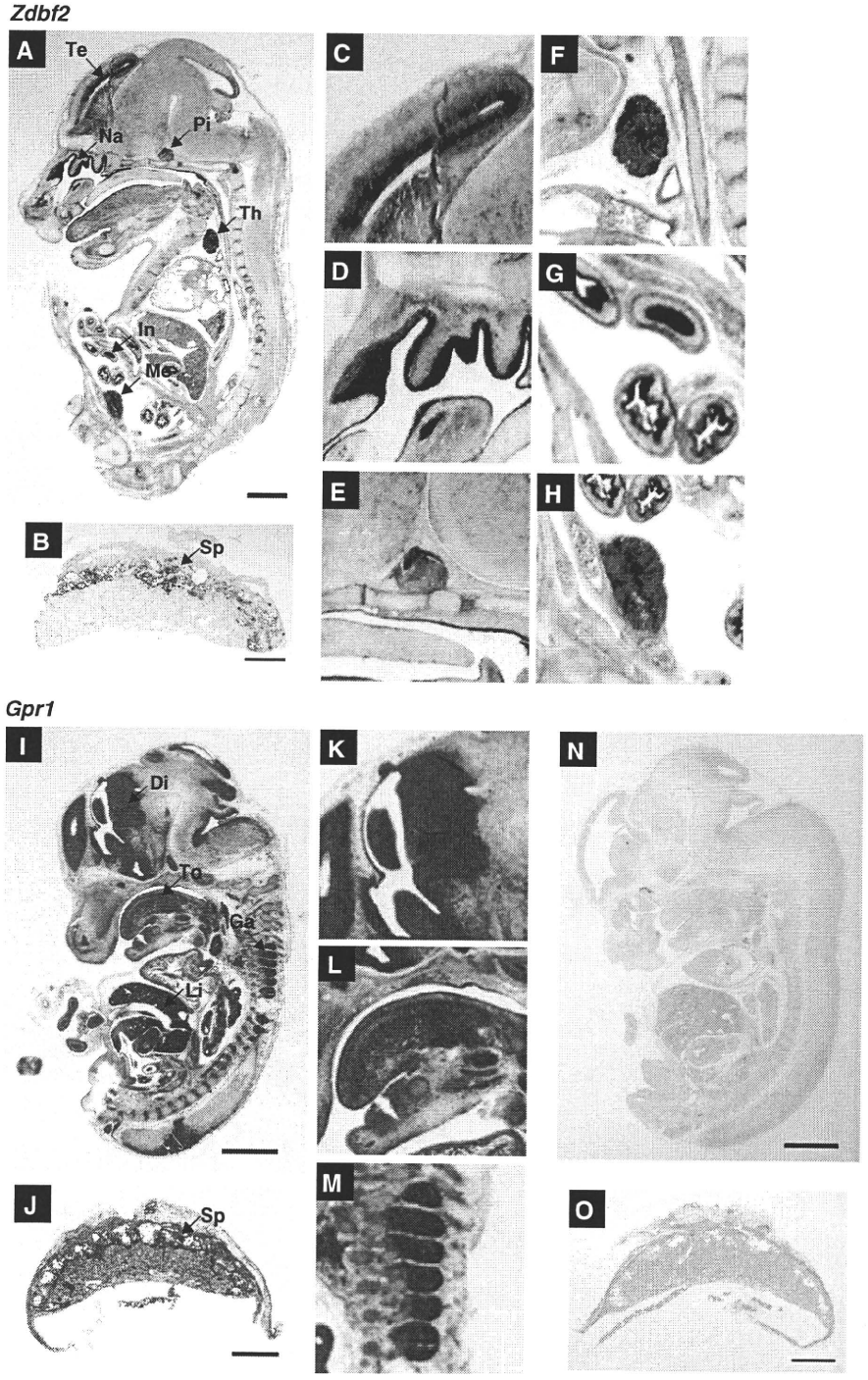

Figure 5. Expression of mouse *Zdbf2* and *Gpr1*. The expression of *Zdbf2* and *Gpr1* were examined in sagittal sections of E13.5 embryo (**A** and **I**) and placenta (**B** and **J**) by *in situ* hybridization. Telencephalon: Te (**C**), nasal epithelium: Na (**D**), pituitary gland: Pi (**E**), thymus: Th (**F**), intestinal epithelium: In (**G**), mesonephrum: Me (**H**), the spongiotrophoblast layer of the placenta: Sp (B), diencephalon: Di (**K**), tongue: To (**L**), dorsal root ganglion: Ga (**M**), liver and the spongiotrophoblast layer of the placenta: Sp (J). No signal was seen with the *Gpr1* sense probe (**N** and **O**). Scale bars indicate 1 mm.

We isolated two regions, which we called DMRh1 and DMRh2 (data not shown, Figure 6A). We examined whether these methylated sequences were DMRs by applying the bisulfite-based PCR methylation assay to genomic DNA isolated from human sperm, blood and placenta. We found that DMRh1 was fully methylated

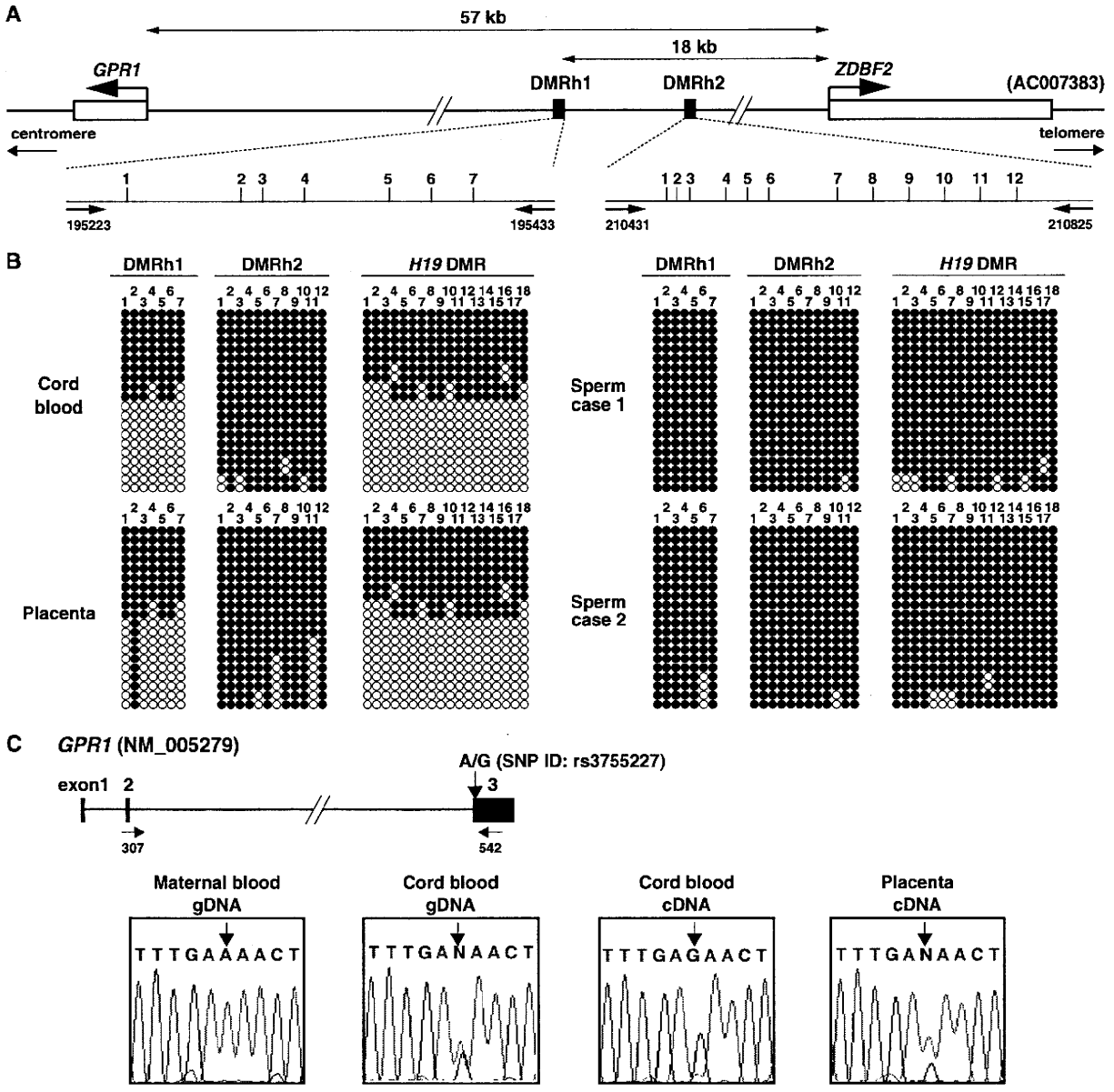

Figure 6. Paternal allele-specific methylation at the region between *GPR1* and *ZDBF2* of human chromosome 2 and imprinting of human *GPR1*. (**A**) Structure of the human region between *GPR1* and *ZDBF2*. Two methylated regions, DMRh1 and DMRh2, identified in meDIP-on-chip of normal human sperm indicated by filled boxes. The vertical bars represent CpG sites. The horizontal arrows represent primer positions. The extent of the regions analyzed in this study and Genbank accession numbers are shown over the line. (**B**) Bisulfite-PCR sequencing of genomic DNA prepared from cord blood, placenta and two cases' sperm. Each row represents a unique methylation profile within the pool of 20 clones sequenced. Closed and open circles represent methylated and unmethylated CpGs, respectively. (**C**) The paternal-specific expression of *GPR1* in human samples. The A/G polymorphic site (SNP ID: rs3755227) in *GPR1* exon 3 is indicated by vertical arrow. Heterozygosity was demonstrated in DNA isolated from cord blood with double peaks in chromatographic sequencing data at the polymorphic residues identified (arrow).

in sperm DNA and ~50% methylated in umbilical cord blood and placental DNA (Figure 6B). In contrast, DMRh2 was fully methylated in all the samples. Part of the DMRh1 sequence (GenBank accession number AC007383; 194613–195967) was similar to part of the mouse DMR1 (GenBank accession number AL669947; 13006–14276) with a 50.6% nucleotide match indicating that we had identified the human homologue of the mouse *Zdbf2* DMR (Supplementary Figure S4).

Human *ZDBF2* is imprinted and expressed only from the paternal allele (26). To determine the allelic expression of *GPR1* (GenBank accession number NM 005279) in human material, we identified an SNP within exon 3 of *GPR1*. We identified the SNPs 3 of 35 cases. We performed RT-PCR analyses on umbilical cord blood and placenta RNA. *GPR1* was expressed from only paternal allele in the three all neonatal leukocytes but not in the placenta (Figure 6C). Both the human and the mouse

GPR1 genes were imprinted and expressed from the paternal genome.

DISCUSSION

In this paper, we report on a novel DNA methylation-based screen for imprinted genes that resulted in the identification of 458 putative DMRs. Of these, 20 were previously characterized DMRs. Several methods for systematical searching for imprinted methylation regions within the mouse genome have been reported. The representative method is restriction landmark genomic scanning with methylation-sensitive restriction endonuclease (RLGS-M), which identified the U2 small nuclear ribonucleoprotein auxiliary factor 35 kDa subunit *(U2afbp-rs)* on mouse chromosome 11 (43), and the *Grf1/Cdc25*^{Mm} on mouse chromosome 9 (44). Another approach based on DNA methylation is called Methylation-sensitive Representational Difference Analysis (Me-RDA/MS-RDA). With this method, two imprinted genes, maternally expressed *Nesp* and paternally expressed *Gnasxl*, were identified at the distal end of mouse chromosome 2 (45,46). In another study using two different methylation-sensitive restriction enzymes, Hin6I (HhaI) and HpaII, three imprinted genes were identified. Interestingly, two of these were located within the intronic regions of other genes (24). Recently, the tiling array technology has been successfully applied to decipher chromatin structure (33,35) using chromatin immunoprecipitation (ChIP-chip) (34). A tiling array approach can provide genome-wide profiling of the methylation pattern in a particular sample when used in combination with a methylated DNA binding column specific to methylated CpG sites (36,37), sodium bisulfite modification (47), and/or the antibody against 5-methylcytosine. In this study, we have demonstrated the power of this technique when applied to studies on genomic imprinting.

The paternally methylated DMRs that we identified on mouse chromosome 1 were located near the imprinted gene, *Zdbf2*. We and another group previously identified *Zdbf2* as an imprinted gene in expression-based screens (26,48) thus validating both approaches. The paternally methylated DMR consisted of three distinct methylated regions interspersed with two non-methylated regions. Similar to the paternal DMRs of *H19* and IG-DMR (*Gtl2*), methylation at the three paternally methylated DMRs was present in the male germline but not in the female germline and was dependent on Dnmt3a, suggesting that all three regions are germ line DMRs. We determined that the *Gpr1-Zdbf2* paternally methylated region spanned 16 kb, which is the longest DMR so far reported (23). We identified a direct repeat sequence in the first *Gpr1-Zdbf2* DMRs. This type of repeat is associated with other imprinted DMRs but its function is still unknown (49,50). When we further characterized the *Zdbf2* domain, we found that *Gpr1/GPR1*, which lies 60 kb from *Zdbf2*, was also paternally expressed. At the human locus, we identified a single, paternally methylated DMR between *GPR1* and *ZDBF2*, and showed that the human *GPR1* gene was also imprinted and paternally expressed in neonatal leukocytes but not in the placenta.

Imprinted genes are regulated by parent-of-origin specific DNA methylation within their DMRs in *cis*. The DMRs on mouse chromosome 1 are paternally methylated. Paternally methylated DMRs are present at only three other imprinted domains, *H19*, IG-DMR (*Gtl2*) and *Rasgrf1* DMRs. DMRs function as imprinting centers, controlling the neighboring imprinted genes (51,52). In the case of the *H19* DMR, and possibly the IG-DMR (*Gtl2*), paternal methylation inhibits the expression of the paternal allele via an insulator that operates as a methylation sensitive boundary (53). The *Gpr1-Zdbf2* DMRs shows more similarity to the *Rasgrf1* DMR as both DNA methylation and active gene expression is from the paternal allele. The imprinted expression of protein coding genes can also be achieved by direct DNA methylation of their promoter (*Peg1*, *Peg3*, *Zac1*) or indirectly, by methylation of the promoter of a long, noncoding antisense RNAs (*Lit1*, *Igf2r*) (54). In the latter case, and in the boundary model, imprinting is achieved by an interplay between the maternally and paternally expressed genes. Currently, there is no evidence of a maternally expressed transcript initiating near the *Gpr1-Zdbf2* DMRs. However, although the EST (1700039I01Rik) was not imprinted in the tissues and at the time points we tested, we cannot exclude imprinting at a different time point or the presence of other imprinted genes lying at a distance from the DMR for this domain.

This work demonstrates the effectiveness of meDIP-on-chip in identifying DMRs. Chromosome 1 was not identified as containing an imprinted domain based on phenotypic studies in mice with maternal or paternal duplications but two approaches have identified an imprinted locus on this chromosome. Conversely, there are regions on mouse chromosome 2 and 12 where imprinted domains are predicted but for which no candidates have been identified (55). Our method has identified a number of novel DMRs providing candidates for these effects. We still do not know which features of DMRs are the most important for attracting germline methylation. For example, CpG-spacing, the presence of repeats, the genomic context of the DMR or a combination of these factors may be involved. Systematic searches will aid in the characterization of common features of paternal and maternal DMRs. These criteria can then be applied to other genomes, including the human genome, to identify novel DMRs. Our method is also suitable for adaptation for other types of epigenetic modification such as a specific histone modification. The identification of new DMRs and imprinted domains will provide novel insights into the mechanism of imprinting and its biological role in mammals.

This work also identified a new imprinted gene that had not been isolated in any expression-based screen. The mouse *Gpr1* encodes a 353 amino acid plasma membrane protein with seven transmembrane domains, which is coupled to the G protein, Gpa2 (56,57). It may therefore play a role in signal transduction. The Gpr1–Gpa2 complex is responsive to glucose and sucrose (58). Several endocrine disorders have been shown to be caused

by either loss- or gain-of-function in G proteins or G-protein-coupled receptors (59). *GNAS* is a complex imprinted locus that produces multiple transcripts. The main transcript derived from *GNAS*, *Gsα*, encodes the α-subunit of the stimulatory guanine nucleotide-binding protein. *Gsα* is expressed biallelically in nearly all tissues and plays essential roles in a multitude of physiologic processes. Other transcripts produced by *GNAS* are expressed exclusively from either the paternal or the maternal *GNAS* allele (60,61). The expression in renal proximal tubules occurs predominantly from the maternal allele and this tissue specific imprinting of *Gsα* is an important role in different kind of pseudohypoparathyroidism (62). We found that the imprinting of *Gpr1* was confined to the embryonic and adult kidney. In others tissues, the gene was not imprinted. This might suggest a functional importance for dosage of *Gpr1* in the development of the kidney.

In summary, using an meDIP method on the whole mouse genome, we identified 458 regions as putative DMRs. We found that the technique successfully identified the majority of known DMRs. The failure to identify two known DMRs was not related to the technique but due to the nature of these sequences, one being highly repetitive and therefore excluded from the array and the second sequence lacking differential methylation in the stem cell material used in the assay. We also further characterized the mouse *Zdbf2* DMR isolated by this technique and found that it had an unusual with a tripartite structure spanning a relatively extensive genomic region. Similar to the *H19* DMR and IG-DMR (*Gtl2*), methylation in the male germline was dependent on Dnmt3a. We have also identified paternal expression of the nearby Gpr1/*GPR1* gene in mouse and human. MeDIP is a powerful, cross-genome method for identifying allele-specific epigenetic marks.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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Assessing loss of imprint methylation in sperm from subfertile men using novel methylation polymerase chain reaction Luminex analysis

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Objective: To assess the clinical value of bisulfite polymerase chain reaction Luminex (BPL), an automated, high-throughput procedure for the detection of alterations in DNA methylation.
Design: Experimental prospective study.
Setting: University research laboratory and private in vitro fertilization (IVF) clinic.
Patient(s): A total of 337 men, 61 with severe oligozoospermia, 67 with moderate oligozoospermia, and 209 with microscopically normozoospermia.
Intervention(s): The ejaculated sperm samples after the routine semen analysis with patients' consent.
Main Outcome Measure(s): Examination of the methylation patterns of eight imprinted loci in sperm DNA, and confirmation with combined bisulfite PCR restriction analysis (COBRA).
Result(s): A total of 47 cases (13.9%) showed abnormal methylation at one or more imprinted loci (18 paternal, 18 maternal, and 11 cases with alterations of both maternal and paternal imprints).
Conclusion(s): The relative ease of the BPL method provides a practical method within a clinical setting to reduce the likelihood of abnormal samples being used in assisted reproduction treatments. (Fertil Steril® 2011;95:129–34. ©2011 by American Society for Reproductive Medicine.)

Key Words: Bisulfite PCR-Luminex methylation analysis, BPL, DNA methylation, genomic imprinting, oligozoospermia, sperm

At differentially methylated regions (DMRs), DNA methylation is a key epigenetic mark that controls the allele-specific expression of imprinted genes (1). Recent studies have identified an increased incidence of Beckwith-Wiedemann syndrome and Angelman syndrome in infants who were conceived by assisted reproduction treatment (ART) (2–4). Because ART involves the isolation, handling, and culture of gametes and early embryos at a time when the epigenetic marks at imprinted loci are relatively vulnerable to external influences (5), some data have been suggestive that imprinting errors may occur during the ART process, both in in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) procedures (6–14). However, our recent work and that of others have suggested that subfertile men, particularly those with oligozoospermia, already carry preexisting imprinting errors in their sperm (15–17).

Southern blotting was the original technique routinely used to analyze DNA methylation (18). This method, which requires a relatively large quantity of DNA (5–10 μg), has largely been superseded by methods involving the sodium bisulfite treatment of genomic DNA, which converts unmethylated cytosine to uracil and leaves the methylated cytosines unconverted. The methylation status of a specific sequence is then measured by the combined bisulfite polymerase chain reaction (PCR) restriction analysis (COBRA) or by DNA sequencing of the PCR product. The combination of COBRA and the sequencing method provides accuracy and sensitivity; nonetheless, there are still limitations with this method, particularly in the expertise required to obtain accurate results, the time needed to achieve a result, and the relative cost, rendering it less suitable for clinical diagnosis. Recently, PCR Luminex was developed as a high-throughput, high-resolution genotyping method to be applied clinically for the detection of different alleles at human leukocyte antigen (HLA) (19) and diabetes mellitus loci (20). This method combines PCR and sequence-specific oligonucleotide probe (SSOP) protocols with the Luminex 100 xMAP flow cytometry dual-laser system to quantitate fluorescently labeled oligonucleotides attached to color-coded microbeads.

We have further developed this technique by combining the PCR amplification-SSOP protocol with Luminex technology. We compared the accuracy of the PCR-Luminex method to the COBRA/sequencing method using the same bisulfite-treated DNA. In a proof-of principle experiment, we applied these techniques to examine DNA methylation at eight DMRs in the sperm DNA of the 337 patients. Our analysis provides further evidence that methylation errors at imprinted loci are more frequent in oligozoospermic men. The BPL methylation analysis is a simple, accurate, rapid approach and thus is suitable for clinical applications.

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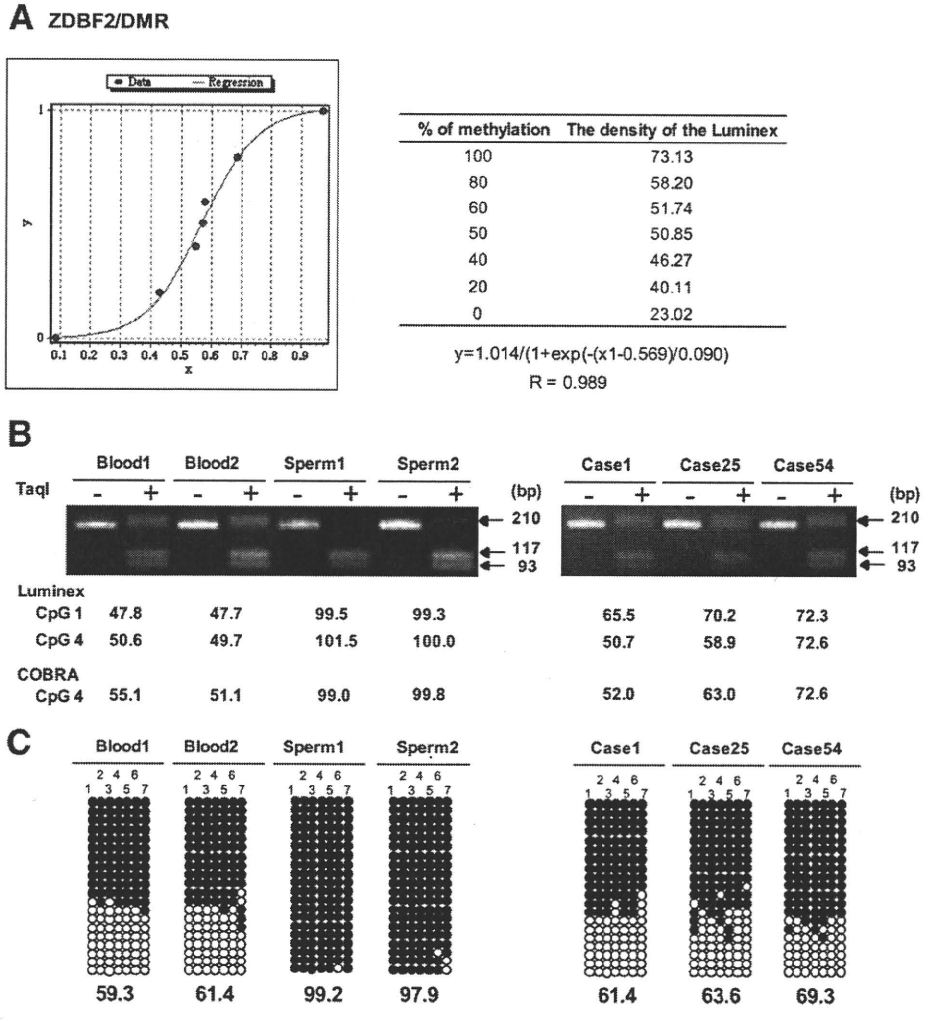
FIGURE 1

Examination of the *ZDBF2* imprinted domain by bisulfite polymerase chain reaction Luminex (BPL) and combined bisulfite polymerase chain reaction restriction analysis (COBRA) assay. (**A**) Mixture of the methylated to unmethylated plasmids (100%, 80%, 60%, 50%, 40%, 20%, and 0 methylated) used to calculate the methylation rate using a regression curve equation. (**B**) DNA methylation analyses by COBRA of genomic DNA prepared from normal sperm and leukocytes (*left*) and from sperm of the oligozoospermic patients (*right*). (**C**) Bisulfite PCR sequencing of genomic DNA prepared from normal sperm and leukocytes (*left*) and from sperm of the oligozoospermic patients (*right*). Each row represents a unique methylation profile within the pool of 20 clones sequenced. Closed and open circles represent methylated and unmethylated CpGs, respectively. The number represents the percentage of methylation by bisulfite sequencing.



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

MATERIALS AND METHODS

Sperm Collection

Ejaculated sperm samples were collected from 337 male patients presenting to a single physician at a private clinic (St. Luke Clinic) with fertility problems. Routine semen analysis (volume, counting, rates of motility, and morphologic abnormality) was performed according to the World Health Organization guidelines (21). Of these patients, 61 had severe oligozoospermia ($<5 \times 10^6$/mL), 67 had moderate oligozoospermia ($5–20 \times 10^6$/mL), and the remaining 209 had a normal sperm count ($\geq 20 \times 10^6$/mL). Purification of motile sperm cells and DNA extraction were performed immediately after the routine examination of the ejaculated sperm, as previously described elsewhere (16). The study was approved by the Tohoku University Medical Department ethics review board, and was performed with patient consent.

BPL Methylation Assay

Sperm DNA samples were treated with sodium bisulfite using an EZ DNA Methylation Kit (Zymo Research, Orange, CA). An in vitro methylated DMR was generated as a control by incubation of a plasmid containing the DMR region with CpG methylase (*Sss*I methylase; New England Biolabs, Ipswich, MA) according to the manufacturer's instructions.

Eight PCR primers sets, biotinylated at their 5'-end, were designed for gene amplification of eight DMRs (Supplementary Table 1, available online; Supplementary Fig. 1, available online). The PCR reaction mix contained 0.2 μM primer, 0.2 mM dNTPs, 1x PCR buffer (50 mM KCl and 10 mM Tris-HCl; pH 8.3), 3 mM $MgCl_2$, 2% dimethyl sulfoxide (DMSO), 0.625 IU of Taq DNA Polymerase (Roche, Tokyo, Japan), and 100–200 ng of bisulfite-treated DNA in a total volume of 25 μL. The PCR conditions were as follows:

TABLE 1

Proportions of imprinting errors at eight differentially methylated regions in the sperm samples.

Microscopic examination	ZDBF2	H19	GTL2	PEG1	LIT1	ZAC	PEG3	SNRPN
Normal (n = 209)	0.0 (0/119)	0.49% (1/204)[a]	0.99% (2/201)[a]	5.43% (7/129)[a]	1.05% (1/95)	0.0% (0/120)	1.57% (2/127)	0.0 (0/124)[a,b]
Moderate (n = 67)	1.67% (1/60)	1.64% (1/61)[b]	10.71% (6/56)[b]	13.79% (4/29)	0.0 (0/7)	1.78% (1/56)[a]	4.00% (1/25)	6.67% (4/60)[a]
Severe (n = 61)	4.25% (2/47)	14.04% (8/57)[a,b]	27.27% (15/55)[a,b]	21.74% (5/23)	11.11% (1/9)[a]	6.55% (4/61)[a]	4.76% (1/21)	3.27% (2/61)
Total (n = 337)	1.32% (3/226)	3.11% (10/322)	7.37% (23/312)	8.84% (16/181)	1.80% (2/111)	2.11% (5/237)	2.31% (4/173)	2.45% (6/245)[b]

Note: Normal = normozoospermia ($\geq 20 \times 10^6$/mL); Moderate = moderate oligozoospermia ($5–20 \times 10^6$/mL); Severe = severe oligozoospermia ($\leq 5 \times 10^6$/mL).

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

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

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40 cycles of 95°C for 20 seconds, 60°C for 30 seconds, and 72°C for 30 seconds using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA).

Oligonucleotide probe sequences (Supplementary Table 1, available online) were synthesized with a terminal amino group and were covalently bound to carboxylated fluorescent microbeads (Multi-Analyte Microsphere Carboxylated; Luminex, Austin, TX) using ethylene dichloride (EDC), following the recommended procedures. These oligonucleotide-labeled microbeads (oligobeads) were mixed together to make an oligobead mixture of 100 oligobeads/μL and were hybridized to the 5'-biotin-labeled PCR amplicons in a total volume of 50 μL per well in a 96-well plate by adding 5 μL of the appropriate oligobead mixture and 5 μL of the PCR amplicons to 40 μL of hybridization buffer (3.75 M TMAC, 62.5 mM TB [pH 8.0], 0.5 mM EDTA, and 0.125% N-lauroylsarcosine). This reaction mixture was first denatured at 95°C for 2 minutes and then hybridized at 48°C for 30 minutes.

After hybridization, the oligobeads were washed in 100 μL of phosphate-buffered saline (PBS)-Tween and pelleted by microcentrifugation at 3,300 rpm for 1 minute using a swing-out microwell plate rotor. Pelleted oligobeads were reacted with a 70-μL aliquot of a 100x diluted solution of streptavidin-phycoerythrin (SA-PE; G&G Science Co., Ltd., Japan) in PBS-Tween. Hybridized amplicons were labeled with SA-PE at 48°C for 15 minutes. Reaction outcomes were measured by the Luminex 100 flow cytometer. Bead populations were detected and identified using the 635-nm laser. The PE fluorescence of the SA-PE-biotin-labeled amplicons that had hybridized to the oligobeads was quantitated using the 532-nm laser. Median fluorescence intensity (MFI) of PE was used to quantify the amount of DNA bound to the oligobeads. The measured data were read using dedicated software. The fluorescence intensity of negative controls was subtracted as background from each of the MFI values to determine the true intensity.

Conventional Bisulfite Treatment PCR Methylation Assay

Methylation assays were performed for the DMRs using COBRA and the bisulfite sequencing techniques as described previously elsewhere (16). An average of 20 clones were sequenced for each individual.

Statistical Analysis

Two proportions were used to analyze the observed data using the difference between two proportions test (Statistica; StatSoft, Tokyo, Japan). $P<.05$ was considered statistically significant. Statistical significance between BPL and COBRA was determined with a Spearman's rank method and Pearson's product-moment correlation coefficient.

RESULTS

Development of BPL Methylation Method

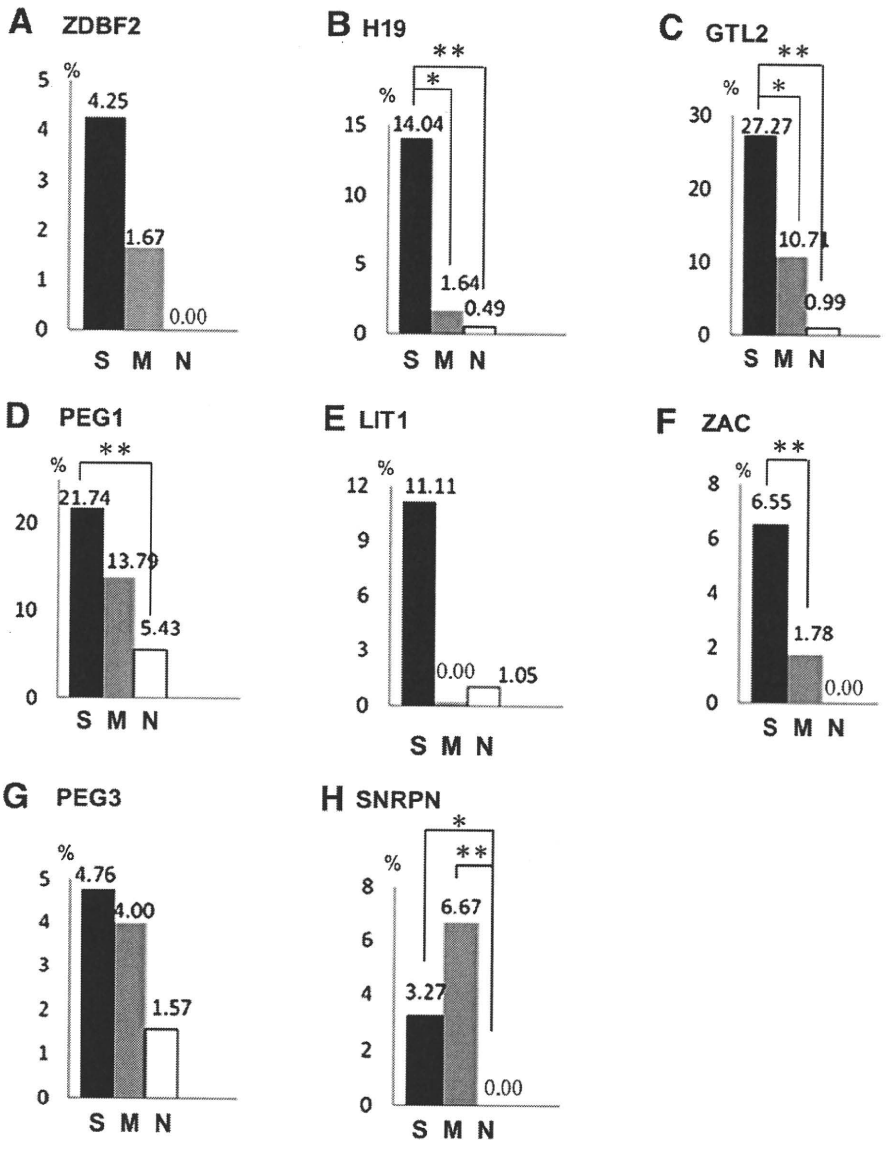
The bisulfite conversion technique essentially generates two different bases, cytosine and uracil (methylation and nonmethylation), in response to the methylation status of the cytosine residue. The PCR-Luminex method can identify a single base substitution by specific hybridization. Therefore, we investigated whether a combination of these techniques might provide a novel way to analyze DNA methylation.

We first evaluated the BPL technique by comparing an in vitro methylated plasmid containing the *ZDBF2* DMR versus an unmethylated version. By mixing the plasmid at ratios of 100%, 80%, 60%, 50%, 40%, 20%, and 0, methylated to unmethylated, we calculated a standard curve for the methylation ratio using a regression curve (as a representative sample at *ZDBF2* DMR CpG site 4: Fig. 1A). The square (R^2 value) of the coefficient at the *ZDBF2* DMR was 0.989. Likewise, R^2 values at the DMR of *H19, GTL2, ZAC, PEG1, PEG3, LIT1,* and *SNRPN* were 0.995, 0.997, 0.992, 0.993, 0.993, 0.988, and 0.993, respectively.

We assessed the methylation status of normal human leukocyte DNA and normal sperm DNA (Fig. 1B, C). A "no DNA" sample was used as a control to eliminate background noise, and BPL values

FIGURE 2

Association between DNA methylation errors and sperm concentrations. Statistically significant differences between the two groups: *$P<.05$; **$P<.01$.



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were then used to calculate the methylation ratio. All imprinted DMRs by BPL were approximately 50% methylated in the somatic cell and fully methylated or unmethylated pattern, as appropriate, in the germ cell.

Validation of BPL Method

We compared the values obtained from the BPL method to those obtained from the COBRA, and performed a statistical analysis with a Spearman's and Pearson's rank correlation. We found that *H19, GTL2, PEG1, ZAC,* and *SNRPN* showed a good correlation but *ZDBF2, PEG3,* and *LIT1* did not. This was due to a few abnormal values. Three samples showed under 90% methylation at the *ZDBF2* DMR in sperm, but all the others were methylated to over 90%. Similarly, the number of samples showing over 10% methylation at *PEG3* and *LIT1* DMRs was four and two, respectively.

We determined the cutoff value of the BPL technique. When the value of the BPL methylation assay and COBRA in the paternally methylated DMR *ZDBF2* was more than 95%, agreement rates were low, and the cases of disagreement were examined in 39.0%. However, in the case of more than 90%, it was high: 100%. Likewise in the paternally methylated DMRs, *H19* and *GTL2* in the case of over 90% were high agreement rates, 92.1% and 96.2%, respectively. On the other hand, in the maternally methylated DMRs,