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(タイトル)	Metabolic Profile and Body Composition in Adults with Prader-Willi Syndrome and Severe Obesity
(タイトル翻訳)	Prader-Willi 症候群と重症肥満を有する成人患者の代謝プロファイルと身体組成
(著者)	Charlotte Höybye, Agneta Hilding, Hans Jacobsson, and Marja Thorén
(書誌事項)	J Clin Endocrinol Metab 2002, 87: 3590–3597
(背景)	Prader-Willi 症候群 (PWS)患者の成人に関する調査では、心血管疾患と重症肥満の罹患率と死亡率の増加について指摘されている。
(目的)	PWS 患者で心血管疾患の罹患率と死亡率の増加の危険因子を特定する。
(対象と方法)	PWS の成人患者 19 名 (男性 10 名、平均年齢 25 歳) の臨床所見、遺伝、内分泌および代謝検査について検討した。また、身長、体重、血圧、BMI などの測定を行った。体脂肪率の測定と空腹時糖負荷試験 (OGTT) も行った。
(結果)	平均 BMI は 35.6kg/m² で、男性 3 名以外すべての患者が高い BMI を示した。全例の骨密度 (BMD) は性別、年齢別の正常値より低かった。4 名が骨粗鬆症、11 名が骨密度減少症であった。 平均の IGF-I は低くかった。18 名のうち 9 名の GH 負荷試験ではピーク値が最低限の 3μg/l よりも低く、9 名は 3.7-16.0μg/l の間の値を示した。すべての患者の陰毛成長は Tanner stage V であった。12 名が性腺機能低下症であった。レプチン濃度と体脂肪率の相関がみられた。血中インスリン濃度は 29-151pmol/l の間であった。IGFBP-1 濃度は 3-88μg/l で、糖尿病を有していた患者 1 名は過高値を示した。OGTT で患者 1 名は糖尿病と診断し、心不全も認められた。 全患者は Holm's の PWS 診断基準を満たし、そのうち 13 名は、メチル化テストで陽性を示し、残り 6 名は陰性であった。この二つのグループを比較した場合、陰性グループには IGF-I 血清濃度が低く、より高い身長 SD 点数を示す傾向があった。血圧、インスリン、ヘモグロビン A、レプチン、脂質レベルが両グループ間で類似していた。
(結論)	PWS では、心血管疾患の罹患率と死亡率の増加がみられた。その危険因子の一つとして、異常な OGTT パターンと GH 欠乏 (GHD) がみられた。このため、成人の PWS 患者のホルモン療法の重要性を強調する。

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(タイトル)	Growth Hormone Treatment of Adults with Prader-Willi Syndrome and Growth Hormone Deficiency Improves Lean Body Mass, Fractional Body Fat, and Serum Triiodothyronine without Glucose Impairment: Results from the United States Multicenter Trial
(タイトル翻訳)	成長ホルモン欠乏を有するPrader-Willi症候群の成人に対する成長ホルモン療法は、耐糖性障害はなく、体重、体脂肪率および血清トリヨードサイロニンを改善する：米国の多施設臨床試験の結果
(著者)	Harriette R. Mogul, Phillip D. K. Lee, Barbara Y. Whitman, William B. Zipf, Michael Frey, Susan Myers, Mindy Cahan, Belinda Pinyerd, and A. Louis Southren
(書誌事項)	J Clin Endocrinol Metab 2008, 93: 1238–1245
(背景)	小児のPrader-Willi症候群 (PWS) ではGH療法の有効性と副作用について広く知られている。PWS成人患者へのGH療法の効果については明らかではない。
(目的)	GH遺伝子機能を欠如するPWS成人患者に対し、GH療法の有効性と安全性を評価する。
(研究デザイン)	6か月間の至適投与とその後6か月間の維持投与によるオープン・ラベル多施設共同試験
(対象)	様々な認知技術、行動特性、および生活環境にある痩せと肥満のPWS成人患者を対象とした。
(介入)	ヒト・リコンビナントGH (Genotropin) を最初に0.2mg/日投与して、毎月0.2mg/日の増分で、許容量 (1.0mg/日を超えない) まで増量した。
(メインな判定法)	体重と体脂肪率は、二元的エネルギーX線吸光法で測定された。
(結果)	6-12か月間GH投与を行い、最終投与量の平均値を0.6mg/日とした30名の患者の体重は42.65±2.25 (SE) から45.47±2.31kg (P≤0.0001) まで増加し、体脂肪率は42.84 ±1.12から39.95 ± 1.34% (P=0.025) まで減少した。38名の研究対象 (糖尿病患者5名を含む) の平均の空腹時血糖値は85.3±3.4 mg/dl、ヘモグロビンA1cは5.5 ±0.2%、空腹時インスリン濃度は5.3 ± 0.6 μU/ml、インスリンの血中濃度曲線化面積 (AUC) が60.4± 7.5 μU/ml、HOMA-IRが1.1 ±0.2で、正常範囲であった。T_3は、すべての患者で正常値で、127.0 ±7.8から150.5 ± 7.8 ng/dl (P= 0.021) まで、26.7%増加した。そのうち、6名 (20%) は、2SDより低かった。緩進行性の足首浮腫は治療中にみられた最も重大な副作用であった (5名) 。
(結論)	この多施設共同研究では、GH療法が身体組成を改良し、T_3を正常化し、成人PWSに対し耐糖性障害がなく、有効であることが証明された。

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(タイトル)	The spectrum of Silver-Russell syndrome: a clinical and molecular genetic study and new diagnostic criteria
(タイトル翻訳)	Silver-Russell 症候群の臨床像 : 臨床経過と分子遺伝学解析による新しい診断基準
(著者)	S M Price, R Stanhope, C Garrett, M A Preece, and R C Trembath
(書誌事項)	J Med Genet 1999, 36: 837–842
(背景)	Silver-Russell 症候群 (SRS) は正常頭囲を保った重症子宮内発育遅延によって特徴づけられる。子宮内発育遅延は貧弱な体型と低い身長を特徴とする。顔面異形症と非対称は典型的な特徴と考えられる。
(目的)	SRS の臨床経過と遺伝学的特徴を研究する。
(対象と方法)	57 名の SRS の患者に対し、妊娠経過、分娩経過、出生後の診察記録を調査した。発育遅延や特別教育についても調査した。身長、体重、頭囲、四肢長と周囲長を測定した。遺伝子解析と染色体解析を行った。
(結果)	57 名のうち、7 名は SRS から除外された。50 名は、男女それぞれ 25 名いた。31 名は典型的な顔面特徴を示し、そのうち、出生時体重 (BW) が−2SD より低かったのが 25 名だった。19 名が軽度の顔面特徴を示し、その内 BW が−2SD より低かったのが 11 名であった。子宮内発育遅延は 29 名にみられた。すべての患者の平均 BW は−2.94SD であった。56%に摂食障害がみられた。発汗と顔色の青白さは 52%にみられた。13 名 (52%) の男性が生殖器手術を受けた。1 名の女性は双角子宮であった。38 名の就学年齢以上の患者のうち、14 名 (38%) が特別教育が必要であった。 17 名 (34%) は身体非対称であり、典型的な顔面特徴も示した。斜指症と短い小指以外に、10 名の典型的顔面特徴をもっていた患者では、終末指関節の関節拘縮症、屈指症をすべての症例でみられた。2 名にカフエ・オレ斑と、1 名に白斑がみられた。 42 例のうち、4 名に UPD7 がみられた。3 名は−2SD より低い BW で、非対称の人はいなかった。1 名に典型的な顔面表現型がみられた。 出生時低体重、典型的顔面特徴および非対称の特徴を同時に持つ患者、1 名を除いて、すべての患者は正常な核型がみられた。
(結論)	突出した額と三角形の顔を持つ低出生体重児では、小指の斜指症を合併した場合、SRS と診断される可能性が高い。UPD7 で、摂食障害や、低血糖症がない患者は、摂食状態や発汗とかんしゃくについて詳しく検査するのが重要である。SRS では UPD7 の頻度は高いが、厳密な診断基準を用いると少なくなるかもしれない。

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(タイトル)	Gastrointestinal Complications of Russell-Silver Syndrome: A Pilot Study
(タイトル翻訳)	Silver-Russell 症候群の胃腸合併症：パイロット・スタディー
(著者)	Jeff Anderson, David Viskochil, Molly O'Gorman, and Chad Gonzales
(書誌事項)	Am J Med Genet 2002, 113: 15-19
(背景)	Silver-Russell 症候群 (SRS) は子宮内と出生後の発育遅延、正常な頭囲、身体非対称と独特な顔面特徴という臨床症状を伴う。SRS と関連した胃腸障害についての報告はあるが、SRS に特異性なものとして明らかになっていない。
(目的)	SRS 症例における胃腸合併症の特徴を明らかにする。
(対象と方法)	SRS と診断された患者の疫学調査: (1) 頻度 (2) 胃腸 (GI) 障害 (3) 栄養摂取、三部構成によって行われた。
(結果)	約 500 部の疫学調査票のうち、135 部が回収された。そのうち 74%は 5%以下の低身長で、79%は子宮内発育遅延 (IUGR) を示した。SRS と GI 合併症の関連を評価するため、身長が 5% (平均より-2SD 下) より高く、小頭症 (平均より-2SD 下) の患者を除いた。除かれたのは 58 名だった。診断基準として、「低身長、正常頭囲、IUGR と非対称」を重要な所見とし、4 つのうち 2 つ以下を満たした患者は除かれた。3 つ満たした患者は低血糖症、三角の顔などの所見より再評価した。 65 名の患者は、GI 合併症の発生率が一般人口よりかなりの増加がしていた。最も頻度が高いものは胃食道逆流病 (GERD) であった。GERD の新生児の発生率は 300 人のうち 1 人だが、被調査者の比率は 34%であった。さらに、25%が逆流性食道炎、25%が食物嫌悪、18%が Nissen fundoplication であった。GI 合併症を有するグループは以下の症状の増加を示した：低血糖症 (36% vs. 25%) (P=0.2389) 、青色強膜 (59% vs. 9%) (P=0.0006) 、腎臓異常 (12% vs. 2%) (P=0.2810) 。
(結論)	この疫学結果では、GI障害がSRSにおいて重要であることを示している。特に、逆流性食道炎とその結果発症するNissen fundoplicationの比率が高かった。これはSRS患者の摂食障害の特徴を示す。GI合併症は、SRSの診断に役立つかもしれない。

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(タイトル)	Molecular Basis of Neonatal Diabetes in Japanese Patients
(タイトル翻訳)	日本の新生児糖尿病の遺伝子解析
(著者)	Shigeru Suzuki, Yoshio Makita, Tokuo Mukai, Kumihiro Matsuo, Osamu Ueda, and Kenji Fujieda.
(書誌事項)	J Clin Endocrinol Metab 2007, 92 (10) : 3979-3985
(背景)	新生児糖尿病 (NDM) は臨床的に一過性のTNDMと永続性のPNDMに分類される。TNDM の場合、インスリンの分泌が生後数か月に自然回復する。一方、PNDM の場合、一生インスリン治療が必要である。この二つの病態の原因遺伝子が異なる。
(目的)	原因となる遺伝子の機能を明らかにし、臨床的特徴との関連性を調べる。
(対象と方法)	31名の日本人患者 (16名がTNDM、15名がPNDM) について、染色体6q24領域の異常と、KCNJ11 と ABCC8 の点突然変異を解析した。また、IPEX症候群を合併する患者1名と、膵臓非形成の患者1名は、FOXP3 と IPF1 の点突然変異について解析した。
(結果)	23 名において NDM 患者の遺伝子解析を行った : 11 名に 6q24、9 名に KCNJ11、2名に ABCC8、1名に FOXP3 に異常がみられた。6q24 異常を示すすべての患者と KCNJ11 変異の患者2名が TNDM であった。新生児の突然変異が5つみられた : 2名 (A174G と C166Y) に KCNJ11、2名 (A90V と N1122D) に ABCC8、1名 (P367L) に FOXP3。6q24 異常と KCNJ11 変異を比較して、いくつかの重要な臨床的な相違点がみられた。6q24 異常の患者の場合、糖尿病の発症が早期、糖尿病性ケトアシドーシスの頻度は低く、巨舌症も高率にみられた。一方、2名の KCNJ11 変異伴う患者はてんかんと発育遅延を示した。
(結論)	6q24 異常と KCNJ11 変異が日本の NDM 発症の主な原因である。臨床像の違いは、遺伝子解析に有用な情報を提供する

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(タイトル)	Neonatal Diabetes: New Insights into Aetiology and Implications
(タイトル翻訳)	新生児糖尿病：病因と病態に関する新しい洞察
(著者)	Julian P.H. Shield
(書誌事項)	Horm Res 2000, 53 (suppl 1) : 7-11
(背景)	新生児糖尿病 (NDM) は生後数週間にみられる高血糖症と定義されている。永続性 (PNDM) と一過性 (TNDM) があり、最近まで、その病気についてほとんど分かっていなかった。
(目的)	NDM の病因と病態について明らかにする。
(対象と方法)	30 名の TNDM 患者の病歴と、6 番染色体の異常について解析した。PNDM に関しては、以前報告された論文を参照した。
(結果)	30 名の発症の平均年齢は生後 3 日目で、出生時体重の平均値は 2.1kg であった。11 名 (37%) はインスリンか、血中'C'ペプチド濃度を測定し、すべて高血糖症と診断、インスリン反応性が極しく低かった。インスリン投与治療期間の平均は 12 週間で、最長では 14 か月であった。13 名 (43%) は、膵島細胞抗体を調べ、陰性を示した。23%が巨舌症を示し、7%が臍ヘルニアを示した。 38%の患者は UPD6 を、38%の患者は、6q24 領域の 6 番父親染色体重複を示した。残り 24%は、6 番染色体の異常を明らかにすることはできなかった。後遺症では、4 歳以上の 18 名のうち、11 名 (61%) が糖尿病の再発を示した。
(結論)	TNDM に関連する遺伝子の解析は、膵臓発生期の機能評価を可能にする。TNDM、子宮内発育遅延、ベータ細胞の機能低下と II 型糖尿病との関連を評価すれば、この遺伝子の更なる評価は、一般人口で、II 型糖尿病への進展に関する評価が可能であるかもしれない。

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
有馬隆博	ヒト卵子・精子・胚のエピジェティクス	森崇英	卵子学	京都大学学術出版会	京都	2011	122-131
有馬隆博	生殖補助医療（ART）とエピジェティクスの異常	日本哺乳動物卵子学会	生命の誕生に向けて（第２版）	近代出版	東京	2010	281-283

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
有馬隆博	ARTにおけるエピジェティクス異常	産婦人科の実際	60	741-750	2011
有馬隆博	母子の健康と環境影響	助産雑誌	65	1000	2011
有馬隆博	ゲノムインプリンティングと発がん	癌と化学療法	38	1745-1749	2011
有馬隆博	胎盤とエピジェネティクス	HORMONE FRONTIER IN GYNECOLOGY	17	349-345	2010

(作成上の留意事項)
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3．日本工業規格Ａ列４番の用紙を用いること。各項目の記入量に応じて、適宜、欄を引き伸ばして差し支えない。

Ⅳ.参考資料

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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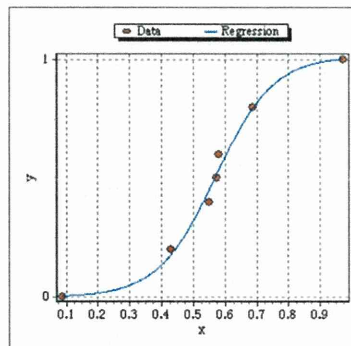
FIGURE 1

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.

A ZDBF2/DMR

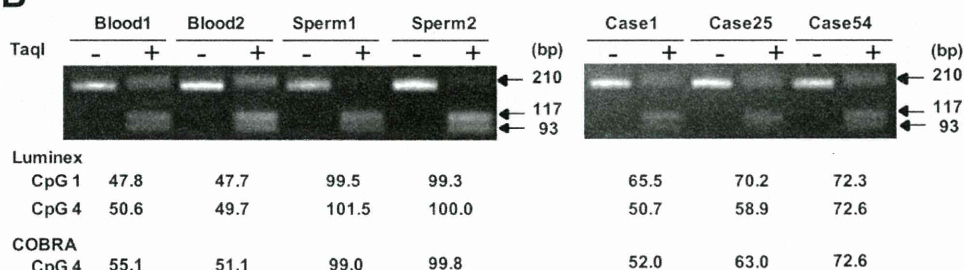


% of methylation	The density of the Luminex
100	73.13
80	58.20
60	51.74
50	50.85
40	46.27
20	40.11
0	23.02

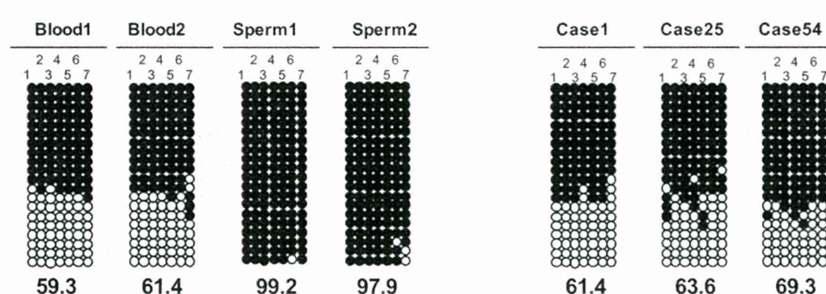
$y=1.014/(1+\exp(-(x1-0.569)/0.090)$

$R = 0.989$

B



C



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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

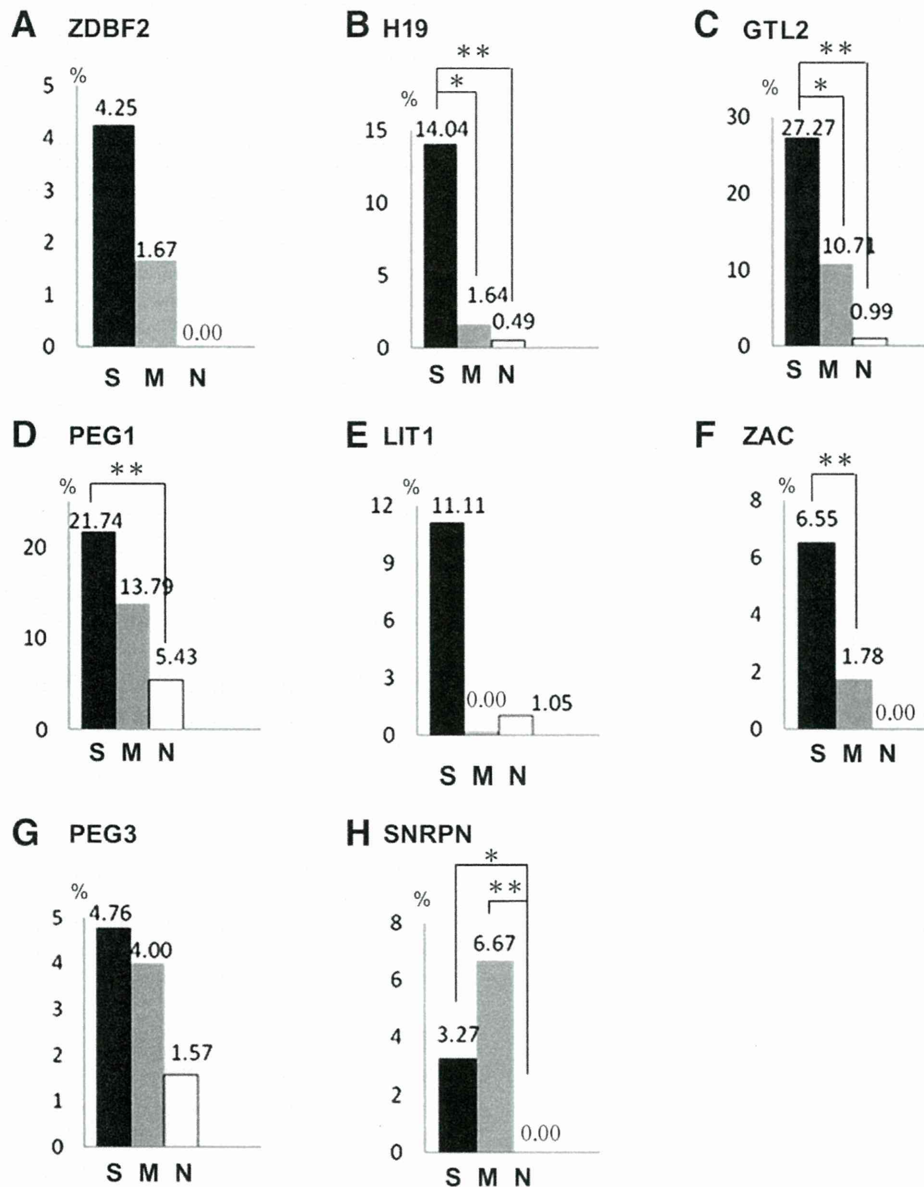
The bisulfite conversion technique essentially generates two different bases, cytosine and uracil (methylation and nonmethylation), in response to the methylation status of the cytosine residue. The PCR-Luminex method can identify a single base substitution by specific hybridization. Therefore, we investigated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

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.

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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.

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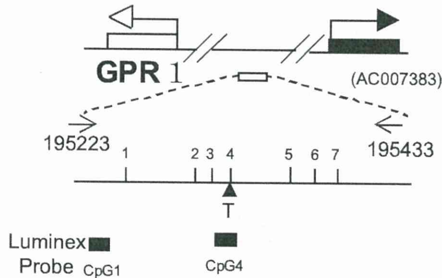
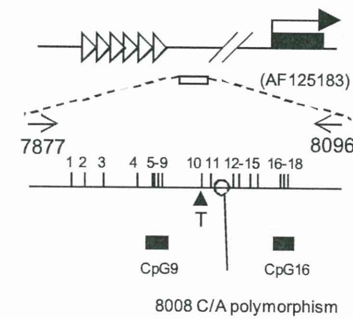
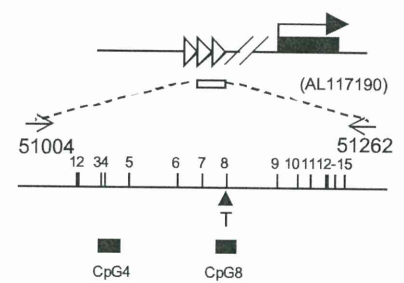
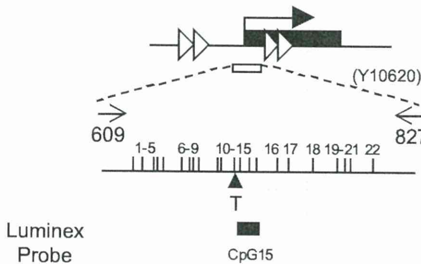
SUPPLEMENTARY FIGURE 1

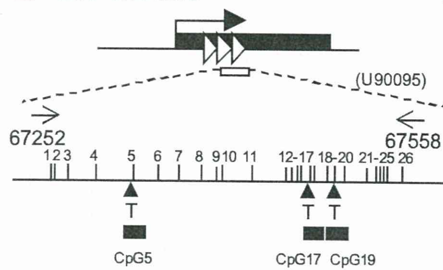
Genomic structures of human differentially methylated regions (DMRs). (**A**) *ZDBF2,* (**B**) *H19,* (**C**) *GTL2,* (**D**) *PEG1,* (**E**) *LIT1,* (**F**) *ZAC,* (**G**) *PEG3,* and (**H**) *SNRPN.* Extent of the regions analyzed in this study and GenBank accession numbers are shown under the line. 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.

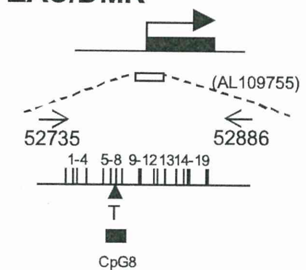


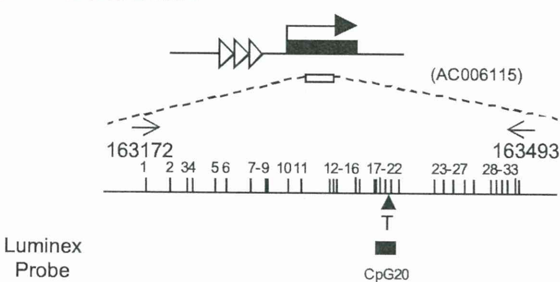


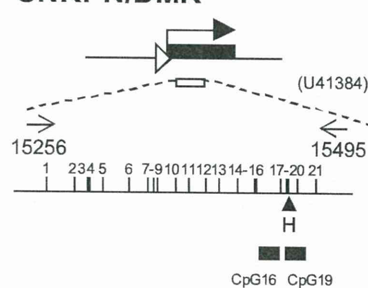








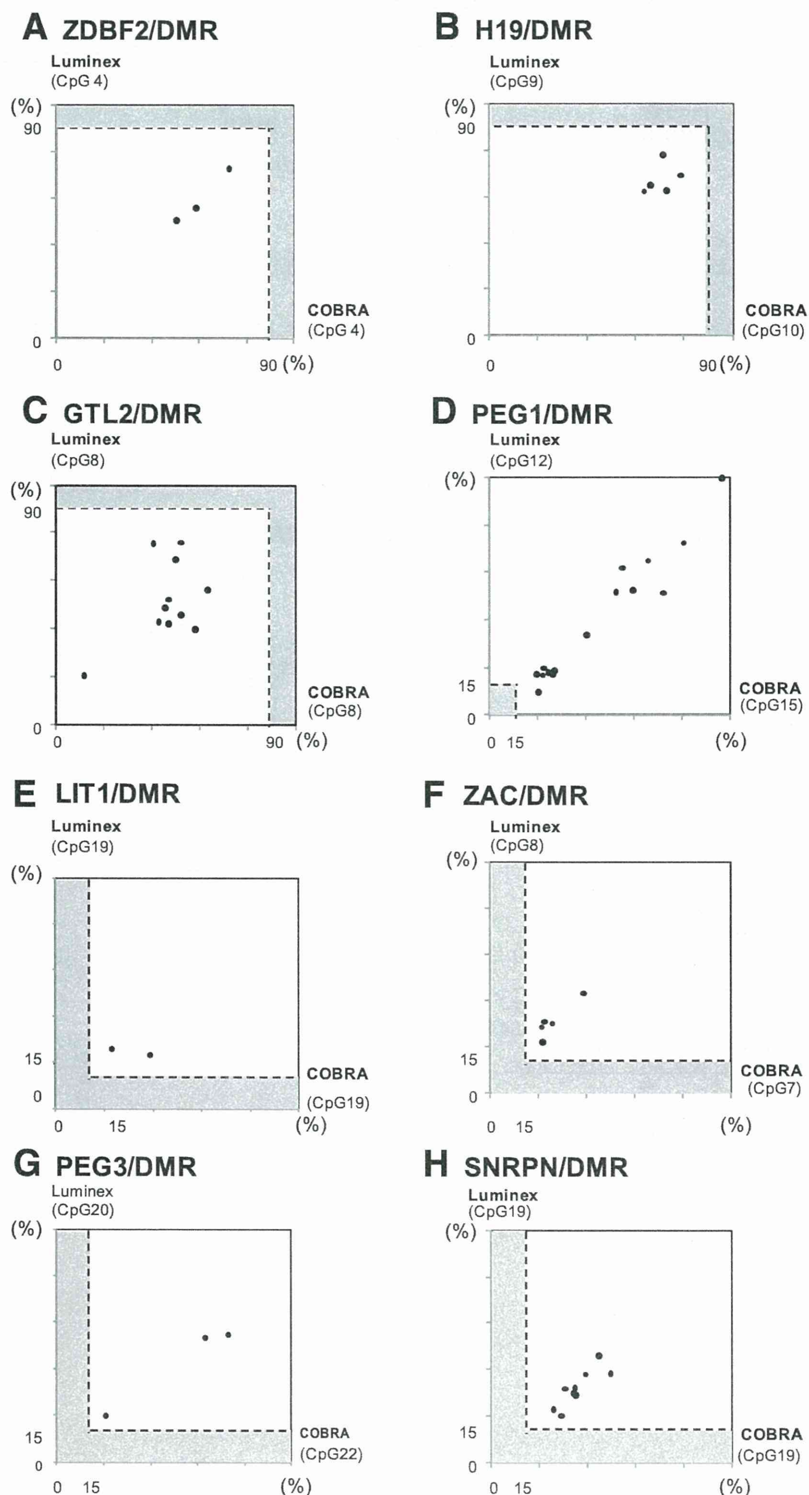




Sato. Imprint methylation loss in oligozoospermia. Fertil Steril 2011.

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).



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

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.

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

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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Re-investigation and RNA sequencing-based identification of genes with placenta-specific imprinted expression

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Within the vertebrate groups, only mammals are subject to a specialized epigenetic process termed genomic imprinting in which genes are preferentially expressed from one parental allele. Imprinted expression has been reported for >100 mouse genes and, for approximately one-quarter of these genes, the imprinted expression is specific to the placenta (or extraembryonic tissues). This seemingly placenta-specific imprinted expression has garnered much attention, as has the apparent lack of conserved imprinting between the human and mouse placenta. In this study, we used a novel approach to re-investigate the placenta-specific expression using embryo transfer and trophoblast stem cells. We analyzed 20 genes previously reported to show maternal allele-specific expression in the placenta, and only 8 genes were confirmed to be imprinted. Other genes were likely to be falsely identified as imprinted due to their relatively high expression in contaminating maternal cells. Next, we performed a genome-wide transcriptome assay and identified 133 and 955 candidate imprinted genes with paternal allele- and maternal allele-specific expression. Of those we analyzed in detail, 1/6 (*Gab1*) of the candidates for paternal allele-specific expression and only 1/269 (*Ano1*) candidates for maternal allele-specific expression were authentically imprinted genes. Imprinting of *Ano1* and *Gab1* was specific to the placenta and neither gene displayed allele-specific promoter DNA methylation. Imprinting of *ANO1*, but not *GAB1*, was conserved in the human placenta. Our findings impose a considerable revision of the current views of placental imprinting.

INTRODUCTION

Genomic imprinting is an essential mechanism in mammalian development that regulates the preferential expression of the paternally or maternally inherited allele of a subset of genes. Within vertebrate lineages, imprinting appears to be restricted to eutherian mammals and marsupials (1). Imprinting arose during mammalian evolution and could thus be linked to placental development and function (2,3). Various theories have been proposed to explain the biological and evolutional significance of this phenomenon (4,5).

One of the key defining features of eutherian mammals is the chorioallantoic placenta. This structure, which infiltrates the maternal uterus, has a pivotal role in embryonic growth and development through regulating the transport of nutrition, gas and waste between fetal and maternal blood (6,7). A large number of imprinted genes are expressed in the placenta and it has been proposed that some of these control the supply of

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nutrients to the fetus (8–11). In contrast, imprinted genes expressed in the embryo may determine nutritional demand by regulating the growth rate of fetal tissues (3). Importantly, low birth weight has implications for postnatal development and has been linked to the development of certain diseases later in life (12), highlighting the critical role of the placenta both in the neonatal period and, more perniciously, in the adult.

In the mouse, X chromosome inactivation does not occur randomly in extraembryonic lineages and genes subject to X-inactivation in female mice display tissue-specific imprinted expression in the placenta but are expressed mosaically in the embryo and adult (13,14). Similarly, there are ~30 autosomal genes, which have been reported to show imprinted expression only in the placenta (or extraembryonic tissues) (15–17). Remarkably, almost all of these genes specifically imprinted in the placenta are active on the maternal allele and repressed on the paternal allele.

The mechanisms for maintaining imprinted expression in the embryo may differ from those at work in the placenta as loss of the maintenance DNA methyltransferase, Dnmt1, results in the relaxation of imprinting of some genes preferentially in the embryo (18,19). For some genes with placenta-specific imprinted expression, imprinted gene expression depends on H3-K9 dimethylation (H3K9me2) and/or H3-K27 trimethylation (H3K27me3) and is impaired in the absence of G9a and EED, which may be a consequence of lineage-specific, temporal dependence on long non-coding RNAs (9,15,16). A comprehensive, whole genome analysis aimed at identifying genes with placenta-specific imprinted expression may provide a clearer picture regarding the requirement of imprinted gene expression in the placenta versus the embryo. However, the mouse placenta consists of contributions from both the mother and the fetus (6) raising the possibility that strategies aimed at identifying novel imprinted genes may be confounded by contaminating material. There are several potential sources of contamination as complete removal of the decidua from the placenta is difficult, maternal cells are also known to exist in the spongiotrophoblast and labyrinth layers (20–22) and the ectoplacental cone is already invaded by maternal blood at embryonic day (E) 6.5 (15).

To assess the importance of this issue, we re-investigated the imprinted status of genes previously reported to show placenta-specific imprinted expression first using an embryo transfer procedure to identify the maternal cell contribution and then using trophoblast stem (TS) cells grown in culture away from the maternal environment. In addition, we performed genome-wide screen to identify all the genes that might fall into this same category, either as contaminants or imprinted genes.

RESULTS

Imprinted gene expression in the placenta without maternal decidua

The expression level of 27 genes, previously reported to show placenta-specific maternal allele expression, was first determined by quantitative polymerase chain reaction (QPCR) in the maternal decidua of E13.5 placenta after dissection (Fig. 1A). Of the 27 genes examined, 6 (*Cntn3*, *Klrb1f*, *Art5*, *Cmah*, *Drd1a*, *Fbxo40*) were expressed at negligible levels in the placenta. Low expression of these genes was also confirmed in the whole transcriptome sequencing as described below. Ten of 21 genes with placenta-specific imprinted expression showed more than 10 times higher expression in the decidua than in the placenta (Fig. 1A). The preferential expression in the decidua was also confirmed using *in situ* hybridization for *Gatm*, *Tfpi2* and *Ampd3* (Fig. 1B).

In order to determine whether there was any remaining maternal contamination after surgical removal of the maternal decidua, we employed an embryo transfer strategy. C3H/HeJ (C3H) embryos were transferred into pseudopregnant C57BL/6 (B6) mice. Placentas derived from this mating strategy are composed of a C3H embryonic component and a B6 maternal component. E13.5 placentas were again collected and the decidua was carefully removed. After removal of the decidua, the remaining material was subjected to genomic DNA amplification over a polymorphism between the C3H and B6 strains spanning the *Gapdh* gene. This revealed that most of maternal cells were removed when dissecting away the deciduas (Fig. 2A).

Single nucleotide polymorphisms (SNPs) between C3H and B6 were used to examine the expression of three genes highly expressed in the decidua and previously reported to be imprinted (*Wt1*, *Gatm* and *Qpct*). When *Wt1* was amplified from genomic DNA obtained from the placenta after removal of the decidua, the peak of the B6 allele was near background level, consistent with the very small level of maternal contamination in this dissected material (Fig. 2B). However, analysis of the cDNA from dissected material revealed predominant expression of the *Wt1* B6 allele (Fig. 2B). A similar pattern was obtained with *Gatm* and *Qpct* (Supplementary Material, Fig. S1A). These data demonstrated that, even after the careful removal of the decidua, there was still sufficient maternal cell contamination to significantly impact expression studies.

The analyses of the placenta-specific imprinting using embryo transfer and TS cells

As described above, maternal cell contamination was a significant factor in the analysis of imprinted gene expression in the mouse placenta. We therefore set up an experiment to ask how many of the genes previously reported to show maternal allele expression in the placenta might have been falsely identified. To distinguish between maternal allele-specific expression and maternal contamination, embryos obtained by crossing Japanese fancy 1 (JF1) females and B6 males ([JF1xB6]F1) were transferred to pseudopregnant B6 recipients. Placentas derived from this embryo transfer experiment were composed of [JF1xB6]F1 embryonic cells and B6 maternal cells. Genes expressed from the maternal allele would carry JF1 SNPs, while genes expressed in contaminating maternal cells would have the B6 SNPs. Genes expressed from the paternal allele also carry B6 SNPs. E13.5 placentas were collected, the decidua was carefully removed as before and the allelic expression was determined. We could confirm that 6 out of

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