表2　BWSにおける遺伝子異常の頻度と症状との関連

遺伝子異常のタイプ	頻度(%)	BWS症状および腫瘍リスクとの関連性
IC2低メチル化	～50	臍帯ヘルニア，片側肥大，ART出生BWS 腫瘍リスク中(～10%) 肝芽腫，Wilms腫瘍以外の腫瘍
IC1高メチル化	2～7	片側肥大 腫瘍リスク高(20～30%) Wilms腫瘍，肝芽腫
patUPD	～20	低血糖・片側肥大 腫瘍リスク高(20～30%) Wilms腫瘍，肝芽腫
*CDKN1C*変異	～10	臍帯ヘルニア，口蓋裂 腫瘍リスク低(<5%)
11番染色体異常 (重複，転座，逆位等)	<2	発達遅滞(重複) 腫瘍リスク不明
上記の異常を認めない	～25	不明 腫瘍リスク不明

8．予後

小児期以降の予後は良好である。

9．フォローアップ

12歳くらいまでは腹部超音波検査で3カ月ごとに腫瘍をスクリーニングする。また，4歳までは2～3カ月ごとにα-fetoprotein(AFP)も測定する。腎奇形に伴う石灰沈着や腎結石に関しては，年1回の頻度で思春期中期まで腹部超音波検査を行う。

10．家族会など

BWS親の会(大森敏秀胃腸科クリニック内)
連絡先：〒362-0075　埼玉県上尾市柏座2-8-2
Tel：090-1435-2301
mail：air@m2.hinocatv.ne.jp
ホームページ：www.beckwith-wiedemann.com
なお，遺伝子解析は筆者の研究室で行っているので，詳細はお問い合わせいただきたい。

Sotos症候群

1．概念

Sotos症候群は，特徴的な顔貌，過成長，学習障害を基本症状とする過成長症候群である(図1D～F)。主な症状として，行動障害，先天性心奇形，新生児黄疸，腎奇形，脊柱側彎，てんかん発作などがある[5]。脳性巨人症ともいわれる。

2．頻度

約14,000出生に1人とされている。95%以上は孤発例であり，家族例の場合は常染色体優性遺伝形式をとる。

3．遺伝子

NSD1(nuclear receptor binding SET domain protein 1)のハプロ不全で発症し，約80～90%の患者で*NSD1*遺伝子の異常を認める[6]。*NSD1*の遺伝子座は5p35で，ヒストンH3リシン36(H3K36)メチル化酵素をコードしている。H3K36メチル化は遺伝子の転写伸長にかかわるが，NSD1の標的遺伝子は明らかでない。日本人症例では，*NSD1*を含む5p35領域の微小欠失が約50%を占めるが，日本人以外の症例では遺伝子内変異が60～80%を占める。5p35欠失症例は遺伝子内変異症例に比べて学習障害が重度である。一方，遺伝子内変異症例のほうが高身長を示す。また，心奇形は5p35欠失症例に多い傾向がある[7]。

4．出生前診断

巨頭症や高身長などは非特異的な所見であるため，超音波検査では正確な診断は難しい。家系内に*NSD1*異常症例が存在する時に限り，羊水およ

表3　Sotos 症候群の臨床症状(Tatton-Brown ら，2005 より引用一部改変)[7]

基本症状 (90%以上の症例でみられる)	・特徴的顔貌 ・学習障害 ・過成長	
主症状 (15～89%の症例でみられる)	・行動障害 ・頭部 MRI/CT の異常 ・てんかん ・骨年齢促進 ・脊柱側彎 ・関節の過弛緩，扁平足	・新生児期の黄疸，筋緊張低下，哺乳不全 ・心奇形 ・腎奇形 ・母体の妊娠高血圧腎症
関連症状 (2～15%の症例でみられる)	・近視 ・遠視 ・乱視 ・斜視 ・白内障 ・眼振 ・コレステリン腫 ・伝音性難聴 ・頭蓋骨癒合 ・歯数不足 ・漏斗胸 ・脊椎異常 ・第2・第3趾間合指症 ・内反尖足 ・拘縮 ・胃食道逆流	・便秘 ・甲状腺機能低下症 ・高カルシウム血症 ・新生児低血糖 ・臍帯ヘルニア ・鼠径ヘルニア ・停留睾丸 ・陰嚢水腫 ・尿道下裂 ・包茎 ・片側肥大 ・発育不全 ・皮膚の色素沈着過剰 ・皮膚の低色素沈着 ・腫瘍 ・血管腫

び絨毛組織を用いた出生前診断が可能である。

5. 出生後診断

臨床診断は基本症状三つ(特徴的顔貌，学習障害，過成長)があれば可能であり，*NSD1* 異常症例の90%以上でこれらの基本症状を呈する(**表3**)。3症状を満たさない場合は，*NSD1* の遺伝子解析により確定診断できる。特徴的顔貌は，Sotos 症候群に最も特異的であり，特に1～6歳にかけて顕著である。頬部紅潮，前頭部の疎な毛髪，前額部の突出，眼瞼裂斜下，細長い顔，細く突き出た下顎を呈する。学習障害については，早期からの発達遅延が非常によくみられる。また，大きな体格，筋緊張低下，協調運動性の低さから運動技能が遅れ，言語発達遅滞もよくみられる。学習障害の程度は軽度から重度まで多様である。過成長については，身長あるいは頭囲が+2 SD 以上を示す。

鑑別診断として Weaver 症候群，Beckwith-Wiedemann 症候群，Simpson-Golabi-Behmel 症候群，Bannayan-Riley-Ruvalcaba 症候群，脆弱 X 症候群などがある。

6. 診療上の留意

学習障害・心奇形・腎奇形・てんかん発作・脊柱側彎についての詳細な病歴を聴取する。小児の場合，診断がついたら心エコーと腎臓の超音波検査を行い，成人の場合は，腎臓超音波で慢性膀胱尿管逆流による腎障害を検査して，重篤な合併症を検出することが重要である。伝音声難聴の検査の検査も行う。

7. 治療

新生児黄疸に対して光線療法を行う。哺乳不全に対しては経鼻チューブ栄養を行うこともある。胃食道逆流に対しては，体位に注意する。各臨床症状については適切な専門家にコンサルトする。

8. 予後

予後はよい。身長は成長とともに目立たなくなるが，巨頭症は成人後も認められる。

9. フォローアップ

幼児期には，1，2年に一度のフォローを行う。小児期の腫瘍リスクは低いため，腫瘍スクリーニングは推奨されない。

10. 家族会など

Show's Home Page：http://www.askashow.com/

Sotos 症候群についての解説や，相談の場として開設。

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics

Aberrant Methylation of H19-DMR Acquired After Implantation Was Dissimilar in Soma Versus Placenta of Patients With Beckwith–Wiedemann Syndrome

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Gain of methylation (GOM) at the H19-differentially methylated region (H19-DMR) is one of several causative alterations in Beckwith–Wiedemann syndrome (BWS), an imprinting-related disorder. In most patients with epigenetic changes at H19-DMR, the timing of and mechanism mediating GOM is unknown. To clarify this, we analyzed methylation at the imprinting control regions of somatic tissues and the placenta from two unrelated, naturally conceived patients with sporadic BWS. Maternal H19-DMR was abnormally and variably hypermethylated in both patients, indicating epigenetic mosaicism. Aberrant methylation levels were consistently lower in placenta than in blood and skin. Mosaic and discordant methylation strongly suggested that aberrant hypermethylation occurred after implantation, when genome-wide de novo methylation normally occurs. We expect aberrant de novo hypermethylation of H19-DMR happens to a greater extent in embryos than in placentas, as this is normally the case for de novo methylation. In addition, of 16 primary imprinted DMRs analyzed, only H19-DMR was aberrantly methylated, except for NNAT DMR in the placental chorangioma of Patient 2. To our knowledge, these are the first data suggesting when GOM of H19-DMR occurs.

Key words: Beckwith–Wiedemann syndrome; H19-DMR; aberrant DNA methylation; after implantation

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Am J Med Genet Part A 158A:1670–1675.

INTRODUCTION

Beckwith–Wiedemann syndrome (BWS) is an imprinting-related condition characterized by macrosomia, macroglossia, and abdominal wall defects (OMIM #130650). The relevant imprinted chromosomal region in BWS, 11p15.5, consists of two independent imprinted domains, *IGF2/H19* and *CDKN1C/KCNQ1OT1*. Imprinted genes within each domain are regulated by two imprinting control regions (ICR), the H19-differentially methylated region (H19-DMR) or KvDMR1 [Weksberg et al., 2010]. Several causative alterations have been identified in patients with BWS: loss of methylation (LOM) at KvDMR1, gain of methylation (GOM) at H19-DMR, paternal uniparental disomy (UPD), *CDKN1C* mutations, and chromosomal abnormality involving 11p15 [Sasaki et al., 2007; Weksberg et al., 2010].

Additional supporting information may be found in the online version of this article.

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Methylation of H19-DMR is erased in primordial germ cells (PGCs) but becomes reestablished during spermatogenesis [Li, 2002; Sasaki and Matsui, 2008]: this methylation regulates the expression of *IGF2* and *H19* by functioning as a chromatin insulator, restricting access to shared enhancers [Bell and Felsenfeld, 2000; Hark et al., 2000]. GOM on the maternal H19-DMR leads to expression of both *IGF2* alleles and silencing of both *H19* alleles. Dominant maternal transmissions of microdeletions and/or base substitutions within H19-DMR have recently been reported in a few patients of BWS with H19-DMR GOM [Demars et al., 2010]. However, when and how the GOM on the maternal H19-DMR occurs is not clear.

Here, we found epigenetic mosaicism in two BWS patients. We also found that GOM at H19-DMR was discordant in blood and skin versus placenta; specifically, methylation levels were lower in placental samples. These findings strongly suggest that aberrant methylation of H19-DMR occurred after implantation. As a result, we expect aberrant de novo methylation happens to a greater extent in embryos than in placentas.

MATERIALS AND METHODS

Patients

Two unrelated patients with sporadic BWS, Patient 1 (BWS047) and Patient 2 (bwsh21-015), were delivered by cesarean in the third trimester of pregnancy. The mothers of both patients conceived naturally. Patient 1 and Patient 2 met clinical criteria for BWS as described by Elliott et al. [1994] and Weksberg et al. [2001], respectively (Table I). The placenta of Patient 1 was large and weighed 1,065 g, but was without any pathological abnormality. The placenta of Patient 2 was also large, weighing 1,620 g, and had an encapsulated placental chorangioma, as reported previously [Aoki et al., 2011]. The standard G-banding chromosome analysis using peripheral blood samples showed no abnormalities in either patient. This study was approved by the Ethics Committee for Human Genome and Gene Analyses of the Faculty of Medicine, Saga University.

Southern Blot Analysis

Genomic DNA was extracted from embryo-derived somatic tissues and the placentas of the patients (Fig. 1). Methylation-sensitive Southern blots with *Bam*HI and *Not*I were employed for KvDMR1, and blots with *Pst*I and *Mlu*I were employed for H19-DMR, as described previously [Soejima et al., 2004]. Band intensity was measured using the FLA-7000 fluoro-image analyzer (Fujifilm, Tokyo, Japan). The methylation index (MI, %) was then calculated (Fig. 1). Southern blots with *Apa*I were used to identify the microdeletion of H19-DMR as described previously [Sparago et al., 2004].

Bisulfite Sequencing and Combined Bisulfite Restriction Analysis (COBRA)

Bisulfite sequencing covering the sixth CTCF binding site (CTS6) was performed. For COBRA, PCR products of each primary imprinted DMR were digested with the appropriate restriction endonucleases and were then separated using the MultiNA Microchip Electrophoresis System (Shimazdu, Japan). The methylation index was also calculated. All PCR primer sets used in this study have been listed in Supplementary Table SI (See Supporting Information online).

DNA Polymorphism Analyses

For quantitative polymorphism analyses, tetranucleotide repeat markers (*D11S1997* and *HUMTH01*) and a triplet repeat marker (*D11S2362*) from 11p15.4–p15.5 were amplified and separated by electrophoresis on an Applied Biosystems 3130 genetic analyzer (Applied Biosystems, NY); data were quantitatively analyzed with the GeneMapper software. The peak height ratios of paternal allele to maternal allele were calculated. A single nucleotide polymorphism (SNP) for the *Rsa*I recognition site in *H19* exon 5 (rs2839703) was also quantitatively analyzed using hot-stop PCR [Uejima et al., 2000]. Band intensity was measured using the FLA-7000 fluoro-image analyzer (Fujifilm).

Mutation Search of H19-DMR

To search for mutations in the binding sites of CTCF, OCT4, and SOX2, we sequenced a genomic region in and around H19-DMR, which included seven CTCF-binding sites, three OCT4 sites, and one SOX2 site.

TABLE I. Clinical Information of BWS Patients

Patient ID	Conception	Birth weight (gestational age)	Clinical features	Karyotype	Placental weight and pathology	Placental–fetal weight ratio
Patient 1 (BWS047)	Natural	4,506 g (36w2d)	macrosomia macroglossia abdominal wall defect hypoglycemia	46,XY	1,065 g no pathological findings	0.236
Patient 2 (bwsh21-015)	Natural	2,540 g (33w5d)	macrosomia macroglossia hypoglycemia renal malformation hepatosplenomegaly	46,XX	1,620 g placental chorangioma	0.638

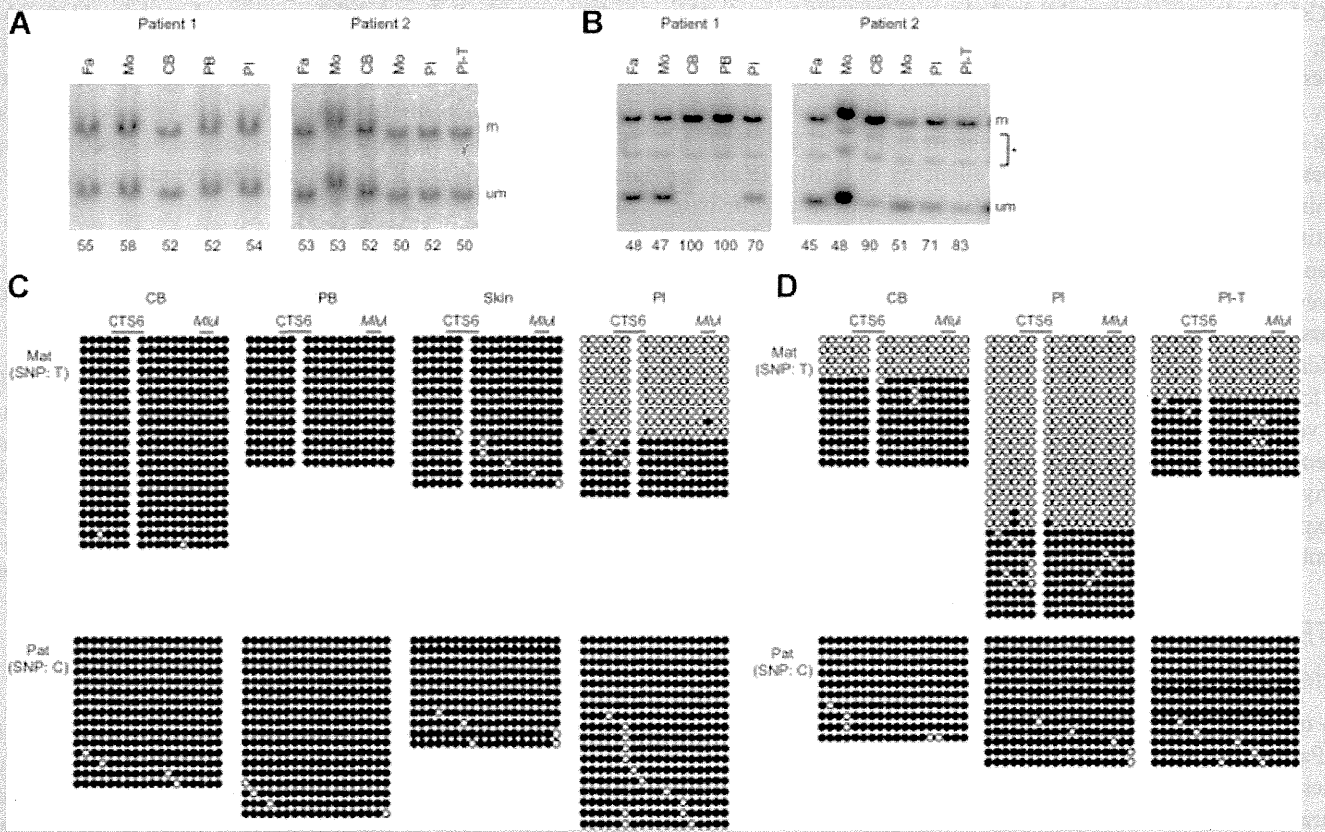

FIG. 1. Methylation analyses of KvDMR1 and H19-DMR. A: Methylation-sensitive Southern blots for KvDMR1. Genomic DNA was extracted from the cord blood, peripheral blood, skin, and placenta of Patient 1 and from the cord blood, placenta, and placental chorangioma of Patient 2. Methylation at KvDMR1 was normal in all samples analyzed. Methylation indices (MI, %) are shown under the figure. B: Methylation-sensitive Southern blots for H19-DMR. The MIs of blood samples were higher than the MIs of placental samples. MI was calculated using the equation [M/(M + U)] × 100, where M is the intensity of the methylated band and U is the intensity of the unmethylated band. C: Bisulfite sequencing of H19-DMR in Patient 1. The two parental alleles were distinguishable by differences in SNPs. Both parental alleles were completely methylated in the cord blood, peripheral blood, and skin samples, and the maternal allele, which is normally unmethylated, was partially methylated in the placenta. D: Bisulfite sequencing of H19-DMR in Patient 2. Methylation of the maternal allele was higher in the cord blood than in the placenta or placental chorangioma. These results were consistent with the results of the Southern blot analysis. We confirmed complete methylation of paternal H19-DMR alleles and complete demethylation of maternal H19-DMR alleles in four normal control placentas that were heterozygous for identifiable SNPs (data not shown). Fa, father; Mo, mother; CB, cord blood; PB, peripheral blood; Pl, placenta; Pl-T, placental chorangioma; m, methylated band; um, unmethylated band; *, nonspecific bands; Mat, maternal allele; Pat, paternal allele; CTS6, sixth CTCF binding site; *MluI*, a restriction site approximately 80 bp downstream of CTS6 assayed by methylation-sensitive Southern blot and COBRA.

RESULTS

We first examined the methylation status of the two ICRs, KvDMR1, and H19-DMR, at 11p15.5 using methylation-sensitive Southern blot analysis. Methylation at KvDMR1 was normal in all samples collected (Fig. 1A); however, methylation at H19-DMR was aberrant (Fig. 1B). In Patient 1, hypermethylation at H19-DMR was complete in cord blood and peripheral blood samples (MI = 100%), and hypermethylation in the placenta was partial (MI = 70%). In Patient 2, H19-DMR was partially hypermethylated in cord blood (MI = 90%) but less so in the placenta and placental chorangioma (MI = 71% and MI = 83%, respectively). For further investigation of differences in methylation between the patients' somatic tissues and placentas, the CTS6 site was subjected to bisulfite sequencing (Fig. 1C and D). We could distinguish the two parental alleles in each patient sample using informative SNPs (rs10732516 and rs2071094). The maternal allele, which is normally unmethylated, was completely methylated in the cord blood, peripheral blood, and skin from Patient 1. However, in placental samples from Patient 1, the maternal allele was only partially methylated: 36% of all CpGs analyzed were methylated. Similar results were observed in Patient 2: the maternal allele in the cord blood was 68% methylated; however, the maternal allele was only 31% and 55% methylated in the placenta and chorangioma samples, respectively. The paternal alleles, which are normally fully methylated, were fully methylated in all samples. These findings supported the results of the Southern blots. Furthermore, we could not find any microdeletions or mutations in or around H19-DMR,

including seven CTCF-binding sites, three OCT4 sites, and one SOX2 site, indicating that there was no genetic cause of the hypermethylation (Fig. 2A and data not shown).

Next, we analyzed polymorphic markers at 11p15.4–p15.5 to determine whether copy number abnormalities or paternal UPD might be involved in these BWS patients. Although smaller PCR products were more easily amplified, paternal–maternal allele ratios in blood samples were between 0.92 and 1.33, indicating that both parental alleles were equally represented in both patients (Fig. 2B). Therefore, we could rule out copy number abnormality and paternal UPD within the patients' blood. We also investigated maternal contamination in the placenta. *D11S1997* and *HUMTH01* for Patient 1 and the *RsaI* polymorphism in *H19* (rs2839703) for Patient 2 were used for this investigation because the mothers were expected to be homozygous for such polymorphisms. Thus, we investigated contamination of our samples by assessing the homozygosity of the polymorphisms in the mothers. The paternal–maternal ratios in Patient 1 were 0.94 and 1.03, indicating an equal contribution of both parental alleles and suggesting no contamination (Fig. 2B). In Patient 2, the ratios were 0.77 and 0.78 in the placenta and chorangioma, respectively, suggesting a small amount of contamination (Fig. 2C). However, such contamination was too

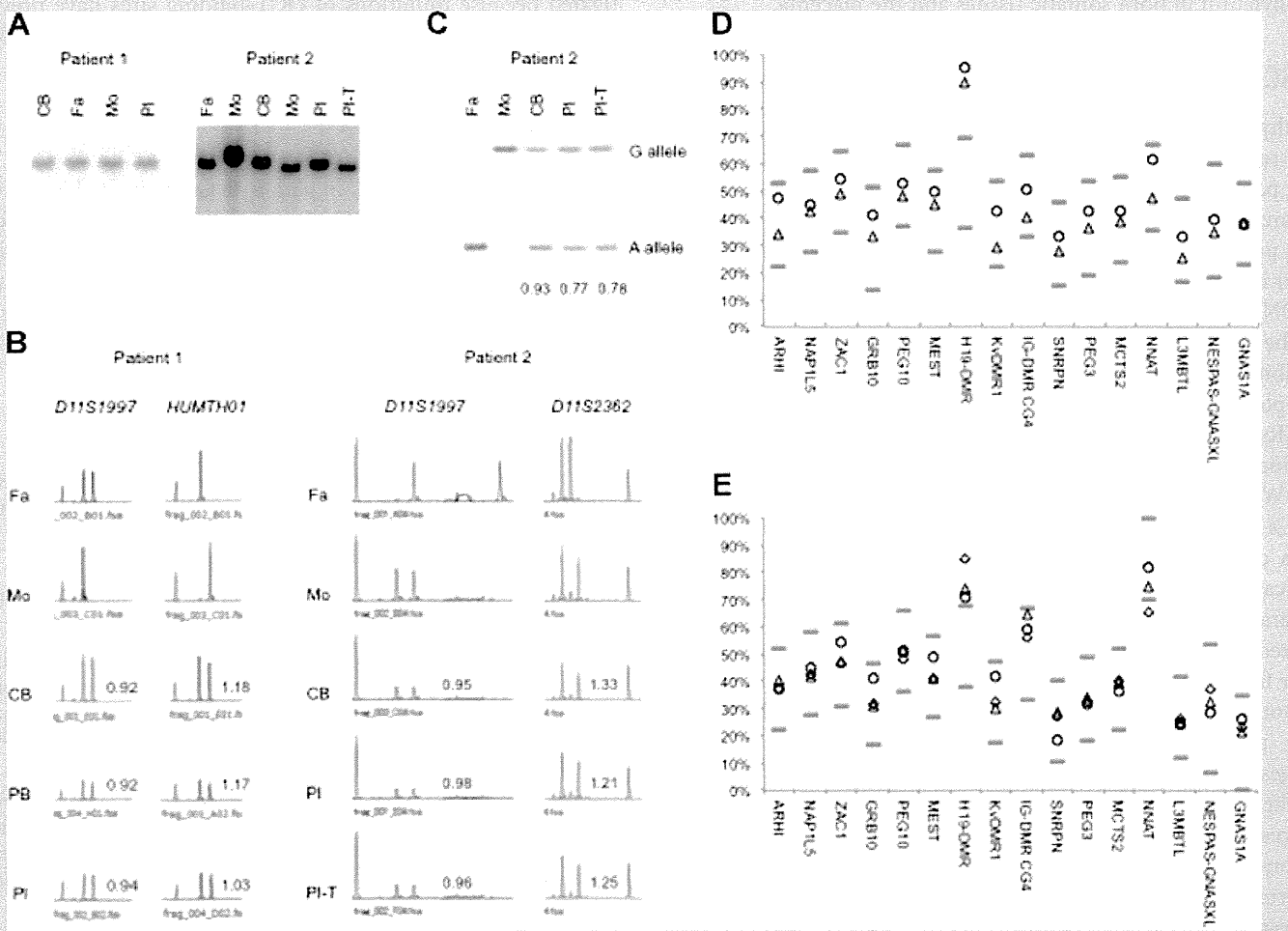

FIG. 2. Microdeletion analysis of H19-DMR, polymorphism analyses, and COBRA of primary imprinted DMRs in embryo-derived and placental samples. A: Southern blots identifying a microdeletion of H19-DMR. A genomic fragment (*7.7* kb) generated by *ApaI* digestion, which included the entire H19-DMR, was evident in all samples, indicating that there was no microdeletion in this DMR. B: Microsatellite markers at 11p15.4–p15.5. The peak heights associated with each parental allele in all samples were quantitatively analyzed. The results indicated that both parental alleles were present and equally represented. C: Hot-stop PCR of an *RsaI* polymorphic site in Patient 2. The ratios of paternal allele to maternal allele are shown under the figure. Although the ratios in the placenta and placental chorangioma are lower than in the cord blood, suggesting a small amount of maternal contamination, this was not enough to affect the results of the methylation analyses. COBRA of cord blood (D) and placentas (E), demonstrating that H19-DMR was hypermethylated. CTS6 is contained within H19-DMR. Methylation at other DMRs was normal in all samples, except for methylation at NNAT, which was aberrant in the placental chorangioma. Cord blood and placentas from 24 normal individuals were used as controls. The upper limit of normal methylation was defined as the higher of these two values: (1) the average of controls + 3 SD, or (2) the average + 15%. Similarly, the lower limit of normal methylation was definite as the lower of these two values: (1) the average of controls − 3 SD, or (2) the average − 15%. The upper and lower limits are indicated by gray bars. ○: Patient 1; △: Patient 2; ◇: placental chorangioma of Patient 2.

small to affect the results of the methylation analyses. In addition, sequence analysis did not show any mutations in *CDKN1C* (data not shown). These findings indicated that H19-DMR was aberrantly hypermethylated in both BWS patients and their associated placentas, but the aberrant methylation was consistently lower in the placenta, and that the H19-DMR GOM was strictly an isolated epimutation.

Finally, we analyzed the methylation status of 16 primary imprinted DMRs scattered throughout the genome-using COBRA (Fig. 2D and E). Only H19-DMR showed aberrant methylation among all primary DMRs in all samples, except for NNAT DMR, which was abnormal only in the placental chorangioma, indicating that the *IGF2/H19* imprinted domain was targeted for aberrant methylation in both somatic tissues and the placenta.

DISCUSSION

Methylation associated with parental imprints are erased in PGC and reestablished during gametogenesis in a sex-specific manner. The paternal pronucleus in the zygote undergoes active demethylation; extensive passive demethylation then ensues on maternal and paternal chromosomes during the pre-implantation period. After implantation, de novo methylation results in a rapid increase in DNA methylation in the inner cell mass (ICM), which gives rise to the entire embryo; in contrast, de novo methylation is either inhibited or not maintained in the trophoblast, which gives rise to the placenta [Li, 2002; Sasaki and Matsui, 2008]. The imprinted DMRs, however, escape these demethylation and de novo methylation events that occur in early embryogenesis. H19-DMR GOM in BWS patients is considered an error in imprint erasure in female PGCs [Horsthemke, 2010]. However, H19-DMR GOM, whether with or without microdeletions within H19-DMR, was partial, indicating a mosaic of normal cells and aberrantly methylated cells [Sparago et al., 2007; Cerrato et al., 2008]. These findings demonstrated that aberrant hypermethylation at H19-DMR was acquired after fertilization, although the precise timing was unknown.

Both participants in this study had isolated GOM at H19-DMR. The partial and variable hypermethylation among samples suggested epigenetic mosaicism. Furthermore, methylation levels in the placentas were lower than those in the blood and skin, suggesting that the aberrant methylation was acquired after implantation—when genome-wide de novo methylation normally occurs. Aberrant de novo methylation at H19-DMR is expected to be more widespread in the embryo than in the placenta, as this is normally the case for de novo methylation [Li, 2002; Sasaki and Matsui, 2008]; this disparity in efficiency could lead to the discordance between hypermethylation in trophoblast-derived placenta and that in embryo-derived blood and skin. This hypothesis is supported by a mouse experiment in which a mutant maternal allele harboring a deletion of four CTCF binding sites was hypomethylated in oocytes and blastocysts, yet was highly methylated after implantation [Engel et al., 2006]. To our knowledge, this is the first evidence demonstrating that aberrant hypermethylation of maternal H19-DMR is acquired after implantation in humans.

We found that of 16 primary imprinted DMRs analyzed, only H19-DMR showed aberrant methylation; even methylation at IG-DMR CG4, another paternally methylated, primary imprinted DMR, was normal in our patients. Although we only studied two patients, this finding indicated that the *IGF2/H19* imprinted domain in both the embryo and placenta was more susceptible than other imprinted domains to aberrant methylation acquired after implantation.

In conclusion, we found that methylation of H19-DMR was discordant in embryo-derived somatic tissue and placenta, strongly suggesting that the aberrant de novo methylation occurred after implantation. However, the precise mechanism of isolated H19-DMR GOM is still unknown. Since no mutations in *CTCF*, an important trans-acting imprinting factor, were found in these patients with isolated GOM at H19-DMR, the potential for mutations in the OCT and SOX transcription factors should be investigated because mutations of OCT-binding sites have previously been found in a few patients with H19-DMR GOM [Cerrato et al., 2008; Demars et al., 2010].

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Characterization of DNA methylation errors in patients with imprinting disorders conceived by assisted reproduction technologies

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BACKGROUND: There is an increased incidence of rare imprinting disorders associated with assisted reproduction technologies (ARTs). The identification of epigenetic changes at imprinted loci in ART infants has led to the suggestion that the techniques themselves may predispose embryos to acquire imprinting errors and diseases. However, it is still unknown at what point(s) these imprinting errors arise, or the risk factors.

METHODS: In 2009 we conducted a Japanese nationwide epidemiological study of four well-known imprinting diseases to determine any association with ART. Using bisulfite sequencing, we examine the DNA methylation status of 22 gametic differentially methylated regions (gDMRs) located within the known imprinted loci in patients with Beckwith-Wiedemann syndrome (BWS, $n = 1$) and also Silver-Russell syndrome (SRS, $n = 5$) born after ART, and compared these with patients conceived naturally.

RESULTS: We found a 10-fold increased frequency of BWS and SRS associated with ART. The majority of ART cases showed aberrant DNA methylation patterns at multiple imprinted loci both maternal and paternal gDMRs (5/6), with both hyper- and hypomethylation events (5/6) and also mosaic methylation errors (5/6). Although our study may have been limited by a small sample number, the fact that many of the changes were mosaic suggested that they occurred after fertilization. In contrast, few of the patients who were conceived naturally exhibited a similar pattern of mosaic alterations. The differences in methylation patterns between the patients who were conceived naturally or after ART did not manifest due to the differences in the disease phenotypes in these imprinting disorders.

CONCLUSION: A possible association between ART and BWS/SRS was found, and we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable.

Key words: assisted reproduction technologies / genomic imprinting / DNA methylation / gametic differentially methylated regions / genomic imprinting disorders

Introduction

Human assisted reproduction technologies (ARTs) are used in the treatment of infertility and involve the manipulation of eggs and/or sperm in the laboratory. Several recent studies have identified an increased incidence of some normally very rare imprinting disorders after ART, including Beckwith-Wiedemann syndrome (BWS: ONIM 130650), Angelman syndrome (AS: ONIM 105830) and Silver-Russell syndrome (SRS: OMIM 180860) but not Prader-Willi syndrome (PWS: OMIM 176270; DeBaun *et al.*, 2003; Gosden *et al.*, 2003; Svensson *et al.*, 2005). Additionally, there are several reports suggesting that epigenetic alterations (epimutations) at imprinted loci occur during the *in vitro* manipulation of the gametes, with both IVF and ICSI approaches implicated (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Moll *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Rossignol *et al.*, 2006; Bowdin *et al.*, 2007; Kagami *et al.*, 2007). However, some studies do not support a link between ART and imprinting disorders (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007).

Epigenetic marks laid down in the male or female germ lines, and which are inherited by the embryos, establish the imprinted expression of a set of developmentally important genes (Surani, 1998). Because imprinted genes are regulated by these gametic epigenetic marks, and by further epigenetic modifications in the somatic cell, they are particularly vulnerable to environmentally induced mutation. One of the best studied epigenetic marks is DNA methylation. DNA methylation is established in either the maternal or paternal germline at discrete genomic loci. This methylation is preserved in the fertilized embryo to generate differentially methylated regions (DMRs) which then signal to nearby genes to establish domains of imprinted chromatin by mechanisms that are not fully understood (John and Lefebvre, 2011). These germline or gametic DMRs (gDMRs) can orchestrate the monoallelic expression of genes over megabases of DNA (Tomizawa *et al.*, 2011) and are reset with every reproductive cycle (Lucifero *et al.*, 2002; Obata and Kono, 2002).

The increased frequency of epimutation(s) at imprinted loci in ART infants has led to the suggestion that ART procedures may induce imprinting error(s). However, these studies are confounded because ART populations are, by their very nature, different from populations who were conceived without the use of ART, with a low fertility rate, an increased frequency of reproductive loss and usually of advanced age, all of which are associated with increased occurrence of fetal and neonatal abnormalities. Furthermore, it is difficult to determine the causality of imprinting errors in any specific abnormality reported after ART. Both IVF and ICSI appear to be associated with an increased relative risk of imprinting disorders (Savage *et al.*, 2011). These procedures are often undertaken for unexpected infertility and require ovarian stimulation, oocyte collection and *in vitro* culture before the embryos are implanted. It has been suggested that infertility and any resulting ovarian stimulation may predispose to epigenetic errors (Sato *et al.*, 2007). Animal studies suggest that *in vitro* embryo culture may be associated with epigenetic alterations. In particular, the large offspring syndrome in cattle undergoing ART is associated with the loss of maternal allele methylation at insulin-like growth factor 2 receptor (*IGF2R*) gDMR (Young *et al.*, 2001) and has phenotypic similarity to BWS. It is still unknown when these imprinting errors arise and what factors predispose to epigenetic changes. Previously, Chang *et al.* (2005) reported no phenotypic differences between BWS patients who were conceived after ART and naturally. However, Lim *et al.* (2009) reported that patients who were conceived after ART had a significantly lower frequency of exomphalos and higher risk of non-Wilms tumor neoplasia. Phenotypic differences between patients who were conceived after ART and naturally are largely unreported, while any changes to phenotype may be altered by the frequency and the degree of epimutations. Studies revealed that some patients with BWS born after ART presented with epimutations that were not restricted to the 11p15 region (Rossignol *et al.*, 2006; Bliek *et al.*, 2009; Lim *et al.*, 2009). Further analysis of abnormal methylation patterns in imprinting disorders may provide clues as to the cause of disease and identify the ART-related risk factor(s).

To address these questions in this study, we engaged in a nationwide epidemiological study of the Japanese population to determine the frequency of four imprinting disorders after natural conception and after ART. We then analyzed the DNA methylation status of 22 gDMRs in BWS and SRS patients conceived by the two routes. Finally, we compared the abnormal methylation patterns and the phenotypes reported for both sets of patients. As a result we found that both BWS and SRS were more frequent after ART and that ART patients exhibited a higher frequency of aberrant DNA methylation patterns at multiple loci with, in some cases, mosaic methylation errors.

Materials and Methods

Nationwide investigation of imprinting disorders

The protocol was established by the Research Committee on the Epidemiology of Intractable Diseases. The protocol consisted of a two-stage postal survey. The first-stage survey was used to estimate the number of individuals with any of the four imprinting diseases: BWS, SRS, PWS and AS. The second-stage survey was used to identify the clinico-epidemiological features of these syndromes.

In the first-stage survey, the pediatric departments of all hospitals were identified based on a listing of hospitals, as at 2008, supplied by the R&D Co. Ltd (Nagoya, Japan). Hospitals were classified into seven categories according to the type of institution and the number of hospital beds. The survey was mailed to a total of 3158 departments in October 2009 with letters of request for participation in recording these diseases. A simple questionnaire was used to ask about the number of patients with any of the four imprinting disorders. Diagnosis was determined by karyotype analyses, genetic analyses and clinical phenotypes by their clinical doctors. In December 2009, a second request was sent to departments that had not responded to the earlier deadline (at the end of November 2009). Following the first-stage survey, we sent acknowledgement letters to departments that had responded.

The second questionnaires were forwarded to the departments that had reported patients with the imprinting disorders on the first questionnaires. Detailed clinical information for the patients with these imprinting disorders was collected, including the age, gender, growth and development pattern, the methods of the diagnosis, the presence of infertility treatment and the methods of ART where applicable. Duplicate results were excluded using the information regarding the patient's age and gender where available. The study was approved by the Ethics Committee of Tohoku University School of Medicine.

Estimation of prevalence of imprinting disorders

The number of patients, who were diagnosed by genetic and cytogenetic testing and by clinical phenotypes, was obtained from data from the departments who responded to the first survey. The 95% confidence interval (CI) was calculated as previously described (Wakai *et al.*, 1997). The prevalence was determined, based on the population of Japan in 2009 (127 510 000) with data from the Statistics Bureau of the Ministry of Internal Affairs and Communications.

DNA preparation

Genomic DNA was obtained from blood or buccal mucosal cell samples from patients with one of the imprinting disorders using standard extraction methods (Kobayashi *et al.*, 2007). For control DNAs, DNA was prepared from the sperm and cord blood samples from unaffected individuals. The study was performed after obtaining patients or their parents' consent.

Bisulfite-treatment PCR including the SNPs

We first searched for single nucleotide polymorphisms (SNPs) within 22 previously reported human gDMRs (Kikyo *et al.*, 1997; Smith *et al.*, 2003; Kobayashi *et al.*, 2006, 2009; Wood *et al.*, 2007) using 20 control Japanese blood DNA samples. PCR primer sets were designed to span these SNPs (Supplementary data, Table SI) and human sperm DNA and blood DNA was used to confirm that these PCR assays detected the methylation status of the 22 DMRs. Paternal DMRs were shown to be fully methylated in sperm DNA, maternal DMRs were fully unmethylated and in blood DNA, both paternal and maternal DMRs showed ~50% methylation (Supplementary data, Fig. S1). The human gDMRs and the non-imprinted repetitive long interspersed nucleotide element (*LINE1*) and *Alu* repetitive sequences were examined by bisulfite sequencing using established protocols (Kobayashi *et al.*, 2007). Briefly, PCR products were purified and cloned into the pGEM-T vector (Promega, Madison, WI, USA). Individual clones were sequenced using M13 reverse primer and an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). On average, 20 clones were sequenced for each sample.

Statistics

The frequency of the manifestation in patients who were conceived after ART was compared with that observed in patients conceived naturally using Fisher's exact test.

Results

Frequency of four imprinting disorders and their association with ART

We first investigated the nationwide frequency of four imprinting disorders (BWS, AS, PWS and SRS) in Japan in the year 2009. Of a total of 3158 departments contacted, 1602 responded to the first-stage survey questionnaire (50.7%). The total number of cases was calculated using a second-stage survey ensuring the exclusion of duplicates (Table I). Using this information, and taking into account the number of patients with suspect clinical signs but without a formal diagnosis, we identified 444 BWS patients (95% CI: 351–538), 949 AS patients (95% CI: 682–1217), 2070 PWS patients (95% CI: 1504–2636) and 326 SRS patients (95% CI: 235–416). From these figures (and using the 2009 population of Japan: 127 510 000) we estimated the prevalence of these syndromes to be 1 in 287 000, 1 in 134 000, 1 in 62 000 and 1 in 392 000, respectively, for BWS, AS, PWS and SRS. Further details are given in Supplementary data, Table SII and Supplementary data, Fig. S2.

Table I The 2009 frequency of four imprinting diseases in Japan in relation to use of assisted reproduction techniques (ART).

Imprinting disorders	Total estimated patient number (95% CI)	The total prevalence of the syndrome	The number of patients after ART/ total (%)
BWS	444 (351–538)	1 in 287 000	6/70 (8.6)
AS	949 (682–1217)	1 in 134 000	2/123 (1.6)
PWS	2070 (1504–2636)	1 in 62 000	4/261 (1.5)
SRS	326 (235–416)	1 in 392 000	4/42 (9.5)

Results of a nationwide epidemiological investigation of four imprinting disorders in Japan, under the governance of the Ministry of Health, Labor and Welfare of the Japanese government. Precise diagnosis was performed using fluorescence *in situ* hybridization and DNA methylation analyses. The type of ART, obtained from the questionnaires, was compared with the frequencies of these diseases and the epimutation rates. BWS, Beckwith-Wiedemann syndrome, AS, Angelman syndrome, PWS, Prader-Willi syndrome; SRS, Silver-Russell syndrome.

Between 1997 and 2008, the period during which the ART babies in this study were born, 0.64–0.98% of the total number of babies born in Japan were born as a result of IVF and ICSI. We ascertained the frequency of ART procedures in the cases of BWS, AS, PWS and SRS via the questionnaire sent to doctors (Table I, Supplementary data, Table SIII). The numbers of patients with PWS and AS we identified was low; however, the frequency of ART in these cases was not dissimilar to that expected, based on the population rate of ART use, with 2/123 (1.6%) cases of AS and 4/261 (1.5%) cases of PWS born after ART. In contrast, for BWS and SRS the frequency of ART was nearly 10-fold higher than anticipated with 6/70 (8.6%) BWS and 4/42 (9.5%) SRS patients born after ART.

After analyzing the second questionnaire, the blood or buccal mucosal cell samples were obtained from 15 individuals with BWS, 23 with SRS, 73 with AS and 29 with PWS. Using polymorphic bisulfite-PCR sequencing, we examined the methylation status of gDMRs within these samples at the imprinted regions implicated in these syndromes. For BWS we assayed *H19* and *KCNQ1OT1* (*LIT1*) gDMRs, for SRS we assayed the *H19* gDMR and for PWS and AS we assayed the *SNRPN* gDMR. For all patients (conceived naturally and with ART), the frequencies of DNA methylation errors (epimutations) corrected were 7/15 (46.7%) for BWS, 9/23 (39.1%) for SRS, 6/73 (8.2%) for AS and 2/29 (6.9%) for PWS. When looking at the ART cases exclusively, epimutation rates were 3/5 (BWS), 3/7 (SRS), 0/2 (AS) and 0/2 (PWS).

Abnormal methylation patterns in the ART and naturally conceived SRS patients with epimutations.

While hypomethylation of *H19* at chromosome 11 is known to be a frequent occurrence in SRS (Bliek *et al.*, 2006), various additional loci at chromosomes 7, 8, 15, 17 and 18 have been implicated as having a

role in this syndrome (OMIM 180860). We first identified SNPs in the previously reported 22 human DMRs using genomic DNA isolated from human sperm and blood from unaffected individuals, which could then be used in bisulfite-PCR methylation assays to assign methylation to the parental allele. We next collected a total of 15 SRS samples, including previously collected samples (ART: 2, naturally conceived: 4), which had DNA methylation errors at the paternal gDMR at *H19*. Five of these were born from ART and 10 were from natural conceptions. We analyzed and compared the DNA methylation status of the 3 other paternal gDMRs and the 19 maternal gDMRs (Supplementary data, Fig. S3, Table, Supplementary data, Table SIV). In four out of the five ART cases, DNA methylation errors were not restricted to the *H19* gDMR, and were present at both maternally and paternally methylated gDMRs. These four cases showed a mixture of hyper- and hypomethylation with mosaic (partial) patterns. In contrast, only 3 of the 10 naturally conceived patients showed DNA methylation errors at loci other than *H19* gDMR.

To determine whether DNA methylation errors occurred in patients at a broader level in the genomes, we assessed the methylation profiles of the non-imprinted *LINE1* and *Alu* elements. We examined a total of 28 CpG sites in a 413-bp fragment of *LINE1* and 12 CpG sites in a 152-bp fragment of *Alu* (Supplementary data, Table SIV), and no significant differences were found in the methylation ratios between patients conceived by ART and naturally.

The abnormal methylation pattern in BWS patients with epimutations

In BWS, hypermethylation of *H19* or hypomethylation of *KCNQ1OT1(LIT1)* at human chromosome 11 are both frequently reported (Choufani *et al.*, 2010). We collected seven BWS samples with DNA methylation errors of the *LIT1* gDMR, one of which was derived from ART patient and six from naturally conceived patients (Supplementary data, Fig. S3, Table II, Supplementary data, Table SIV). In the one ART (ICSI) case, we identified four additionally gDMR methylation errors, again present at both maternally and paternally methylated gDMRs and with mixed hyper- and hypomethylation patterns. Furthermore, the methylation error at the *NESPAS* DMR was mosaic in this patient. One of the six naturally BWS cases had similar changes. Although we had only one BWS case conceived by ART, widespread methylation errors were similar to those for the DNA methylation error pattern in SRS.

Phenotypic differences between ART patients and those conceived naturally

The increased frequency of DNA methylation errors at other loci in the ART cases suggested that the BWS and SRS cases born after ART might exhibit additional phenotypic characteristics. However, when we compared in detail the clinical features from both categories of conception (Supplementary data, Table SV), we found no major differences between ART and naturally conceived patients with BWS and SRS.

Discussion

Our key finding from this study was a possible association between ART and the imprinting disorders, BWS and SRS. We did not find a similar association with PWS and AS but our numbers were quite low in this study and a larger due to the questionnaire return rate and relative rarity of the diseases, international study will be required to reach definitive conclusions. Furthermore, factors such as PCR and/or cloning bias in the bisulfite method and correction for changing rate of ART over time must be considered when analyzing any results.

In addition to the possible association between ART and BWS/SRS, we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART shown by us and others is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable. The process of ART exposes the developing epigenome to many external influences, which have been shown to influence the proper establishment and maintenance of genomic imprints, including hormone stimulation (Sato *et al.*, 2007), *in vitro* culturing (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003), cryopreservation (Emiliani *et al.*, 2000; Honda *et al.*, 2001) and the timing of embryo transfer (Shimizu *et al.*, 2004; Miura and Niikawa, 2005). Furthermore, we and others have also shown that some infertile males, particularly those with oligozoospermia, carry pre-existing imprinting errors in their sperm (Marques *et al.*, 2004; Kobayashi *et al.*, 2007; Marques *et al.*, 2008) which might account for the association between ART and imprinting disorders.

Imprinting syndromes and their association with ART

We report the first Japanese nationwide epidemiological study to examine four well-known imprinting diseases and their possible association with ART. We found that the frequency of ART use in both BWS and SRS was higher than anticipated based on the nationwide frequency of ART use at the time when these patients were born. Several other reports have raised concerns that children conceived by ART have an increased risk of disorders (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Maher *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Lim and Maher, 2009). However, the association is not clear in every study (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007). The studies reporting an association were mainly from case reports or case series whereas the studies where no association was reported were cohort studies. Therefore, the differences in the epidemiological analytical methods might accounts for the disparity in findings.

Owing to the rare nature of the imprinting syndromes, statistical analysis is challenging. In addition, the diagnosis of imprinting diseases is not always clear cut. Many of the syndromes have a broad clinical spectrum, different molecular pathogenesis, and the infant has to have reached a certain age before these diseases become clinically detectable. It is therefore likely that some children with these diseases are not recorded with the specific diagnosis code for these syndromes. Nonetheless, in this study we were examining the relationship between ART and the imprinting syndromes and these confounding factors are likely to apply equally to both groups.

Both BWS and SRS occurred after ART but our numbers for PWS and AS were low, precluding any definitive conclusion for these two disorders. However, while most cases of BWS and SRS are caused by an epimutation, epimutations are very rare in PWS and AS (only 1–4%) and ART would not be expected to increase chromosome 15

Table II Abnormal methylation in patients with SRS and BWS.

Case	ART	Abnormal methylation			
SRS					
SRS-1	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated	PEG10 hypermethylated (mosaic)	GRB10 hypermethylated; ZNF597 hypomethylated
SRS-2	IVF-ET	H19 hypomethylated (mosaic)			
SRS-3	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated (mosaic)		
SRS-4	IVF-ET	H19 hypomethylated	GRB10 hypermethylated		
SRS-5	IVF-ET	H19 hypomethylated (mosaic)	INPP5F hypermethylated		
SRS-6		H19 hypomethylated			
SRS-7		H19 hypomethylated (mosaic)	ZNF597 hypermethylated (mosaic)	ZNF331 hypomethylated (mosaic)	
SRS-8		H19 hypomethylated			
SRS-9		H19 hypomethylated (mosaic)			
SRS-10		H19 hypomethylated			
SRS-11		H19 hypomethylated (mosaic)	PEG1 hypermethylated		
SRS-12		H19 hypomethylated			
SRS-13		H19 hypomethylated (mosaic)	FAM50B hypomethylated		
SRS-14		H19 hypomethylated			
SRS-15		H19 hypomethylated			
BWS					
BWS-1	ICSI	LIT1 hypomethylated	ZDBF2 hypermethylated	PEG1 hypermethylated	NESPAS hypomethylated (mosaic)
BWS-2		LIT1 hypomethylated			
BWS-3		LIT1 hypomethylated			
BWS-4		LIT1 hypomethylated			
BWS-5		LIT1 hypomethylated			
BWS-6		LIT1 hypomethylated	ZDBF2 hypomethylated	ZNF331 hypomethylated (mosaic)	
BWS-7		LIT1 hypomethylated			

ET, embryo transfer. Summary of the abnormal methylation patterns in the ART conceived and naturally conceived patients with Silver-Russell syndrome (SRS) and Beckwith-Wiedemann syndrome (BWS) with epimutations. Numbers in parentheses show the results of the methylation rates obtained using bisulfite-PCR sequencing. The % of DNA methylation of 22 gDMRs in all patients with SRS and BWS examined are presented in Supplementary data, Table SIV. Depictions in red represent DMRs normally exclusively methylated on the maternal allele, while blue represent paternally methylated sites.

deletions or uniparental disomy, consistent with our findings. Prior to this investigation, there was some evidence for an increased prevalence of BWS after ART but less evidence for an increased prevalence of SRS, with five SRS patients reported linked to ART (Svensson *et al.*, 2005; Bliek *et al.*, 2006; Kagami *et al.*, 2007; Galli-Tsinopoulou *et al.*, 2008). Our population-wide study provides evidence to suggest that both BWS and SRS occur more frequently after ART in the Japanese population.

Mechanisms of epimutation in the patients conceived by ART

By performing a comprehensive survey of all the known gDMRs in a number of patients with BWS and SRS, we found that multiple loci were more likely to be affected in ART cases than those conceived naturally. Lim *et al.* (2009) have reported a similarly increased frequency of multiple errors after ART, with 37.5% of patients conceived with ART and 6.4% of naturally conceived patients displaying abnormal methylation at additional imprinted loci. However, while Bliek *et al.* (2009) reported alterations in multiple imprinted loci in 17 patients out of 81 BWS cases with hypomethylation of *KCNQ1OT1*(*LIT1*) ICR, only 1 of the cases with multiple alterations was born after ART. Similarly, Rossignol *et al.* (2006) reported that 3 of 11 (27%) ART-conceived patients and 7 of 29 (24%) naturally conceived patients displayed abnormal methylation at additional loci. In these four earlier studies, not all gDMRs were assayed and it may be that by doing so, these incongruities will be resolved.

The pattern of cellular mosaicism we observed in some patients suggested that the imprinting defects occurred after fertilization rather than in the gamete as DNA methylation alterations arising in the gamete would be anticipated to be present in every somatic cell. This suggested the possibility that the DNA methylation errors occurred as a consequence of impaired maintenance of the germline imprints rather than a failure to establish these imprints in the germline or a loss of these imprints in the sperm or oocytes *in vitro*. Furthermore, some patients conceived by ART with SRS and BWS showed

alterations at both maternally and paternally methylated gDMRs suggesting that the defects were not limited to one parental germline. The mechanisms controlling the protection of imprinted loci against demethylation early in the development remain unclear. Our data suggested that this protection may fail in ART resulting in the tissue-specific loss of imprints, though it remains unclear if this ever occurs naturally. Potential factors involved could include the culture conditions for the newly fertilized oocyte and the length of exposure to specific media or growth factors, as part of the ART procedure. Some of the naturally conceived patients also had abnormal methylation at both maternally and paternally methylated gDMRs, which were in some cases mosaic. This could indicate that fertility issues arise as a consequence of pre-existing mutations in factors required to protect and maintain imprints early in life and it may therefore be possible to identify genetic mutations in these factors in this group of patients.

Clinical features

In our large-scale epidemiological study, we found differences in the frequency of some classic features of SRS and BWS between patients conceived by ART and those conceived naturally. We found that 7/7 (100%) ART conceived SRS patients showed body asymmetry, whereas only 30/54 (55.5%) who were conceived naturally possessed this feature. Similarly in BWS, earlobe creases were present in 4/7 (57.1%) ART conceived cases and 44/89 (49.4%) naturally conceived, bulging eyes in 3/7 (42.8%) versus 21/89 (23.6%), exomphalos in 6/7 (85.7%) versus 61/89 (68.5%) and nephromegaly in 2/7 (28.6%) versus 18/89 (20.2%), respectively. It is therefore possible that the dysregulation of the additional genes does modify the typical SRS and BWS phenotypes (Azzi *et al.*, 2010). BWS patients with multiple hypermethylation sites have been reported with complex clinical phenotypes (Bliek *et al.*, 2009) and a recently recognized BWS-like syndrome involving overgrowth with severe developmental delay was reported after IVF/ICSI (Shah *et al.*, 2006).

In our study patients with diagnosed imprinting disorders that presented with defects at additional loci (i.e. other than the domain responsible for that disorder) did not display additional phenotypes not normally reported in BWS or SRS. Since we were effectively selecting for classic cases of BWS and SRS in the first instance, it is possible that there are individuals born through ART showing entirely novel or confounding phenotypes that were not identified in our survey. Alternatively, as many of the alterations we observed showed a mosaic pattern, it is possible that mosaic individuals have more subtle phenotypes. In light of this new information on mosaicism, we may be able to use our knowledge of the individual's epigenotype to uncover these subtle changes.

This study, and the work of our colleagues, highlights the pressing need to conduct long-term international studies on ART treatment and the prevalence of imprinting disorders, particularly as the use of ART is increasing worldwide. It remains to be seen if other very rare epigenetic disorders will also have a possible association with the use of ART. Furthermore, it is not yet known what other pathologies might be influenced by ART. For example, in addition to general growth abnormalities, many imprint methylation errors also lead to the occurrence of various cancers (Okamoto *et al.*, 1997; Cui *et al.*, 1998). Further molecular studies will be required to understand the pathogenesis of these associations, and also to identify preventative methods to reduce the risk of occurrence of these syndromes following ART.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

H.H., H.O., N.M., F.S. and A.S. performed the DNA methylation analyses. M.K., K.N. and H.S. collected the samples of the patients. K.N. did the statistical analyses. H.H., M.V.D.P., R.M.J. and T.A. wrote this manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

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Human Chorionic Gonadotropin Induces Human Macrophages to Form Intracytoplasmic Vacuoles Mimicking Hofbauer Cells in Human Chorionic Villi

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Key Words
Hofbauer cells · Macrophages · Human chorionic gonadotropin · Luteinizing hormone/chorionic gonadotropin receptor · Chorionic villi · Placenta

Abstract
The most characteristic morphological feature of macrophages in the stroma of placental villi, known as Hofbauer cells, is their highly vacuolated appearance. They also show positive immunostaining for human chorionic gonadotropin (hCG) and express messenger ribonucleic acid of the luteinizing hormone/chorionic gonadotropin receptor with a deletion of exon 9 (LH/CG-R Δ9). Maternal hCG enters fetal plasma through the mesenchyme of the placental villi and promotes male sexual differentiation in early pregnancy; therefore, excess hCG may induce aberrant genital differentiation and hCG must be adjusted at the fetomaternal interface. We hypothesized that hCG is regulated by Hofbauer cells and that their peculiar vacuoles are involved in a cell-specific function. To assess the morphological modification and expression of LH/CG-R Δ9 in human macrophages after hCG exposure, the present study examined phorbol 12-myristate 13-acetate (PMA)-treated THP-1 cells, a human monocyte-macrophage cell line. hCG induced transient vacuole formation in PMA-treated THP-1 cells, morphologically mimicking Hofbauer cells. Immunocytochemistry showed that PMA-treated THP-1 cells incorporated hCG but not luteinizing hormone or follicle-stimulating hormone. Western blotting analyses demonstrated that PMA-treated THP-1 cells expressed an immunoreactive 60-kDa protein, designated as endogenous LH/CG-R Δ9. hCG induced a transient reduction in the LH/CG-R Δ9, which was synchronous with the appearance of cytoplasmic vacuoles. In conclusion, human macrophages regulating hCG via cytoplasmic LH/CG-R Δ9 mimic the morphological characteristics of Hofbauer cells. Their vacuoles may be associated with their cell-specific function to protect the fetus from exposure to excess maternal hCG during pregnancy.

Introduction

Resident tissue macrophages differentiated from blood monocytes adapt to their local environment to perform specific functions in organs and tissues [Hume et al., 2002]. Macrophages in the stroma of placental villi, known as Hofbauer cells, are characterized by a peculiar vacuolated form [Castellucci and Kaufmann, 2006],

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Abbreviations used in this paper

bp	base pairs
CD	cluster of differentiation
FSH	follicle-stimulating hormone
hCG	human chorionic gonadotropin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HE	hematoxylin and eosin
HRP	horseradish peroxidase
LDL	low-density lipoprotein
LH	luteinizing hormone
LH/CG-R	luteinizing hormone/chorionic gonadotropin receptor
LH/CG-R Δ9	luteinizing hormone/chorionic gonadotropin receptor with a deletion of exon 9
mRNA	messenger ribonucleic acid
PMA	phorbol 12-myristate 13-acetate
RT-PCR	reverse transcription-polymerase chain reaction
TBST	Tris-buffered saline containing 0.05% Tween 20

which is not observed in human macrophages distributed in other organs. Despite the identification of Hofbauer cells more than 100 years ago, their functions and the physiological roles of the vacuoles are still not well understood.

Hofbauer cells are found in the stroma adjacent to human chorionic gonadotropin (hCG)-producing trophoblasts and fetal capillaries, and their cytoplasmic vacuoles may be associated with phagocytic activity [Ingman et al., 2010]. Moreover, they may maintain host defense to prevent pathogens, toxins and immunological complexes from reaching the fetus [Frauli and Ludwig, 1987; Ingman et al., 2010]. Hofbauer cells show positive immunohistochemical staining with hCG [Katabuchi et al., 1989, 1994], thus suggesting that hCG is phagocytosed from the surrounding environment because macrophages are thought to be unable to synthesize hCG [Castellucci and Kaufmann, 2006]. Fetal plasma concentrations of total hCG are only 3% of the maternal plasma levels, ranging from 30 to 2,800 mIU/ml between 8 and 20 weeks of gestation [Clements et al., 1976]. Circulating hCG in fetal blood is necessary for the differentiation of the fetal genitalia; therefore, excess hCG may induce aberrant genital differentiation of the fetus [Huhtaniemi et al., 1977; Takasugi et al., 1985; Matzuk et al., 2003]. We thus hypothesized that macrophages in placental villi regulate hCG to prevent the fetus from excess maternal hCG and the peculiar cytoplasmic vacuoles are related to the cell-specific function of Hofbauer cells.

Khan et al. [2000] first demonstrated that human macrophages could take up and degrade hCG by culturing human peritoneal macrophages. Sonoda et al. [2005] confirmed the degradation of hCG by phorbol 12-myristate 13-acetate (PMA)-treated THP-1 cells, which were established from human acute monocytic leukemia cells as a human monocyte-macrophage lineage cell line. They subsequently revealed that both PMA-treated THP-1 cells and Hofbauer cells expressed only a messenger ribonucleic acid (mRNA) encoding the variant type of luteinizing hormone/chorionic gonadotropin receptor (LH/CG-R) with a deletion of exon 9 (LH/CG-R Δ9) and that the ability of PMA-treated THP-1 cells to degrade hCG was impaired by transfection of full-length LH/CG-R mRNA [Sonoda et al., 2005]. Although these results suggested that endogenous LH/CG-R Δ9 was involved in the regulation of hCG in human macrophages, they could not confirm whether LH/CG-R Δ9 protein was produced from the endogenous gene and regulated hCG in human macrophages. The current study assessed the morphological changes and expression of LH/CG-R Δ9 in PMA-treated THP-1 cells after hCG exposure.

Materials and Methods

THP-1 Cell Culture and Gonadotropin Treatment

THP-1 cells were obtained from the American Type Culture Collection. They adhere to plastic dishes in culture medium and change into amoeboid cells with the characteristics of macrophages when treated with PMA (Sigma-Aldrich, Japan). THP-1 cells were first maintained in suspension cultures in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Japan) containing 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin (Gibco), and 50 mg/ml streptomycin sulfate (Gibco) at 37°C in 5% CO_2 and 95% air. The cells were incubated in 60-mm dishes (BD Falcon, Japan) at a concentration of 1×10^6 cells/ml for 24 h in medium supplemented with 1.6×10^{-7} M PMA to induce their differentiation into macrophages. The cells were extensively washed with phosphate-buffered saline (pH 7.2), then cultured in RPMI-1640 medium containing 10% fetal bovine serum again and subsequently exposed to 1,000 mUI/ml of hCG (Mochida, Japan), luteinizing hormone (LH; Sigma-Aldrich, Japan) or follicle-stimulating hormone (FSH; Sigma-Aldrich). The concentration of hCG was determined according to the reported fetal plasma concentrations [Clements et al., 1976]. LH and FSH were used at the same concentration to compare the differences with hCG. Liquid culture media containing gonadotropin were removed by suction at the following defined periods; 1, 15, 30 min, 1, 2 or 3 h. The adherent cells in all dishes were extensively washed with phosphate-buffered saline, and then were used in the subsequent experiments.

Paraffin Embedding of Cell Block Specimens

The adherent cells without or with gonadotropin treatment were detached from the dishes using a cell scraper (BD Falcon) with 500 μl of trypsin-ethyleneaminetetraacetic acid solution (Gibco), and fixed in 10% neutral buffered formalin. The cells were suspended in 1% sodium arginate and solidified by the addition of 1 M calcium chloride. Finally, gelatinous specimens containing PMA-treated THP-1 cells without or with gonadotropin treatment were embedded in paraffin and cut into 4-μm sections.

Tissue Collection

Tissues of the chorionic villi were obtained from a normal pregnant woman undergoing an artificial abortion in early pregnancy. Informed consent was obtained from the patient. Ethical approval was also obtained and this study was performed according to the guidelines of the Ethics Committee in Faculty of Life Science, Kumamoto University. The tissue sample was fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin and cut into 4-μm sections.

Hematoxylin and Eosin Staining, Immunocytochemistry and Immunofluorescence Analyses

A paraffin-embedded section of chorionic villi and 6 paraffin-embedded sections of cell blocks were stained with hematoxylin and eosin (HE). A paraffin-embedded section of chorionic villi was immunohistochemically stained with 3.7 μg/ml of mouse anti-cluster of differentiation (CD) 68 monoclonal antibody (Dako, Japan). Nine paraffin-embedded sections of cell blocks were immunocytochemically stained with 3.7 μg/ml of mouse anti-CD68 monoclonal antibody (Dako), 14.2 μg/ml of rabbit anti-hCG-β polyclonal antibody (Dako), 0.1 μg/ml of mouse anti-LH-β monoclonal antibody (Thermo Scientific, Japan), 0.4 μg/ml of mouse anti-FSH-β monoclonal antibody (Thermo Scientific, Japan) or 1.0 μg/ml of rabbit anti-human LH/CG-R polyclonal antibody which was raised against amino acids 28–77 (exon 1) mapping to an extracellular domain of LH/CG-R of human origin (Santa Cruz Biotechnology, Calif., USA). For immunocytochemistry, horseradish peroxidase-labeled goat anti-rabbit or mouse immunoglobulins were used for 60 min as secondary antibodies. The peroxidase activity was visualized with Immpact DAB Diluent (Vector Laboratories, USA). Nuclear staining was performed with 1% methyl green in water. For immunofluorescence analyses, anti-rabbit or mouse Alexa Fluor 488 were used for 90 min as secondary antibodies. 4′,6-Diamidino-2-phenylindole (Roche, Japan) was used for detection of nuclei. Sections were viewed with a Biorevo BZ-9000 fluorescence microscope (Keyence, Japan). Negative controls were prepared by replacing the primary antibody with nonimmune antiserum from rabbits or mice.

Isolation of mRNA and Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from cells and the chorionic villi obtained from the placenta by using RNeasy Mini Kit (Qiagen, Japan), according to the manufacturer's protocols. Complementary deoxyribonucleic acid (cDNA) was synthesized using the SuperScript III Transcriptor First Strand cDNA Synthesis System for RT-PCR (Invitrogen, Japan), according to the manufacturer's instructions.

PCR was performed using KOD-Plus-Neo kit (Toyobo Co., Japan) on a Thermal Cycler PTC-200 (Bio-Rad, Japan). Two different primers for PCR were designed specific for hCG-β gene 7 and 8 products, respectively (all forward then reverse primer): hCG-β1 5′-TCACTTCACCGTGGTCTCCG-3′ and 5′-TGCAGCACGCGGGTCATGGT-3′ [423 base pairs (bp)] with 10 s at 98°C, 30 s at 60°C and 30 s at 68°C for 35 cycles; hCG-β2 5′-TGGCTGTGGAGAAGGAGGGCTGC-3′ and 5′-GGAAGCGGGGGTCATCACAGGTC-3′ (300 bp) with 10 s at 98°C, 30 s at 64°C and 30 s at 72°C for 35 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for control with same condition as hCG-β1.

Western Blot Analysis

The adherent cells without or with gonadotropin treatment were solubilized in 500 μl of Laemmli sample buffer (Bio-Rad, Japan) containing Complete Mini-Protease Inhibitors (Roche, Japan) and β-mercaptoethanol (1:20). The protein concentrations were determined by a Bradford assay [Bradford, 1976]. A total of 20 μl of protein extracts from these cells were loaded onto a reducing 10% sodiumdodecyl sulfate-tris(hydroxymethyl)aminomethane (Tris) polyacrylamide gel (Bio-Rad), and transferred to nitrocellulose membranes (Bio-Rad). The membranes were briefly washed in Tris-buffered saline containing 0.05% Tween 20 (TBST) and nonspecific binding sites were blocked by immersing the membranes in skim milk (Yukijirushi, Japan) for 1 h at room temperature on an orbital shaker. The membranes were shortly rinsed in two changes of TBST, and washed once for 15 min and twice for 5 min in fresh changes of TBST. The membranes were subjected to 1.0 μg/ml of rabbit anti-human LH/CG-R polyclonal antibody in blocking reagent for 1 h at room temperature. The membranes were washed and incubated with the peroxidase-labeled goat anti-rabbit IgG for 1 h at room temperature. The membranes were washed 3 × 10 min in fresh changes of TBST. Bound antibodies were detected by a chemiluminescent detection system (Amersham, Japan) as recommended by the manufacturer's protocol. Amersham hyper film ECL (GE Healthcare, Japan) was used to capture the chemiluminescence (exposure for 1, 5 min, and up to 15 min).

Results

hCG-Induced Morphological Change in PMA-Treated THP-1 Cells

There were CD68-positive coarsely vacuolated cells in the stroma of the human chorionic villi in early gestation (fig. 1a, b), and it is generally agreed that these cells are macrophages, known as Hofbauer cells. We confirmed that CD68, a human macrophage marker, was also expressed in PMA-treated THP-1 cells (fig. 1c). In the cell blocks of PMA-treated THP-1 cells, small cells with scanty cytoplasm were observed without hCG and 15 min after the addition of hCG (fig. 2a, b). When PMA-treated THP-1 cells were exposed to hCG for 30 min, multiple cytoplasmic vacuoles of various sizes appeared in the cytoplasm (fig. 2c). These cells mimicked the structure of Hofbauer cells in early pregnancy. The vacuoles were decreased in number and partly became larger in size at