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ARTICLE

Low frequency of imprinting defects in ICSI children born small for gestational age

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Although there is an increased frequency of low birth weight after assisted reproduction, the mechanisms underlying this association are unclear. We have proposed that some of the children conceived by intracytoplasmic sperm injection (ICSI) with low birth weight might have an epimutation (faulty methylation pattern) in one of the imprinted genes involved in fetal growth control, eg, *KCNQ1OT1*, *PEG1*, *PEG3*, *GTL2*, *IGF2/H19* and *PLAGL1*. Using bisulfite DNA sequencing and sequence-based quantitative methylation analysis (SeQMA), we determined the methylation pattern of these genes in buccal smears from 19 ICSI children born small for gestational age (SGA, birth weight <3rd percentile) and from 29 term-born normal weight children after spontaneous conception. We detected clear hypermethylation of *KCNQ1OT1* and borderline hypermethylation of *PEG1* in one and the same ICSI child. The other children and the parents of the affected child have normal methylation patterns. Imprinting defects appear to be a rare finding in ICSI children born SGA. Methylation of the paternal *KCNQ1OT1* and *PEG1* alleles may be a previously unrecognized cause of SGA. The epimutations found in the SGA child, whose father had oligozoospermia, probably result from an imprint erasure defect in the paternal germ line and therefore appear to be linked to the fertility problem of the father and not to *in vitro* fertilization/ICSI.
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Introduction

Children born after *in vitro* fertilization/intracytoplasmic sperm injection (ICSI) have an increased risk of premature birth, low birth weight, sex chromosome abnormalities, malformations and possibly imprinting disorders.[1] Epigenetic defects (faulty gene expression states) appear to contribute to some of these problems.[2] At present, it is unclear whether the defects are linked to the subfertility of the parents or the technique.

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In humans and other placental mammals, approximately 100 genes are subject to genomic imprinting. Imprinted genes are expressed from one of the parental alleles only and appear to control resource acquisition of the embryo and fetus.[3,4] They operate in the placenta, the fetus or both. Paternally expressed genes such as *PLAGL1*, *PEG1* (paternally expressed gene 1), *IGF2, KCNQ1OT1* and *PEG3* enhance fetal growth, whereas maternally expressed genes such as *GTL2* and *CDKN1C* appear to restrict fetal growth. Rare overgrowth syndromes in humans and animals are caused by the loss of maternal gene expression (*CDKN1C*[5] in Beckwith–Wiedemann syndrome and *IGF2R* in animals with large offspring syndrome[6]) or gain of maternal gene expression (*IGF2* in Beckwith–Wiedemann syndrome[7]). Reduced paternal *IGF2* expression is found in ~50% of patients with Silver–Russell syndrome, which is characterized by pre- and postnatal growth retardation.[8,9] On the

basis of the findings in these rare syndromes, it is possible that the dysregulation of imprinted genes may contribute to low birth weight in children.

DNA methylation at differentially methylated regions (DMRs) is one of the regulatory mechanisms controlling allele-specific expression of imprinted genes. Parental methylation imprints are erased in primordial germ cells, differentially established during spermatogenesis and oogenesis, and maintained during postzygotic development. Errors in imprint erasure, imprint establishment and imprint maintenance lead to aberrant imprints (imprinting defects[10]). As these defects do not affect the DNA sequence, they represent primary epimutations. We have investigated whether ICSI children born small for gestational age (SGA) have epimutations at one or more imprinted loci controlling fetal growth.

Materials and methods

Patients and controls

Children who were born SGA (birth weight <3rd percentile) were recruited from a prospective study on ICSI.[11] Among 2055 singletons born in the study group, we identified 60 SGA children. In 38 cases, the parents had previously given consent to be contacted again. In total, 19 parents agreed to send buccal smears from their child and themselves. At this time, the children were between 4 and 7 years old. Week of gestation, birth weight and birth size of the SGA children are listed in Table 1. For controls, we collected samples from 29 age-matched spontaneously conceived children with a normal birth weight.

DNA preparation

DNA was extracted and purified from buccal cells using the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Bisulfite treatment of genomic DNA

Bisulfite treatment of genomic DNA was modified from established protocols. Genomic DNA (500 ng in 50 μl) was denatured by adding 5.5 μl freshly prepared 3 M NaOH and incubating the solution at 37°C for 15 min. For complete denaturation, the samples were incubated at 95°C for 2 min and immediately cooled on ice. The bisulfite solution was prepared by dissolving 8.5 g of sodium bisulfite in 15 ml degassed water, adding 900 μl of a 50 mM hydroquinone solution, and adjusting the pH to 5.1 with 1000 μl of 10 M NaOH. The bisulfite solution (500 μl) was added to the denatured DNA, mixed and incubated at 50°C for 16 h in the dark. The DNA was recovered by using the Wizard DNA Clean-Up System (Promega) followed by elution in 50 μl water. Subsequently, 5.5 μl of 3 M NaOH was added, and the samples were incubated for 15 min at 37°C. The solution was then neutralized by adding 55 μl of 6 M NH_4OAc pH 7.0. The DNA was ethanol precipitated, washed in 70% ethanol, dried and resuspended in 8 μl water.

Sequence-based quantitative methylation analysis

Sequence-based quantitative methylation analysis (SeQMA) allows determining the degree of methylation of all CpGs in a given sequence.[12]

First, genomic DNA is treated with sodium bisulfite, which converts unmethylated cytosine residues to uracil, whereas methylated cytosine residues remain unchanged. This results in specific sequence changes. PCR is performed on the bisulfite-treated DNA and the products are subjected to the *Taq* cycle sequencing procedure with fluorescence-labeled dideoxynucleotides. The sequencing products are subjected to capillary gel electrophoresis and analyzed with the GeneScan software (ABI). By comparing thymine peak areas at CpG sites with genomic thymine peak areas at control sites (non-CpG sites), the degree of methylation can be determined. Thymine residues that are present in the original sequence have an invariable peak height and area, whereas thymine peak heights and areas at CpG sites are variable after bisulfite treatment depending on the methylation status (Figure 2).

An aliquot of bisulfite-treated genomic DNA (1 μl) was used for each PCR in a total volume of 25 μl. For improved sequencing results, we used primers with a tag (forward 5′-CTTGCTTCCTGGCACGAG-3′ and reverse 5′-CAGGAAA CAGCTATGAC-3′), which contain cytosine and guanine residues. PCR conditions were as follows: 95°C for 10 min, 35 cycles of 95°C for 20 sec, annealing for 20 s (annealing temperatures are given in Table 2), 72°C for 30 s; finally 7 min at 72°C. The PCR products were gel purified by Wizard SV® Gel and PCR Clean-Up System (Promega). The sequence reaction was performed with Big Dye Terminators (BigDye® Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and the forward tag primer. Cycle sequencing conditions were as follows: 96°C for 1 min, 25 cycles of 96°C for 10 s, 56°C for 5 and 60°C for 4 min. After adding 1 μl of GeneScan-LIZ 500 size standard, the sequencing products were subjected to fragment analysis on an ABI

Table 1 Week of gestation, birth weight and birth size of the 19 ICSI children

Sample	1	2	3	4	11	12	13	14	21	22	23	24	31	32	33	34	41	42	43
Week of gestation	38	40	39	36	37	38	33	38	na	38	38	40	36	25	39	36	37	35	38
Birth weight (g)	2120	2240	2400	2070	2200	2250	1400	2200	2270	2490	2360	2450	1990	510	2440	1635	1810	1650	2170
Birth size (cm)	45	45	46	44	45	46	40.5	48	na	46	47	48	43	30	49	44.5	43	40	46

na, not available.

Table 2 Investigated loci and primer sequences used for bisulfite PCR

Gene	*Primer*	*Primer sequence (5′–3′)*	*Annealing temperature (°C)*	*Product size (bp)*	*Number of CpGs*	*Accession number*
KCNQ1OT1	LIT1-Not1-Ftag	CTTGCTTCCTGGCACGAGTTTATAGGTTT TTATATYGAGGGTTTATAGTAG	57	156	4	U90095
	LIT1-Not1-RM13	CAGGAAACAGCTATGACAAATAAACY RAAAACACRAACCAATTCTCTAC				
PEG1	PEG1-Ftag	CTTGCTTCCTGGCACGAGTYGTTGTTGG TTAGTTTTGTAYGGTT	58	184	10	AC144863
	PEG1-RM13	CAGGAAACAGCTATGACCCAACC ACACCCCCTCRTTCCCAC				
PEG3	PEG3-Ftag	CTTGCTTCCTGGCACGAGTTATTAGT TTAGGGTGGATATTTTT	56	177	6	AC006115
	PEG3-RM13	CAGGAAACAGCTATGACACTAATTAACTA ACACAAAAACCCC				
PLAGL1	ZAC1-P1-inFtag	CTTGCTTCCTGGCAC-GAGGGGGTTTTTTTTTGTTAYGTG	59	244	8	AL109755
	ZAC1-P1-RM13	CAGGAAACAGCTATGACCCCAACCRTAT CTAAATCAAAACT				
GTL2	GLT2-3.2-Ftag	CTTGCTTCCTGGCACGAGAAGAGGGA ATAGTTTTGAGATTTTT	59	247	8	AL117190
	GTL2-3.2-RM13	CAGGAAACAGCTATGACTAACCCCTCA CTAACCTTATCACA				
IGF2/H19	H19-in-Ftag	CTTGCTTCCTGGCACGAGGGTAY GGAATTGGTTGTAGTTGTGG	56	204	8	AF087017
	H19-CTCF-RM13	CAGGAAACAGCTATGACATATCCTATTC CCAAATAACCCC				

Y = C or T; R = G or A; The forward primer for the *PEG1* locus was taken from Sato *et al*[13] and the reverse primer for the *ZAC1* locus was taken from Valleley *et al*[14] and both primers were provided with a tag.

3100 Genetic Analyzer using the GeneScan software (Applied Biosystems). Analyzed loci and primer sequences are listed in Table 2. Primers were designed with the help of MethPrimer (http://www.urogene.org/methprimer/) and all of them are within the core region of the corresponding DMR.

Raw data of each CpG peak area (a uracil/thymine target residue generated by bisulfite treatment from a CpG dinucleotide) were exported from the GeneScan software. The lack of cytosine residues outside CpG islands indicates that there was complete conversion of unmethylated cytosines to thymines. The relative peak area (RPA) for each CpG was determined by calculating the ratio of each single CpG peak area to the sum of the nearest control peak areas in the sample (peak area CpG/Σcontrol peak areas = RPA). Control peaks were chosen from thymine residues in the original sequence, which are in close vicinity to the respective CpG. To define a normal 'expected value', we averaged data from five standard DNA samples by calculating the mean RPA for each CpG peak (corresponding to each probe or locus). These samples are from healthy individuals who have normal methylation levels. We compared each RPA to the mean RPA to obtain a percentage value denoted RPA ratio (RPA/mean RPA = RPA ratio). The RPA ratios correspond to 50% of methylation (RPA ratio × 50%). For assessment of methylation status in target samples, RPAs and RPA ratios were calculated in the same way (RPAs were compared with the expected RPAs). RPA ratios were averaged to calculate RPA ratio *N* for each target sample. The obtained quantitative values (*N* values) show the degree of non-methylation and thus the degree of methylation. As suggested by Dikow *et al*[12] *N* values >0.6 indicate hypomethylation, whereas values <0.4 indicate hypermethylation. Values between 0.4 and 0.6 indicate a normal methylation.

The degree of methylation in the standard DNA samples at the different loci analyzed was set to 50%. Thus, in comparison to the ICSI samples, we get relative values for methylation status and can detect methylation abnormalities.

Combined bisulfite restriction analysis

To confirm the SeQMA results, combined bisulfite restriction analysis (COBRA) was performed. PCR products for *KCNQ1OT1* and *PEG1* (see above) were digested with *Bst*UI, which only cleaves the recognition site, if the original DNA was methylated. Gel-purified PCR products (150 ng) were digested with the enzyme and electrophoresed on a 3% agarose gel.

Genomic sequence analysis

We designed primers for *KCNQ1OT1* and *PEG1* for unmodified genomic DNA, so that the PCR products span the regions analyzed by SeQMA. For each PCR, 140 ng genomic DNA was used in a total volume of 25 μl. PCR primers LITnDNA5 forward (GAGACCTTGCCCGGGTTC)

and LITnDNA4 reverse (ACTGGCTGGGTGTG-AGGT) were used to amplify a 304 bp *KCNQ1OT1* region. PCR conditions were as follows: 95°C for 2 min, 35 cycles of 95°C for 30 s, 61°C for 30 s, 72°C for 1 min; finally 5 min at 72°C. The PCR products were purified by Microcon (Millipore). The sequence reaction was performed with Big Dye Terminators (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) using the forward and reverse primers, respectively. Cycle sequencing conditions were as follows: 96°C for 1 min, 25 cycles of 96°C for 10 s, 60°C for 5 s and 60°C for 4 min. For *PEG1*, PCR primers PEG1: forward (CCGGAGTGGCTGTAGCTG) and PEG1: reverse (CGCCTGTCGGTAGAGTTTTC) were used to amplify a 306-bp region. PCR and cycle sequencing conditions were the same as for *KCNQ1OT1*.

Results

Using a quantitative methylation assay, we studied six DMRs associated with imprinted loci in 19 ICSI/SGA (cases) and 29 age-matched control children. The surveyed regions contain 4 CpGs (*KCNQ1OT1*), 6 CpGs (*PEG3*), 8 CpGs (*IGF2/H19*, *PLAGL1* and *GTL2*) and 10 CpGs (*PEG1*), respectively. We designated a case as abnormally methylated, if the value was outside the 40–60% range and not observed in any of the 29 control children. By these criteria, only one ICSI/SGA child (no. 33) had abnormal methylation levels (hypermethylation of *KCNQ1OT1* and *PEG1*; Figure 1). A few cases had *KCNQ1OT1* methylation levels slightly less than 40%, but so had some control children. Two control children, but no cases had *GTL2* methylation levels slightly higher than 60%.

Child no. 33 had 74% methylation of *KCNQ1OT1* and 63% methylation of *PEG1* (Figure 1). As judged by single-site methylation analysis (Figure 2), all CpGs tested showed the same degree of hypermethylation. Similar findings (72 and 60%, respectively) were made in another independent SeQMA experiment (not shown) and confirmed by an independent method (COBRA; Figure 3). The parents of child no. 33 had normal methylation levels (P1 and P2 in Figure 1).

As we were aware of a single nucleotide polymorphism (SNP) in the primer-binding site of the primer LIT1-Not1-RM13 (rs11023840), which might bias the PCR and thus confound the SeQMA and COBRA analyses, we had included a wobble in the sequence (see Materials and methods). The fact that child no. 33 and her mother are heterozygous for this SNP (Supplementary Figure 1), yet only the child shows abnormal *KCNQ1OT1* methylation levels, argues against a confounding role of this SNP in our analysis. To search for other variants in the *KCNQ1OT1* and *PEG1* primer-binding sites, we sequenced the two loci in unmodified genomic DNA. As shown in Supplementary Figure 1, child no. 33 is homozygous for the reference sequence, apart from the rs11023840 SNP in the LIT1-Not1-RM13 primer.

Child no. 33 was conceived by ICSI because of male factor infertility with a low sperm count (4 million sperms/ml). She was born after 39 weeks of gestation and had a birth weight of 2440 g and a length of 49 cm. She showed catch-up growth after birth and has developed normally (Supplementary Figure 2). She has no phenotypic abnormalities.

Discussion

The present study was designed to determine whether imprinting defects contribute to SGA in ICSI children. The patients were recruited from a prospective study on the outcome of ICSI.[11] Among 19 ICSI/SGA children, we identified only one child with putative imprinting defects of *KCNQ1OT1* and *PEG1*. None of the children had Silver–Russell syndrome, which often results from hypomethylation of *IGF2/H19*.[8] Our results show that imprinting defects are not frequent in ICSI/SGA children, at least not at the six loci investigated in this study. However, our study is limited by the fact that we could investigate only one tissue (buccal smear) at only one time point. Most importantly, we did not have newborn and placental material. Owing to the fact that imprinting defects are often mosaic (see discussion below), the frequency of imprinting defects might actually be higher than observed in our study.

By SeQMA, one child had 72–74% methylation of *KCNQ1OT1* and 60–63% methylation of *PEG1*. The parents were normal at these loci. The latter finding and our genomic sequence analysis exclude the possibility that our analysis was confounded by an SNP, and indicate that the child has a *de novo* hypermethylation. Although the results were confirmed by an independent method (COBRA), the findings in *PEG1* are borderline. We note, however, that child no. 33 has the highest degree of *PEG1* methylation among all 48 children. The results between two SeQMA experiments are reassuringly similar, showing that there is little inter-experiment variation, but there appears to be a certain degree of inter-individual variation in methylation, which makes the detection of mosaic imprinting defects difficult.

Do our findings make sense? Hypermethylation of *KCNQ1OT1* and *PEG1* means that the paternal allele is methylated, which is normally unmethylated. *PEG1* is located in 7q32, encodes a member of the alpha/beta hydroxylase fold family and is paternally expressed in human fetal tissues. The promoter region is methylated on the maternal chromosome and unmethylated on the paternal chromosome.[15] Maternal uniparental disomy 7 (complete methylation at this locus) is associated with pre- and postnatal growth retardation, but the role of *PEG1* in this condition is unknown. Lefebvre *et al*[16] showed that

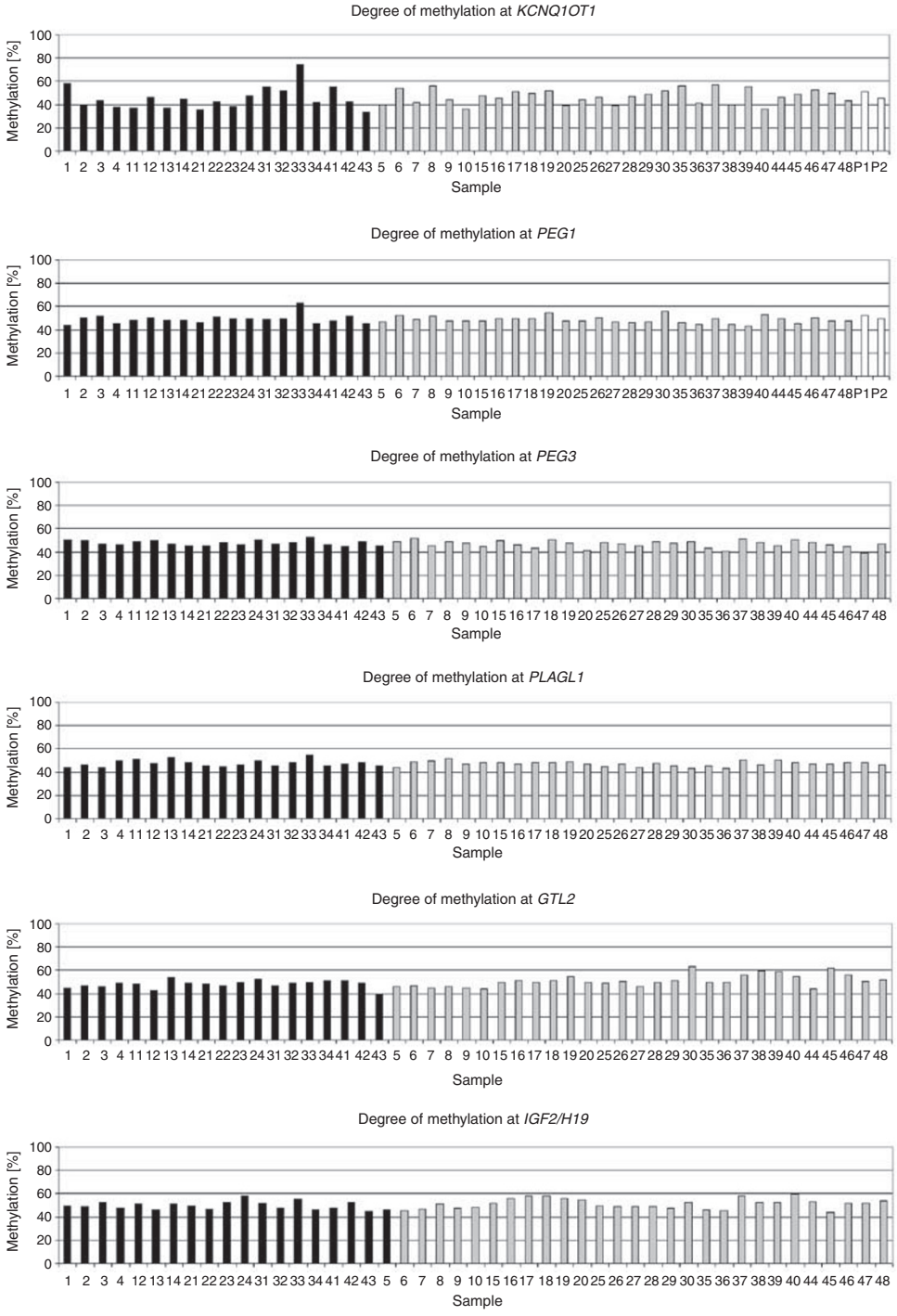

Figure 1 Methylation analysis by sequence-based quantitative methylation analysis (SeQMA). The histograms show the degree of methylation at six loci in intracytoplasmic sperm injection (ICSI)/small for gestational age (SGA) children (black bars), control children (gray bars) and the parents of child no. 33 (P1 and P2, white bars; *KCNQ1OT1* and *PEG1* only). Child no. 33 is hypermethylated at *KCNQ1OT1* and possibly at *PEG1*. Her parents have normal methylation levels. Because of insufficient material of sample no. 11, we could not perform SeQMA for the locus *IGF2/H19* for this sample.

heterozygous mice that inherited a mutant allele from the paternal germ line were smaller and lighter, but otherwise fertile.

CDKN1C is located in 11p15, encodes the cyclin-dependent kinase inhibitor p57KIP2 and is maternally expressed. Regulation of imprinted *CDKN1C* expression is

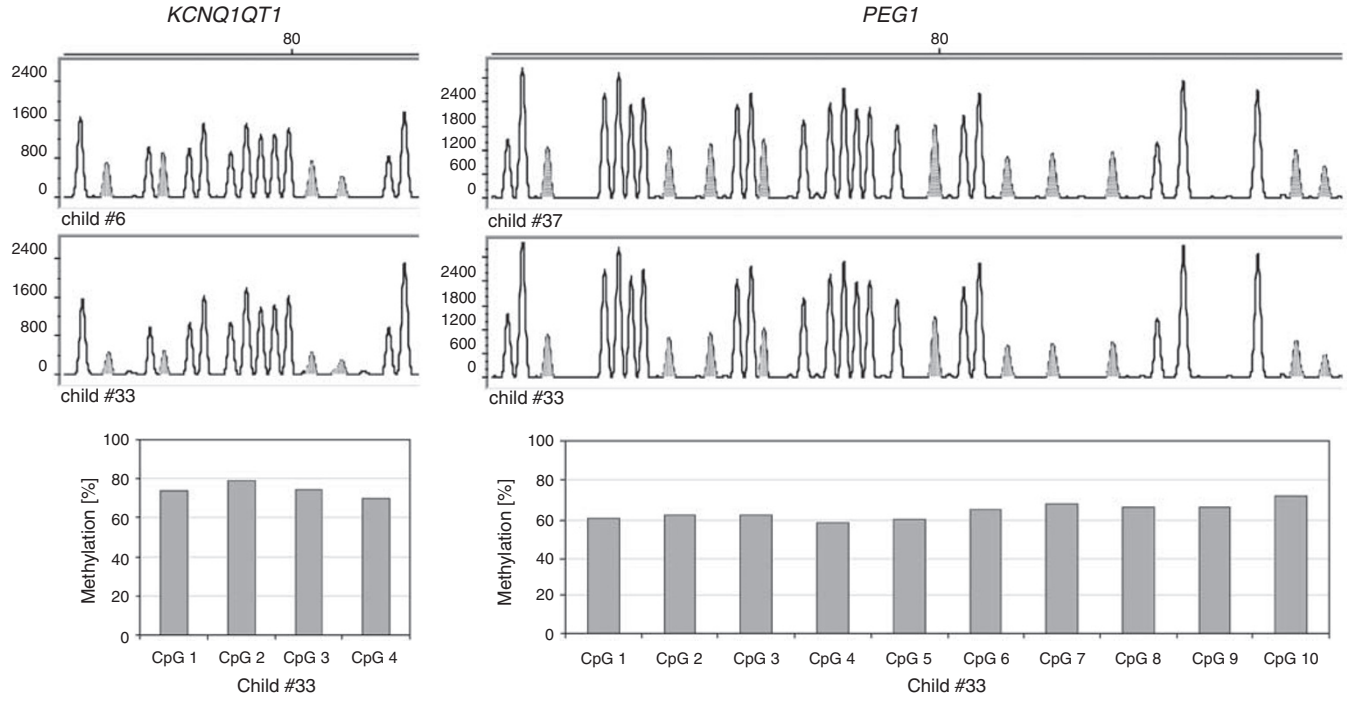

Figure 2 Single-site methylation analysis. Sequencing reactions were subjected to GeneScan analysis. The upper four panels show the T channel outputs for *KCNQ1OT1* (left) and *PEG1* (right). Thymine residues resulting from bisulfite conversion of CpGs to TpGs are indicated in gray. Compared to children of the control group, child no. 33 is hypermethylated at the *KCNQ1OT1* CpGs and borderline hypermethylated at the *PEG1* CpGs.

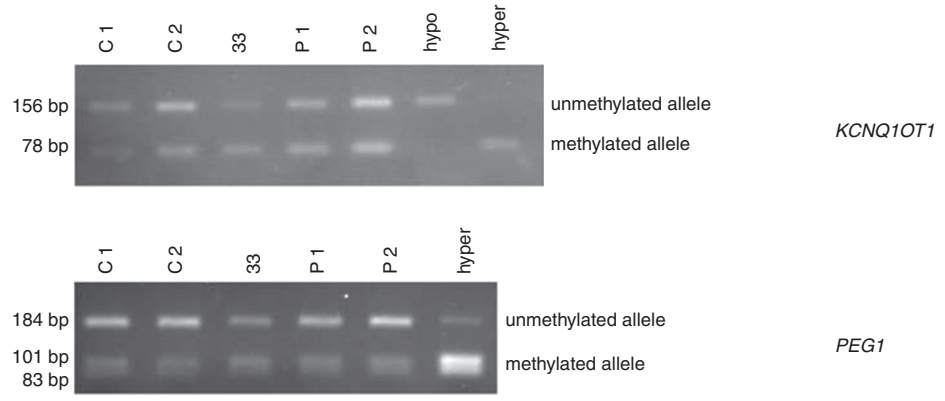

Figure 3 Methylation analysis by combined bisulfite restriction analysis (COBRA). *Bst*UI cleaves the bisulfite PCR products only, if the original DNA was methylated. In child no. 33, the relative intensity of the unmethylated *KCNQ1OT1* and *PEG1* bands is lower when compared to normal control (C1 and C2) and the parents (P1 and P2). Hypo, strongly hypomethylated control; hyper, strongly hypermethylated DNA sample. For *PEG1*, no hypomethylated DNA sample was available.

regulated by the maternally methylated *KvDMR1* within intron 10 of the *KCNQ1* gene, which also regulates expression of the paternally expressed *KCNQ1OT1* transcript.[17] Loss of *CDKN1C* expression is associated with overgrowth (Beckwith–Wiedemann syndrome[5]). Maternal duplication of 11p15 is associated with growth retardation,[18,19] although this effect could also involve the *IGF2/H19* cluster. Up to now hypermethylation of the *KCNQ1OT1* locus has not been reported in humans, but probably leads to overexpression of *CDKN1C* and thus may cause fetal growth retardation. A study of Fitzpatrick *et al*[20] demonstrated that a paternal inheritance of a deletion

of *Kvdmr1* results in overexpression of six maternally expressed genes, including *Cdkn1c*. The main feature of these knockout mice is growth retardation.

In summary, we note that the methylation changes of *KCNQ1OT1* and *PEG1* in child no. 33 are in the expected direction.

Assuming the methylation changes are authentic, what might be the cause? In general, epimutations after assisted reproduction may be linked to the fertility problems of the parents (father or mother) or the procedure. On the basis of studies in patients with Angelman syndrome,[21,22] Beckwith–Wiedemann syndrome[23–26] and animal models,[6] it has been suggested that superovulation[13,27] or cell culture[23,24] might affect methylation at certain imprinted loci. These procedures are unlikely to be the reason for the abnormal methylation pattern in child no. 33. In the aforementioned studies, loss of methylation at maternal alleles at the *SNRPN* and *KCNQ1OT1* loci had been found. In child no. 33, we have found hypermethylation of the paternal allele of *KCNQ1OT1* and possibly *PEG1*.

A study of Kobayashi *et al*[28] revealed that sperm from infertile men carry a higher risk of transmitting incorrect primary imprints to their offspring. Moreover, they found out that errors were more frequent at maternally methylated DMRs in the ejaculated sperm than at paternally methylated DMRs. Aberrant methylation of several maternally methylated loci such as *PEG1*, *KCNQ1OT1*, *PLAGL1*, *PEG3* and *SNRPN* in the ejaculated sperm are probably the result of errors in imprint erasure. Transmission of the aberrant imprint might thus be promoted during male infertility treatment.[28–30] Therefore, it is likely that a sperm of the affected child's father, who had oligozoospermia, carried an incorrect imprint (hypermethylation at *KCNQ1OT1* and *PEG1*), which was transmitted to the child.

Interestingly, Kagami *et al*[31] found partial hypermethylation of *PEG1* in a patient with Silver–Russell syndrome conceived after *in vitro* fertilization. Furthermore, four of the eight abnormally methylated cytosines were also methylated in the father. It was inferred, therefore, that the paternal *PEG1* allele with mildly hypermethylated DMR was further methylated and transmitted to the patient.

If child no. 33 was conceived from a sperm carrying methylated *KCNQ1OT1* and *PEG1* alleles, why does she not have 100% methylation at these loci? Partial hypermethylation of these loci in child no. 33 reflects a mosaic situation, that is, the presence of hypermethylated cells and normal cells. Mosaic hypermethylation may result (i) from the postzygotic gain of methylation at these loci, possibly as a failure of a cell to protect the unmethylated paternal alleles against *de novo* methylation or (ii) from the postzygotic loss of an aberrant methylation imprint. It is difficult to link these defects to the assisted reproduction technology procedure.

On the basis of the methylation changes found in sperm of infertile men, including the *PEG1* locus, it is tempting to speculate that the methylation changes in child no. 33 are linked to the fertility problem of the father. The following scenario would be compatible with the published data and our findings. Child no. 33 was conceived from a sperm that was abnormally methylated at *KCNQ1OT1* and *PEG1*, probably as a result from a failure to erase the maternal methylation imprint during spermatogenesis. It has been shown in patients with Prader–Willi syndrome and a sporadic imprinting defect, that the chromosome carrying the imprint defect is always inherited from the paternal grandmother,[32] strongly suggesting that defects in imprint erasure do occur. In contrast to a real maternal methylation imprint, the aberrant methylation pattern may not include each CpG within the DMR. As a consequence, this abnormal imprint may be less stable during postzygotic development, leading to complete loss in some cells. As such cells would have a proliferative advantage relative to a cell with *KCNQ1OT1* and *PEG1* hypermethylation, this cell line would outgrow the affected cell line. This scenario is speculative, but would explain the catch-up growth in this child.

In summary, imprinting defects appear to be a rare finding in ICSI children born SGA, at least at the loci investigated in this study. To the best of our knowledge, hypermethylation of *KCNQ1OT1* has never been observed before in a normal or a growth-retarded child. It may represent a rare stochastic event or be linked to paternal subfertility, but not to *in vitro* fertilization/ICSI. Given the rare frequency of imprinting defects and the instability of defective imprints at certain loci, it will be necessary to collect newborn and placental tissue and to examine more children.

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