MHR-Basic Science of Reproductive Medicine Vol.14, No.2 pp. 67–73, 2008
Advance Access publication on January 4, 2008 doi:10.1093/molehr/gam093

Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia

C.J. Marques[1], P. Costa[1], B. Vaz[1], F. Carvalho[1], S. Fernandes[1], A. Barros[1,2] and M. Sousa[1,2,3,4]

[1]Department of Genetics, Faculty of Medicine, Porto 4200-319, Portugal; [2]Centre for Reproductive Genetics Alberto Barros, Porto 4100-009, Portugal; [3]Lab Cell Biology, Institute of Biomedical Sciences Abel Salazar, University of Porto, Largo Prof. Abel Salazar 2, Porto 4099-003, Portugal

[4]Correspondence address. Tel: +351-22-206-22-17; Fax: +351-22-206-22-32; E-mail: msousa@icbas.up.pt

Genomic imprinting marks in the male germ line are already established in the adult germinal stem cell population. We studied the methylation patterns of *H19* and *MEST* imprinted genes in sperm of control and oligozoospermic patients, by bisulphite genomic sequencing. We here report that 7 out of 15 (46.7%) patients with a sperm count below 10×10^6/ml display defective methylation of *H19* and/or *MEST* imprinted genes. In these cases, hypomethylation was observed in 5.54% (1.2–8.3%) and complete unmethylation in 2.95% (0–5.9%) of *H19* clones. Similarly, for the CTCF-binding site 6, hypomethylation occurred in 4.8% (1.2–8.9%) and complete unmethylation in 3.7% (0–6.9%) of the clones. Conversely, hypermethylation occurred in 8.3% (3.8–12.2%) and complete methylation in 6.1% (3.8–7.6%) of *MEST* clones. Of the seven patients presenting imprinting errors, two had both *H19* hypomethylation and *MEST* hypermethylation, whereas five displayed only one imprinted gene affected. The frequency of patients with *MEST* hypermethylation was highest in the severe oligozoospermia group (2/5 patients), whereas *H19* hypomethylation was more frequent in the moderate oligozoospermia (2/5 patients). In all cases, global sperm genome methylation analysis (LINE1 transposon) suggested that defects were specific for imprinted genes. These findings could contribute to an explanation of the cause of Silver–Russell syndrome in children born with *H19* hypomethylation after assisted reproductive technologies (ART). Additionally, unmethylation of the CTCF-binding site could lead to inactivation of the paternal *IGF2* gene, and be linked to decreased embryo quality and birth weight, often associated with ART.

Keywords: genomic imprinting; male infertility; oligozoospermia; DNA methylation

Introduction

Genomic imprinting is a mechanism that regulates gene expression in a parental origin-dependent way, leading to monoallelic gene expression. Evidence suggesting the mouse paternal genome is essential for normal development of extraembryonic tissues and the maternal genome for embryogenesis (Barton *et al*., 1984) came after finding that androgenotes and gynogenotes could not develop to term due to poor embryo and trophectoderm development, respectively (McGrath and Solter, 1984; Surani *et al*., 1984). The differential expression of parental genomes was later described as affecting only some chromosomes or chromosome regions, indicating the existence of a form of chromosome imprinting affecting gene activity (Cattanach and Kirk, 1985). After fertilization in mice, the paternal genome undergoes active demethylation and the maternal genome undergoes passive demethylation, without affecting imprinting marks. Thus, the functional differences between parental genomes are inheritable from the gametes and retained following genome activation at the embryo two-cell stage (Surani *et al*., 1986). At the blastocyst stage, the inner cell mass genome becomes hypermethylated, whereas in trophectoderm it remains undermethylated (Santos and Dean, 2004). After this first reprogramming, demethylation of the whole-genome and erasure of imprinting marks occur in primordial germ cells, by the time they migrate into genital ridges (Hajkova *et al*., 2002). In the human germ line, re-establishment of paternal imprints starts prenatally and is completed postnatally at the pachytene stage (Kerjean *et al*., 2000), whereas maternal imprints are acquired postnatally being completed by metaphase II (Geuns *et al*., 2003).

Imprinted genes play important roles in embryo development, placental function, neurological processes and behaviour. Imprinting deregulation might cause Beckwith–Wiedemann (11p15.5), Prader–Willi and Angelman (15q11-q13) human syndromes, as well as cancer (Takai *et al*., 2001; Ulaner *et al*., 2003; Arnaud and Feil, 2005). Due to an increased risk of birth of children with imprinting syndromes after assisted reproduction techniques (ART) in association with hypomethylation of the maternal allele, the maintenance and/or establishment of maternal imprints was suggested to be potentially affected during ovarian hormonal hyperstimulation and/or embryo culture (Arnaud and Feil, 2005). However, the recent demonstration of imprinting defects in cases of disrupted spermatogenesis raised the possibility that they could be associated with infertility itself (Marques *et al*., 2004).

To further underscore the impact of disturbed spermatogenesis on the risk of producing gametes with genomic imprinting defects, the imprinting marks of sperm from controls and infertile males with different degrees of oligozoospermia were here studied using bisulphite modification and cloning analysis. Two imprinted genes were chosen, *H19* and *MEST/PEG1* (Mesodermal-specific transcript/Paternally expressed gene 1). *H19* encodes for an untranslated RNA, being

methylated (repressed) in the paternal allele and unmethylated (expressed) in the maternal allele (Bartolomei *et al*., 1991; Zhang and Tycko, 1992). Paternal methylation of *H19* is acquired asynchronously, as it first occurs in the paternal allele (Davis *et al*., 1999). Thus, even in the absence of methylation, parental alleles can retain their identity, possibly through other epigenetic marks such as histone modifications (Delaval *et al*., 2007). Nevertheless, both alleles become methylated before the onset of meiosis (Davis *et al*., 1999). *H19* is physically and functionally linked to the *IGF2* gene, being reciprocally expressed in endoderm and mesoderm tissues during the fetal life and strongly down-regulated after birth (Gabory *et al*., 2006). They share common enhancers, located downstream of *H19*, whose activity is regulated by a differentially methylated region (DMR) upstream of the *H19* gene. In the maternal allele, *H19* is unmethylated, which allows the CTCF insulator protein (CCCTC-binding factor) to bind to the DMR. This prevents access of *IGF2* to the common enhancers, thus inhibiting *IGF2* and promoting *H19* expression. In the paternal allele, *H19* is methylated and binding of CTCF is blocked, thus inactivating *H19* and promoting *IGF2* expression (Arney, 2003). Oppositely, *MEST* is methylated and repressed in the maternal allele, and unmethylated and expressed in the paternal allele (Kaneko-Ishino *et al*., 1995; Nishita *et al*., 1996). It is mainly expressed in mesodermal tissues (Kaneko-Ishino *et al*., 1995) and is implicated in maternal behaviour (Lefebvre *et al*., 1998). In females, *MEST* becomes remethylated during meiosis (Lucifero *et al*., 2004), whereas in males it remains unmethylated (Kerjean *et al*., 2000; Lucifero *et al*., 2002).

We here show, by bisulphite genomic sequencing analysis of 788 clones, that sperm of patients with <10 million spermatozoa per ml of semen present complete unmethylation of *H19* (along with the CTCF-binding site) and complete methylation of *MEST* imprinted genes. In order to investigate if hypomethylation of *H19* could be linked to a decreased level of global methylation of the sperm genome, the methylation status of LINE1 transposon was then analysed. LINE1 (L1) elements are retrotransposons that account for 5–10% of the human genome (Woodcock *et al*., 1997). Methylation of CpGs within this element is important for maintaining transcriptional inactivation of potentially functional L1 elements and for inhibiting L1 transposition (Burden *et al*., 2005). L1 elements seem to be resistant to the global demethylation that occurs in primordial germ cells, which suggests that methylation might be passed from one generation to the other. However, *de novo* methylation still occurs in the male germ line, resulting in complete methylation of these elements in sperm (Sanford *et al*., 1987; Lane *et al*., 2003; Lees-Murdock *et al*., 2003). On the contrary, L1 elements are sensitive to the reprogramming events occurring in the zygote and blastocyst stages (Lane *et al*., 2003). The present data shows that methylation errors occurring during spermatogenesis are specific for imprinted genes and associate with disturbed spermatogenesis.

Materials and Methods

Patient recruitment and classification

Under informed consent of the patients and ethics committee approval, 25 samples of semen were obtained from males undergoing routine spermiogram analysis: 5 with normal semen parameters (NZ: $\geq 20 \times 10^6$ sperm/ml, $\geq 15\%$ sperm normal morphology and $\geq 25\%$ sperm rapid progressive motility; undergoing fertility treatments due to infertility of female factor) and 20 with oligozoospermia (OZ), 5 with mild (OZ1: $10–20 \times 10^6$/ml), 5 with moderate (OZ2: $5–10 \times 10^6$/ml), 5 with severe (OZ3: $1–5 \times 10^6$/ml) and 5 with very severe (OZ4: $<1 \times 10^6$/ml) oligozoospermia. Among the 20 oligozoospermic samples, 14 had asthenozoospermia and 6 had normal sperm rapid progressive motility (1 with mild, 1 with moderate, 3 with severe and 1 with very severe oligozoospermia). Morphology was abnormal in all samples, with 0% sperm normal morphology in very severe oligozoospermia. All males had normal karyotypes and absence of Y-chromosome microdeletions (Pinho *et al*., 2005).

Purification of sperm from the ejaculate

After liquefaction (37°C, 30 min), the semen was centrifuged (437*g*, 20 min) using Suprasperm gradients (MediCult, Copenhagen, Denmark; 1 ml semen, 2 ml 55%, 2 ml 80%, 1 ml 90%). The pellet was washed (2×10 min, 437*g*) in sperm preparation medium (SPM, MediCult), slightly covered with 0.1–1 ml SPM and incubated at 37°C with 5% CO_2 in humidified air, for 1 h, to collect the swim-up fraction. The purity of the fraction was confirmed by phase contrast microscopy (Nikon, Tokyo, Japan). In cases with very low sperm concentration, 200–300 sperm cells were isolated by micromanipulation using an inverted microscope (Nikon), equipped with Hoffman optics, thermic stage and micromanipulators (Narishige, Nikon), and micropipettes with 7 μm of internal diameter (SweMed, Billdal, Sweden).

DNA extraction and modification with sodium bisulphite

Sperm DNA was decondensed and extracted by adding 20 μl of alkaline lysis buffer, 1 M KOH (Merck, Darmstadt, Germany) and 0.05 M dithio-threitol (Invitrogen, Carlsbad, CA, USA), followed by incubation at 80°C during 20 min. The reaction was stopped by adding 20 μl of neutralizing buffer, 0.9 M Tris–HCl (Sigma, Steinheim, Germany), 0.3 M KCl and 0.2 M HCl, pH 8.3 (Merck). Extracted DNA was then treated and modified with a sodium bisulphite procedure using the CpGenome DNA Modification Kit (Chemicon International, Temecula, USA), according to manufacturer's instructions. Bisulphite converts unmethylated cytosines to uracil, whereas 5-methylcytosines (5-MeC) remain unaltered. Only sequences with $>95\%$ of non-CpG cytosines converted and without unconverted cytosines adjacent to CpGs were validated.

DNA amplification

Modified DNA was amplified by PCR for *H19* and *MEST* imprinted genes (Kerjean *et al*., 2000) and for LINE-1 transposon. For *H19*, 18 CpGs of the DMR were analysed within a sequence of 322 bp (GenBank Accession Number AF087017; nucleotides 6006-6328). This sequence includes the CTCF-binding site 6 (Fig.1, CpG 4–8). In the sequence, CpG 7 (GenBank Accession number AF125183, position 7966) behaves as a polymorphic site as it may display either a cytosine or a thymine (C/T). Because CpG 7 is not informative in terms of methylation after bisulphite modification, it was not considered for the quantitative analysis. For *MEST*, the amplified region included part of the first exon and part of the first intron from the DMR (GenBank Accession Number Y10 620; nucleotides 609-898), containing 22 CpGs and spanning a 289 bp sequence. For LINE-1 transposon, a region with 19 CpGs within the CpG island (GenBank Accession Number X58 075; nucleotides 113–357) was amplified using the following primers: F113 5'-ttattagggagtgttagatagtggg-3′ and R357 5′-cctctaaaccaaatataaaatataatctc-3' (designed with MethPrimer, Li and Dahiya, 2002).

A PCR reaction (50 μl) was prepared for each gene and their respective negative controls (DNA extraction, bisulphite treatment, PCR reaction). The reagent mixture contained 1× buffer with 1.5 mM $MgCl_2$ (Qiagen, Hilden, Germany), 0.12 mM of each dNTP (Invitrogen), 0.5 μM of each primer (Thermo Electron, Ulm, Germany), 1.5 U HotStarTaq enzyme (5 U/μl, Qiagen) and different amounts of the modified DNA according to the gene, 6 μl (*H19*) and 4 μl (*MEST*, LINE 1). The PCR conditions were: initial strand denaturation (15 min, 95°C), followed by 45 amplification cycles (denaturation, 1 min, 94°C; primer annealing, 1 min, 60°C; strand elongation, 1 min, 72°C) and a final extension (20 min, 72°C).

DNA cloning

Amplified products were purified with the GFX PCR-DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, England), according to manufacturer's instructions. Purified PCR products were cloned with the TOPO TA cloning kit (Invitrogen), using the *E. coli* Mach1-T1 (Invitrogen) bacteria strain and the pCRII vector (Invitrogen), according to manufacturer's instructions. Selection of bacterial clones that incorporated the plasmid containing the fragment of interest was performed using selective LB growth medium with ampicillin (100 μg/ml, AppliChem,

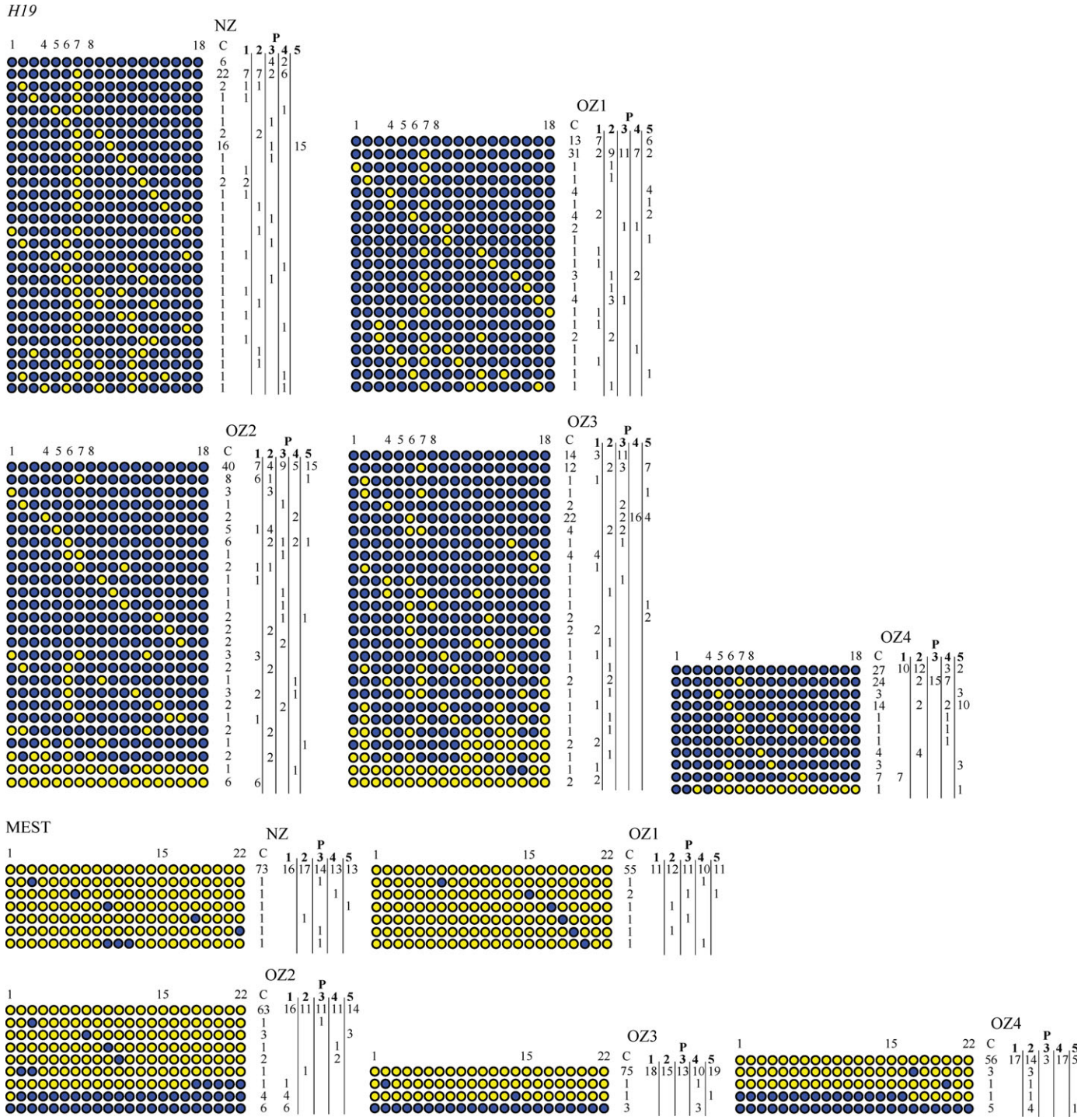

Figure 1: Methylation patterns of *H19* (18 CpGs) and of *MEST/PEG1* (22 CpGs) in sperm. Normozoospermia (NZ). Oligozoospermia: mild, moderate, severe and very severe (OZ1-OZ4). CpGs: methylated (blue), unmethylated (yellow), normal C/T polymorphism (CpG 7), CTCF-binding site 6 (CpGs 4–8). Number of clones (C). Patient codes (P) with number of clones per methylation patterns

Darmstadt, Germany) and X-Gal (8 μg/ml of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside; Sigma). For each sample, ∼20 positive clones were selected for sequencing analysis.

DNA sequencing

For each sample, ∼20 clones from H19, MEST and LINE 1 PCR products were sequenced using the BigDye Terminator Cycle Sequencing v1.1 Ready Reaction kit (Applied Biosystems) and analysed in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Direct sequencing of all samples prior to cloning was also performed to assure the absence of a bias in cloning and to determine the overall level of methylation. The methylation status of all CpGs present in the sequences was analysed manually and using the BiQ Analyzer software (Bock *et al*., 2005).

For *H19* gene, clones with >50% of the CpGs unmethylated were considered hypomethylated and for *MEST* gene, clones with >50% of the CpGs methylated were considered hypermethylated, as suggested before (Davis *et al*., 1999).

Statistics

For each group, the mean of the percentages obtained from the five patients and the SEM were analysed using Microsoft Excel Analysis ToolPak (Windows). Statistical analysis was performed using raw data (number of clones) obtained from each of the five patients, for each group, and data was analysed with two-sided Fisher's exact test (SPSS 15, Windows) for comparison between groups. Differences with *P*-values <0.05 were considered significant.

Results

Direct sequencing

Analysis of *H19* and *MEST* methylation, by direct sequencing, has shown similar results to those obtained in a previous study (Marques *et al*., 2004). *H19* was completely methylated in four of the five NZ patients with one patient presenting CpG 10 unmethylated. *H19* hypomethylation was found in one of the 10 patients with moderate oligozoopermia (OZ1+OZ2) and in three of the 10 patients with severe oligozoospermia (OZ3+OZ4). Regarding *MEST* gene, direct sequencing of all patients analysed have shown *MEST* completely unmethylated.

Methylation status of H19

A total of 419 clones were studied for *H19* gene, 72 for NZ and 347 for oligozoospermic samples distributed into four subgroups, 76 for OZ1, 101 for OZ2, 84 for OZ3 and 86 for OZ4 (Fig. 1). The analysed sequence contains 18 CpGs within the DMR of *H19* including one polymorphic site at CpG 7 (C/T) and the CTCF-binding site 6 (CpGs 4–8) (Fig. 1). Figure 2 summarizes the results obtained regarding complete methylation, hypomethylation (>50% of unmethylated CpGs) and complete unmethylation of *H19* gene. Complete methylation of *H19* was found in all groups, with a mean of 39.9% in NZ, 59.8% in OZ1, 48.3% in OZ2, 28.5% in OZ3 ($P = 0.001$ to OZ1; $P = 0.024$ to OZ2) and 61.2% in OZ4 ($P < 0.001$ to OZ3). Hypomethylation and complete unmethylation of *H19* gene were only found in oligozoospermia (Fig. 2). Hypomethylation occurred in 6% of the clones from OZ2 ($P = 0.021$ to OZ1), 8.6% of the clones from OZ3 ($P = 0.014$ to OZ1) and in 1.1% of the clones from OZ4 ($P = 0.033$ to OZ3) samples. Complete unmethylation occurred in 4.3% of the clones from OZ2 ($P = 0.038$ to OZ1; $P = 0.032$ to OZ4) and in 2.2% of the clones from OZ3 (Fig. 2). In *H19*, the number of unmethylated CpGs varied between 1–4 in NZ, 1–3 in OZ1, 1 to all in OZ2 and OZ3, and 1–14 in OZ4 (Fig. 1).

Per individual, complete methylation of *H19* gene was found in 23 of the 25 (92%) cases, in all five cases with OZ1, OZ2 and OZ4, and in four of the five cases in NZ and OZ3. Hypomethylation and complete unmethylation were only found in oligozoospermia cases with a sperm count below 10×10^6/ml. Hypomethylation was found in 5/15 cases: 2/5 with OZ2, 2/5 with OZ3 and 1/5 with OZ4. Complete unmethylation was found in 2/15 cases: 1/5 with OZ2 and 1/5 with OZ3 (Fig. 1).

Methylation status of CTCF-binding site 6

Figure 3 summarizes the results obtained regarding complete methylation, hypomethylation (>50% of unmethylated CpGs) and complete unmethylation of the CTCF-binding site. The CTCF-binding site (Fig. 1: CpGs 4–8) was completely methylated in clones from all groups: 87.9% in NZ, 83.8% in OZ1, 66.9% in OZ2 ($P = 0.036$ to OZ1), 46.4% in OZ3 ($P < 0.001$ to OZ1; $P = 0.01$ to OZ2) and 69.2% in OZ4 ($P = 0.03$ to OZ1; $P = 0.019$ to OZ3). Hypomethylation and complete unmethylation of the CTCF-binding site were only found in oligozoospermia (Fig. 3). Hypomethylation occurred in 7.7% of the clones from OZ2 ($P = 0.011$ to OZ1), 3.3% of the clones from OZ3 and in 1.1% of the clones from OZ4 ($P = 0.022$ to OZ2). Complete unmethylation was found in 5.6% of the clones from OZ2 ($P = 0.02$ to OZ1; $P = 0.016$ to OZ4) and in 3.3% of the clones from OZ3 samples. The number of unmethylated CpGs at the CTCF-binding site varied between 1–2 in NZ, 1 in OZ1, 1–4 in OZ2 and OZ3, and 1–3 in OZ4 (Fig. 1).

Per individual, complete methylation of CTCF-binding site 6 was found in 24/25 (96%) cases, in all 5 cases of NZ and with OZ1, OZ2 and OZ4, and in 4/5 cases with OZ3. Hypomethylation and complete unmethylation were only found in oligozoospermia cases with a sperm count below 10×10^6/ml. Hypomethylation was found in 5/15 cases: 3/5 with OZ2, 1/5 with OZ3 and 1/5 with OZ4. Complete unmethylation was found in 3/15 cases: 2/5 with OZ2 and 1/5 with OZ3 (Fig. 1).

Methylation status of MEST

A total of 369 clones were studied for *MEST* gene (22 CpGs within the DMR), 79 for NZ and 290 for oligozoospermic samples distributed into four subgroups: 62 for OZ1, 82 for OZ2, 80 for OZ3 and 66 for

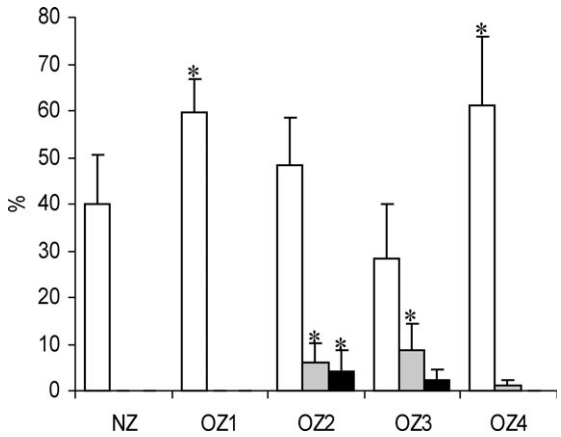

Figure 2: Percentages of clones with complete methylation (white bars), hypomethylation (grey bars) and complete unmethylation (black bars) of *H19* gene. Data are represented as mean ± SEM ($n = 5$ patients from each group). Statistically significant differences to the control (NZ) are represented (*$P <$ 0.05). Clones that present between 0–50% of abnormal methylation are not represented. This information is shown in Supplementary Data, Table S1

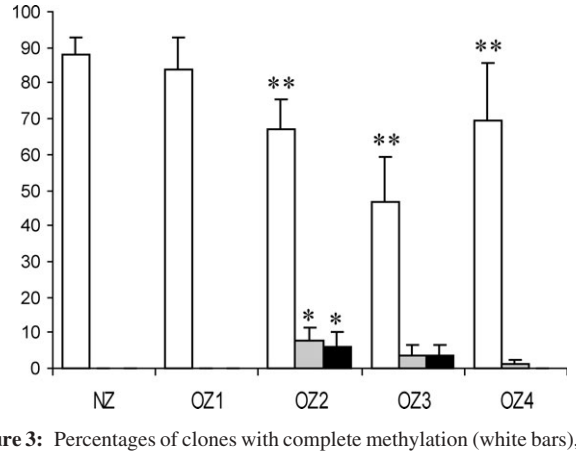

Figure 3: Percentages of clones with complete methylation (white bars), hypomethylation (grey bars) and complete unmethylation (black bars) of CTCF-binding site 6. Data are represented as mean ± SEM ($n = 5$ patients from each group). Statistically significant differences to the control (NZ) are represented (*$P < 0.05$; **$P < 0.01$). Clones that present between 0–50% of abnormal methylation are not represented. This information is shown in Supplementary Data, Table S2

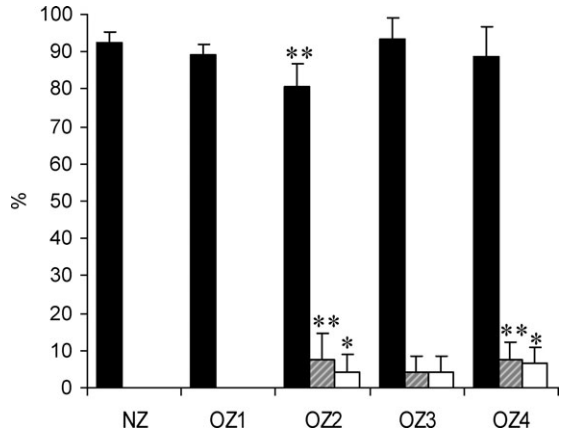

Figure 4: Percentages of clones with complete unmethylation (black bars), hypermethylation (grey stripped bars) and complete methylation (white bars) of *MEST* gene. Data are represented as mean ± SEM ($n = 5$ patients from each group). Statistically significant differences to the control (NZ) are represented (*$P < 0.05$; **$P < 0.01$). Clones that present between 0–50% of abnormal methylation are not represented. This information is shown in Supplementary Data, Table S3

OZ4 (Fig. 1). Figure 4 summarizes the results obtained regarding complete unmethylation, hypermethylation (>50% of methylated CpGs) and complete methylation of *MEST* gene. Clones with complete unmethylation of *MEST* were found in all groups (Fig. 4), 92.5% in NZ, 89.1% in OZ1, 80.7% in OZ2, 93.3% in OZ3 ($P = 0.003$ to OZ2) and 88.8% in OZ4. Hypermethylation and complete methylation of *MEST* gene were only found in oligozoospermia (Fig. 4). Hypermethylation was present in 7.4% of the clones from OZ2 ($P = 0.005$ to OZ1), 4.3% of the clones from OZ3 and 7.7% of the clones from OZ4 ($P = 0.028$ to OZ1). Complete methylation was found in 4.4% of the clones from OZ2 ($P = 0.037$ to OZ1), 4.3% of the clones from OZ3 and 6.8% of the clones from OZ4 (Fig. 4). In *MEST*, the number of methylated CpGs varied between 1–3 in NZ, 1 in OZ1 and 1–22 in OZ2, OZ3 and OZ4 (Fig. 1).

Per individual, complete unmethylation of *MEST* gene was found in all cases of NZ and with OZ1, OZ2, OZ3 and OZ4. Hypermethylation and complete methylation were only found in oligozoospermia cases with a sperm count below 10×10^6/ml. Hypermethylation was found in 4/15 cases, 1/5 with OZ2, 1/5 with OZ3 and 2/5 with OZ4. Complete methylation was found in 4/15 cases: 1/5 with OZ2, 1/5 with OZ3 and 2/5 with OZ4 (Fig. 1). In comparison, whereas for *H19* imprinted gene, including the CTCF-binding site 6, the highest number of cases with hypomethylation (2/5) was in the OZ2 subgroup, for the *MEST* imprinted gene the highest number of cases affected with hypermethylation (2/5) were in the OZ4 subgroup. Comparisons between both imprinted genes further revealed that two patients, one from OZ2 (Patient 1) and one from OZ4 (Patient 5), presented both *H19* hypomethylation and *MEST* hypermethylation (Fig. 1).

Methylation status of LINE-1 transposon

For human LINE-1 transposon, the individual that presented more hypomethylation of the *H19* gene in each of the five groups was selected to test if that was associated with global demethylation of the sperm genome or if it was specific of the imprinted gene. In total, 1425 CpG were analysed (84 clones) (Table I). The level of methylation of LINE-1 was high in all groups, 78.5% in NZ, 77.6% in OZ1, 83.8% in OZ2 ($P = 0.046$ to OZ1), 69.3% in OZ3 ($P = 0.027$ to NZ; $P = 0.018$ to OZ1; $P < 0.001$ to OZ2) and 73.9% in OZ4 ($P = 0.003$ to OZ2) (Table I). The patient that presented the highest methylation on this element was the same that also presented *H19* and CTCF completely unmethylated and *MEST* completely methylated (Fig. 1, Patient 1).

Table I. Methylation status of LINE-1 in human sperm.

Groups	Clones, *n*	Total CpGs, *n*	Methylated CpGs, *n*
NZ	11	195	153 (78.5%)
Oligozoospermia			
OZ1	19	340	264 (77.6%)
OZ2	17	315	264 (83.8%)
OZ3	20	280	194 (69.3%)
OZ4	17	295	218 (73.9%)

Discussion

We here report for the first time that infertile male patients with a sperm count below 10×10^6/ml display defective methylation of imprinted genes. For *H19* imprinted gene, hypomethylation occurred in 5.5% (1.2–8.3%) and complete unmethylation in 2.95% (0–5.9%) of the clones. Similarly, for *H19*-CTCF-binding site 6, hypomethylation occurred in 4.8% (1.2–8.9%) and complete unmethylation in 3.7% (0–6.9%) of the clones. Conversely, for *MEST* imprinted gene, hypermethylation occurred in 8.3% (3.8–12.2%) and complete methylation in 6.1% (3.8–7.6%) of the clones. Finally, in all cases, global sperm genome methylation analysis by using bisulphite genomic sequencing of LINE1 transposon confirmed that these defects are specific of the imprinted genes (the patient that presented the highest methylation on this element was the same that also presented *H19* and CTCF completely unmethylated and *MEST* completely methylated).

The main resetting of *H19* methylation has been postulated to be already finished in spermatogonia after birth (Kerjean *et al*., 2000; Hartmann *et al*., 2006; Oakes *et al*., 2007). Accordingly, previous studies in sperm of mice and normozoospermic individuals have shown that the DMR of the *H19* gene is completely methylated (Kerjean *et al*., 2000; Li *et al*., 2004) or presents a reduced number of unmethylated CpGs (Olek and Walter, 1997; Davis *et al*., 1999; Frevel *et al*., 1999; Lucifero *et al*., 2002). On the contrary, oligozoospermic patients were shown to have changed methylation profiles of *H19* (24%, 23/96 patients), including of the CTCF-binding site 6 (11.5%, 11/96 patients) (Marques *et al*., 2004). Therefore, we here confirm that unmethylation of the DMR and CTCF-binding site 6 of *H19* occurs in human sperm from cases with disrupted spermatogenesis, probably in association with loss of control of DNA methyltransferase activity. Furthermore, the present cloning analysis revealed that 25% (5/20) of all oligozoospermic patients had hypomethylated (>50% of unmethylated CpGs) and 10% (2/20) complete unmethylated *H19* clones, including hypomethylation (25%, 5/20 of patients) and complete unmethylation (15%, 3/20 patients) of the CTCF-binding site 6. Although deletion of the CTCF-binding sites was demonstrated not to affect the establishment of paternal methylation marks nor the expression of *H19* and *IGF2* during mice spermatogenesis (Engel *et al*., 2006), deletions within the DMR disrupted *H19* and *IGF2* expression in a tissue specific manner (Thorvaldsen *et al*., 2002). As CTCF binding protects the maternal allele from acquiring methylation and is required for normal preimplantation development (Fedoriw *et al*., 2004; Rand *et al*., 2004), acquisition of sperm *H19* methylation, in cases with disturbed spermatogenesis, might thus be further blocked by CTCF binding.

The *MEST* gene is highly expressed in human placenta, where it might play a role in angiogenesis (Mayer *et al*., 2000). Mice deficient

for *MEST* present growth retardation, increased perinatal and postnatal lethality and abnormal maternal behaviour (Lefebvre *et al*., 1998), suggesting that this gene might play an important role in growth and behaviour. Studies on mice and human sperm have shown that *MEST* gene is completely unmethylated (Kerjean *et al*., 2000) or has one CpG methylated (Lucifero *et al*., 2002). We first show here, by cloning sequence analysis, that *MEST* gene is hypermethylated and completely methylated (20%, 4/20 of all oligozoospermic patients) in cases of disrupted spermatogenesis and that these defects aggravate with the severity of the spermiogenic failure. Erroneous methylation of this gene could be due to incomplete erasure of methylation marks during germ line development or could be acquired *de novo*, either during formation of spermatozoa in the seminiferous tubules or through abnormal epididymal function (Ariel *et al*., 1994). Interestingly, one patient presented both complete unmethylation of *H19* and complete methylation of *MEST*, suggesting that a problem with the identification of the identity of the germ line could be occurring.

Imprinting errors associated with ART have been traditionally related to hypomethylation of the maternal allele, either due to ovarian hormonal stimulation or *in vitro* embryo culture (Arnaud and Feil, 2005). The present study indicates that human sperm from patients with disturbed spermatogenesis present a significant increase in abnormal paternal imprinting marks, due to hypomethylation (*H19*) and hypermethylation (*MEST*), suggesting that genomic imprinting defects might underlie infertility by itself. Furthermore, because sperm with hypomethylation of *H19* simultaneously present hypomethylation of the CTCF-binding site 6, infertile patients carry a significant higher risk of causing biallelic inactivation of the *IGF2* gene in the human embryo, which could have a direct negative impact on embryo quality, pregnancy and birth weight rates. In fact, other studies have demonstrated that sperm DNA global methylation is essential for normal embryo development and pregnancy outcome in mice (Kelly *et al*., 2003) and humans (Benchaib *et al*., 2005). Also, absence of *IGF2* expression has been related with low birth weight in mice (DeChiara *et al*., 1991; Engel *et al*., 2004) and could underlie the higher rates of this condition in children conceived by ART (Schieve *et al*., 2002). A very recent study, byKobayashi *et al*. (2007), using combined bisulphite restriction analysis, which permits the identification of the methylation status of only few CpGs, reported imprinting errors, such as *H19* unmethylation and *MEST* methylation, in sperm from oligozoospermic patients, albeit not indicating a threshold for the appearance of these errors. Additionally, the authors show that abnormal methylation patterns present in one patient are not transmitted to the offspring. However, children presenting Silver–Russel syndrome due to hypomethylation of *H19* and conceived by ART have been reported (Kallen *et al*., 2005; Svensson *et al*., 2005; Bliek *et al*., 2006).

In conclusion, we here show, by bisulphite cloning sequencing, the occurrence of genomic imprinting defects in human sperm, by erroneous *H19* hypomethylation and *MEST* hypermethylation. Additionally, we first report a threshold of 10 million sperm per ml of semen, below which there is a risk of gametes carrying these imprinting defects. Since these patients also presented clones with normal *H19* methylation and *MEST* unmethylation, our data also demonstrate that different patterns of sperm methylation might occur in the same patient and that cases presenting spermatozoa with severe imprinting errors can also produce sperm with normal imprinting marks. This fact suggests that misregulations leading to errors in the establishment of imprinting marks are not widespread throughout the seminiferous tubules. Accordingly, abnormal methylation patterns of imprinted genes in cases with disrupted spermatogenesis might derive from different imprinting marks in the testicular adult germinal stem cell pool, or from defects occurring during progenitor diploid germ cell expansion or haploid germ cell differentiation.

Supplementary material

Supplementary material is available at *Molehr Journal* online (http://molehr.oxfordjournals.org).

Acknowledgements

We acknowledge Drs Cristiano Oliveira and José Manuel Teixeira da Silva (MD, Gynecology and Obstetrics) for couple evaluation (CRGAB) and Joaquina Silva (MD), Paulo Viana, Ana Gonçalves, Mariana Cunha and Cláudia Osório for spermiogram analysis (CRGAB). We are also thankful to Dr Philippe Arnaud (Institute of Molecular Genetics of Montpellier), and Dr Raquel Seruca and Luis Cirnes (IPATIMUP, University of Porto) for their help with bisulphite modification.

Funding

This work was partially supported by the Portuguese Foundation for Science and Technology (FCT) with a PhD fellowship (SFRH/BD/19 967/2004 to C.J.M.), a Young Researcher fellowship (POCI/SAU-MMO/60 555/04 to P.C.) and Research grants (POCI/SAU-MMO/60 709/60555/59 997/04; UMIB to M.S.).

References

Ariel M, Cedar H, McCarrey J. Developmental changes in methylation of spermatogenesis-specific genes include reprogramming in the epididymis. *Nat Genet* 1994;**7**:59–63.

Arnaud P, Feil R. Epigenetic deregulation of genomic imprinting in human disorders and following assisted reproduction. *Birth Defects Res C Embryo Today* 2005;**75**:81–97.

Arney KL. H19 and Igf2—enhancing the confusion? *Trends Genet* 2003;**19**:17–23.

Bartolomei MS, Zemel S, Tilghman SM. Parental imprinting of the mouse H19 gene. *Nature* 1991;**351**:153–155.

Barton SC, Surani MA, Norris ML. Role of paternal and maternal genomes in mouse development. *Nature* 1984;**311**:374–376.

Benchaib M, Braun V, Ressnikof D, Lornage J, Durand P, Niveleau A, Guerin JF. Influence of global sperm DNA methylation on IVF results. *Hum Reprod* 2005;**20**:768–773.

Bliek J, Terhal P, van den Bogaard MJ, Maas S, Hamel B, Salieb-Beugelaar G, Simon M, Letteboer T, van der Smagt J, Kroes H *et al.* Hypomethylation of the H19 gene causes not only Silver–Russell syndrome (SRS) but also isolated asymmetry or an SRS-like phenotype. *Am J Hum Genet* 2006;**78**:604–614.

Bock C, Reither S, Mikeska T, Paulsen M, Walter J, Lengauer T. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics* 2005;**21**:4067–4068.

Burden AF, Manley NC, Clark AD, Gartler SM, Laird CD, Hansen RS. Hemimethylation and non-CpG methylation levels in a promoter region of human LINE-1 (L1) repeated elements. *J Biol Chem* 2005;**280**:14413–14419.

Cattanach BM, Kirk M. Differential activity of maternally and paternally derived chromosome regions in mice. *Nature* 1985;**315**:496–498.

Davis TL, Trasler JM, Moss SB, Yang GJ, Bartolomei MS. Acquisition of the H19 methylation imprint occurs differentially on the parental alleles during spermatogenesis. *Genomics* 1999;**58**:18–28.

DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 1991;**64**:849–859.

Delaval K, Govin J, Cerqueira F, Rousseaux S, Khochbin S, Feil R. Differential histone modifications mark mouse imprinting control regions during spermatogenesis. *EMBO J* 2007;**26**:720–729.

Engel N, West AG, Felsenfeld G, Bartolomei MS. Antagonism between DNA hypermethylation and enhancer-blocking activity at the H19 DMD is uncovered by CpG mutations. *Nat Genet* 2004;**36**:883–888.

Engel N, Thorvaldsen JL, Bartolomei MS. CTCF binding sites promote transcription initiation and prevent DNA methylation on the maternal allele at the imprinted H19/Igf2 locus. *Hum Mol Genet* 2006;**15**:2945–2954.

Fedoriw AM, Stein P, Svoboda P, Schultz RM, Bartolomei MS. Transgenic RNAi reveals essential function for CTCF in H19 gene imprinting. *Science* 2004;**303**:238–240.

Frevel MA, Sowerby SJ, Petersen GB, Reeve AE. Methylation sequencing analysis refines the region of H19 epimutation in Wilms tumor. *J Biol Chem* 1999;**274**:29331–29340.

Gabory A, Ripoche MA, Yoshimizu T, Dandolo L. The H19 gene: regulation and function of a non-coding RNA. *Cytogenet Genome Res* 2006;**113**:188–193.

Geuns E, De Rycke M, Van Steirteghem A, Liebaers I. Methylation imprints of the imprint control region of the SNRPN-gene in human gametes and preimplantation embryos. *Hum Mol Genet* 2003;**12**:2873–2879.

Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, Walter J, Surani MA. Epigenetic reprogramming in mouse primordial germ cells. *Mech Dev* 2002;**117**:15–23.

Hartmann S, Bergmann M, Bohle RM, Weidner W, Steger K. Genetic imprinting during impaired spermatogenesis. *Mol Hum Reprod* 2006;**12**:407–411.

Kallen B, Finnstrom O, Nygren KG, Olausson PO. In vitro fertilization (IVF) in Sweden: risk for congenital malformations after different IVF methods. *Birth Defects Res A Clin Mol Teratol* 2005;**73**:162–169.

Kaneko-Ishino T, Kuroiwa Y, Miyoshi N, Kohda T, Suzuki R, Yokoyama M, Viville S, Barton SC, Ishino F, Surani MA. Peg1/Mest imprinted gene on chromosome 6 identified by cDNA subtraction hybridization. *Nat Genet* 1995;**11**:52–59.

Kelly TL, Li E, Trasler JM. 5-aza-2′-deoxycytidine induces alterations in murine spermatogenesis and pregnancy outcome. *J Androl* 2003;**24**:822–830.

Kerjean A, Dupont JM, Vasseur C, Le Tessier D, Cuisset L, Paldi A, Jouannet P, Jeanpierre M. Establishment of the paternal methylation imprint of the human H19 and MEST/PEG1 genes during spermatogenesis. *Hum Mol Genet* 2000;**9**:2183–2187.

Kobayashi H, Sato A, Otsu E, Hiura H, Tomatsu C, Utsunomiya T, Sasaki H, Yaegashi N, Arima T. Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. *Hum Mol Genet* 2007 (advance access published 17 July, 2007, doi:10.1093/hmg/ddm187).

Lane N, Dean W, Erhardt S, Hajkova P, Surani A, Walter J, Reik W. Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. *Genesis* 2003;**35**:88–93.

Lees-Murdock DJ, De Felici M, Walsh CP. Methylation dynamics of repetitive DNA elements in the mouse germ cell lineage. *Genomics* 2003;**82**:230–237.

Lefebvre L, Viville S, Barton SC, Ishino F, Keverne EB, Surani MA. Abnormal maternal behaviour and growth retardation associated with loss of the imprinted gene Mest. *Nat Genet* 1998;**20**:163–169.

Li JY, Lees-Murdock DJ, Xu GL, Walsh CP. Timing of establishment of paternal methylation imprints in the mouse. *Genomics* 2004;**84**:952–960.

Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* 2002;**18**:1427–1431.

Lucifero D, Mertineit C, Clarke HJ, Bestor TH, Trasler JM. Methylation dynamics of imprinted genes in mouse germ cells. *Genomics* 2002;**79**:530–538.

Lucifero D, Mann MR, Bartolomei MS, Trasler JM. Gene-specific timing and epigenetic memory in oocyte imprinting. *Hum Mol Genet* 2004;**13**:839–849.

Marques CJ, Carvalho F, Sousa M, Barros A. Genomic imprinting in disruptive spermatogenesis. *Lancet* 2004;**363**:1700–1702.

Mayer W, Hemberger M, Frank HG, Grümmer R, Winterhager E, Kaufmann P, Fundele R. Expression of the imprinted genes MEST/Mest in human and murine placenta suggests a role in angiogenesis. *Dev Dyn* 2000;**217**:1–10.

McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* 1984;**37**:179–183.

Nishita Y, Yoshida I, Sado T, Takagi N. Genomic imprinting and chromosomal localization of the human MEST gene. *Genomics* 1996;**36**:539–542.

Oakes CC, La Salle S, Smiraglia DJ, Robaire B, Trasler JM. Developmental acquisition of genome-wide DNA methylation occurs prior to meiosis in male germ cells. *Dev Biol* 2007;**307**:368–379.

Olek A, Walter J. The pre-implantation ontogeny of the H19 methylation imprint. *Nat Genet* 1997;**17**:275–276.

Pinho MJ, Neves R, Costa P, Ferrás C, Sousa M, Alves C, Almeida C, Fernandes S, Silva J, Ferrás L *et al.* Unique (Yq12;1q12) translocation with loss of the heterochromatic region of chromosome 1 in a male with azoospermia due to meiotic arrest. *Hum Reprod* 2005;**20**:689–696.

Rand E, Ben-Porath I, Keshet I, Cedar H. CTCF elements direct allele-specific undermethylation at the imprinted H19 locus. *Curr Biol* 2004;**14**:1007–1012.

Sanford JP, Clark HJ, Chapman VM, Rossant J. Differences in DNA methylation during oogenesis and spermatogenesis and their persistence during early embryogenesis in the mouse. *Genes Dev* 1987;**1**:1039–1046.

Santos F, Dean W. Epigenetic reprogramming during early development in mammals. *Reproduction* 2004;**127**:643–651.

Schieve LA, Meikle SF, Ferre C, Peterson HB, Jeng G, Wilcox LS. Low and very low birth weight in infants conceived with use of assisted reproductive technology. *N Engl J Med* 2002;**346**:731–737.

Surani MA, Barton SC, Norris ML. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* 1984;**308**:548–550.

Surani MA, Barton SC, Norris ML. Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embryonic genome. *Cell* 1986;**45**:127–136.

Svensson J, Bjornstahl A, Ivarsson SA. Increased risk of Silver–Russell syndrome after in vitro fertilization? *Acta Paediatr* 2005;**94**:1163–1165.

Takai D, Gonzales FA, Tsai YC, Thayer MJ, Jones PA. Large scale mapping of methylcytosines in CTCF-binding sites in the human H19 promoter and aberrant hypomethylation in human bladder cancer. *Hum Mol Genet* 2001;**10**:2619–2626.

Thorvaldsen JL, Mann MR, Nwoko O, Duran KL, Bartolomei MS. Analysis of sequence upstream of the endogenous H19 gene reveals elements both essential and dispensable for imprinting. *Mol Cell Biol* 2002;**22**:2450–2462.

Ulaner GA, Vu TH, Li T, Hu JF, Yao XM, Yang Y, Gorlick R, Meyers P, Healey J, Ladanyi M *et al.* Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site. *Hum Mol Genet* 2003;**12**:535–549.

Woodcock DM, Lawler CB, Linsenmeyer ME, Doherty JP, Warren WD. Asymmetric methylation in the hypermethylated CpG promoter region of the human L1 retrotransposon. *J Biol Chem* 1997;**272**:7810–7816.

Zhang Y, Tycko B. Monoallelic expression of the human H19 gene. *Nat Genet* 1992;**1**:40–44.

Submitted on October 15, 2007; resubmitted on December 6, 2007; accepted on December 19, 2007