Aberrant methylation of multiple imprinted genes in embryos of tamoxifen-treated male rats

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Abstract

Genomic imprinting is an epigenetic phenomenon known to regulate fetal growth and development. Studies from our laboratory have demonstrated that treatment of adult male rats with tamoxifen increased postimplantation loss around mid gestation. Further studies demonstrated the aberrant expression of transcripts of several imprinted genes in the resorbing embryos at days 11 and 13 of gestation including IGF2. In addition, decreased methylation at the *Igf2-H19* imprint control region was observed in spermatozoa and in resorbing embryos sired by tamoxifen-treated males. In this study, methylation analysis of the imprinted genes, which were found to be differentially expressed, was done using EpiTYPER in the spermatozoa of tamoxifen-treated rats and in postimplantation embryos sired by tamoxifen-treated rats. Differentially methylated regions (DMRs) for most imprinted genes have not been identified in the rats. Hence, initial experiments were performed to identify the putative DMRs in the genes selected for the study. Increased methylation at CpG islands present in the putative DMRs of a number of imprinted genes was observed in the resorbing embryos sired by tamoxifen-treated male rats. This increase in methylation is associated with the downregulation of most of these genes at the transcript level in resorbing embryos. No change in the methylation status of these genes was observed in spermatozoa. These observations suggest that a deregulation of mechanisms protecting unmethylated alleles from a wave of *de novo* methylation occurs following implantation.

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Introduction

Parental allele-specific expression of a subset of genes is known as genomic imprinting. Approximately 100 imprinted genes are known in mammals, and most of them have been implicated in embryo growth and development (Bartolomei & Ferguson-Smith 2011). CpG methylation is a major component of the germline-specific epigenetic modifications implicated in monoallelic expression of imprinted genes. Imprinted genes contain differentially methylated regions (DMR), which ensure their allele-specific expression (Constancia *et al.* 1998). Methylation at primary DMRs is established in the germline, and once acquired, these imprints are maintained in all the somatic lineages (Constancia *et al.* 1998), whereas methylation at secondary DMRs is established after fertilization and could be tissue specific (Constancia *et al.* 1998). Four imprinted genes, *Igf2-H19*, *Dlk1-Meg3* (*Gtl2*), *Rasgrf1* and *Zdbf2*, are known to be methylated in the male germline (Arnaud & Feil 2005, Kobayashi *et al.* 2009). In the male germline, methylation is initiated by embryonic day (E) 17.5 and is completed after birth by the pachytene stage of meiosis (Oakes *et al.* 2007). In the female germline, methylation is only initiated after birth during oocyte growth;

imprints are acquired synchronously at different imprinted loci by metaphase II (Lucifero *et al.* 2004). Once acquired, the imprint marks are maintained and read throughout embryo development.

In addition to CpG methylation, non-CpG methylation, mainly involving methylation at CpA and CpT dinucleotides, was first demonstrated in cultured murine ES cells (Ramsahoye *et al.* 2000). Thereafter, non-CpG methylation was found primarily at CpA dinucleotides and to a lesser extent at CpTs dinucleotides, in both non-imprinted genes such as *Nf1* (Haines *et al.* 2001) and imprinted genes (Tomizawa *et al.* 2011). A significant amount of non-CpG methylation has been reported in mouse oocytes, albeit to a lesser extent in blastocysts, and was negligible in sperm and E12.5 mouse embryos (Tomizawa *et al.* 2011). The functional significance of non-CpG methylation is, however, not yet known. In this study, we therefore restricted ourselves to analyzing CpG methylation at imprinted genes.

In recent years, evidence that methylation defects in sperm can cause aberrant embryo development is accumulating (Nanassy & Carrell 2008, Jenkins & Carrell 2012). In rodents, paternal exposure to drugs or environmental chemicals has been associated with epimutations and defects in embryo development. Studies by

Anway *et al.* (2005) have demonstrated that exposure of pregnant female rats to endocrine disruptors like vinclozolin (an antiandrogenic compound) or methoxychlor (an estrogenic compound), before and during embryonic sex determination, resulted in male offspring with reduced spermatogenetic capacity. The F₁ adult males exhibited reduced testicular germ cell number and viability, and an increased incidence of infertility. The testicular phenotype appeared to be transmitted, transgenerationally through the F₁–F₄ generations, mainly involving alterations in the DNA methylation patterns of the male germline. Recently, it has been reported that the sperm obtained from methoxychlor-treated adult mice showed a decrease in methylation at the *Meg3* DMR and an increase in methylation at *Peg3*, *Snrpn*, and *Mest* DMRs (Stouder & Paoloni-Giacobino 2011).

Chronic administration of cytosine analogs 5-azacytidine and 5-aza-2-deoxycytidine to adult male rats has been associated with decreased sperm counts, abnormal testicular histology, and an increase in preimplantation embryo loss (Doerksen & Trasler 1996, Doerksen *et al.* 2000, Kelly & Trasler 2004). In addition, treatment with 5-aza-2-deoxycytidine also induced DNA hypomethylation in rat male germ cells (Kelly *et al.* 2003). Sperm from 5-aza-2-deoxycytidine-treated male mice also exhibited a decrease in methylation of the *Rasgrf1* DMR (Oakes *et al.* 2007). These studies in rodents demonstrate that the acquisition and maintenance of DNA methylation patterns in the male germline are crucial for spermatogenesis and embryogenesis.

Treatment with drugs such as tamoxifen has been shown to reduce fertility and affect the sperm quality of adult male rats (Gill-Sharma *et al.* 1993, Motrich *et al.* 2007). Tamoxifen, a selective estrogen receptor modulator, is widely used for the treatment of breast cancer and is prescribed to patients with gynecomastia and oligozoospermia (Noci *et al.* 1985, Parker *et al.* 1986, Cuzick *et al.* 2003). Studies from our laboratory have shown that tamoxifen treatment of adult male rats at a dose of 0.4 mg/kg per day (equivalent to human tamoxifen therapy) for 60 days results in an increase in pre- and postimplantation loss (POL) but the rate of fertilization is unaltered (Balasinor *et al.* 2001). Histology of the resorptions (at E20) showed the presence of trophoblast outgrowths, which are suggestive of early placentation, leading us to conclude that the POL might have commenced around E9–E11 (Balasinor *et al.* 2002). Embryos at E13 have just started resorbing and are not dead; hence, we refer to them as ‘resorbing embryos’ hereafter.

We previously reported a decrease in methylation at the *Igf2*–*H19* imprinting control region (ICR) in sperm and embryos sired by tamoxifen-treated male rats, as well as a decrease in the *Igf2* transcript and protein levels (Kedia *et al.* 2004, Pathak *et al.* 2009, 2010). Furthermore, microarray studies comparing the expression profiles of normal vs resorbing embryos at E11 and E13 from both control and treated groups showed downregulation of a number of other imprinted genes: *Plagl1*, *Peg3*, *Peg5*,

Ascl2, *Cdkn1c*, *Phlda2*, *Igf2r*, *Grb10*, *Gpc3* and *Sgce* (Kedia-Mokashi *et al.* 2011). In this study, we aimed to decipher the cause of the aberrant expression of imprinted genes in these embryos. We studied the methylation status of nine imprinted genes, four known to be expressed from the paternal allele (*Dlk1*, *Plagl1*, *Peg5* and *Peg3*) and five known to be expressed from the maternal allele (*Igf2r*, *Grb10*, *Kcnq1*, *Ascl2* and *Cdkn1c*) in the sperm of tamoxifen-treated rats and the embryos (at E13) sired by them. The global DNA methylation levels in these embryos were also assessed.

Materials and Methods

Animals

Randomly bred adult male (75-day-old) and female (90-day-old) rats of the Holtzman strain weighing 250–300 g and maintained at the temperature of 22–23 °C, humidity 50–55%, and 14 h light:10 h darkness cycle were used for the study. Food and water were available *ad libitum*. The study was approved by the Institute’s Animal Ethics Committee for ‘Care and Use of Laboratory Animals for Biomedical Research’.

Treatment and mating schedule

Tamoxifen citrate tablets (Hetero Healthcare Ltd, Hyderabad, India) containing 10 mg tamoxifen was uniformly suspended in water by sonication and administered orally to adult male rats. Tamoxifen was administered at a concentration of 0.4 mg/kg day, 5 days a week for 60 days, between 0900 and 1100 h via a rat feeding tube. Control male rats were administered water only. Thirty-eight male rats per group were used in the study. Control or tamoxifen-treated male rats were mated with normal cycling female rats a week before the completion of the 60-day treatment regimen. The mating design used a ratio of one male to two females. The day of sperm-positive vaginal smear was considered as day ‘0’ of gestation. Gravid females were killed on E13, by asphyxiation with CO₂. The number of implantation sites, normal and resorbing embryos (distinguished based on the size), were noted, snap-frozen, and stored at –80 °C until further use. The POL was calculated using the following formula:

$$\frac{\text{No. of implantation sites} - \text{No. of normal embryos}}{\text{No. of implantation sites}} \times 100$$

Sample collection and DNA extraction

Sperm preparation

After mating, control or treated male rats were perfusion cleared by 0.01 M PBS, pH 7.4, containing 1 ml heparin (5000 IU) through the heart (Hess 1990). The epididymis was excised and collected in sterile PBS. Sperm were collected from the cauda epididymis. The cauda were thoroughly washed in PBS (136 mM NaCl, 2.6 mM KCl, 6.4 mM Na₂HPO₄, and 1.28 mM KH₂PO₄, pH 7.4), and after giving three to four superficial cuts, the cauda were incubated at 37 °C

for 30–45 min. The supernatant containing sperm was filtered through a 40 µm cell strainer, centrifuged, and aliquots of 10×10^6 sperm were stored at -80°C (Pathak *et al.* 2009).

DNA was extracted from 10×10^6 sperm, suspended in 100 µl PBS (supplied in the kit), and processed from the sperm lysis step onward, using HiPurA Sperm Genomic DNA Purification Spin Kit (HiMedia, Mumbai, India) according to manufacturer's protocol. The DNA was eluted in HPLC grade water and the concentration was determined spectrophotometrically. Sperm samples from ten control rats and nine tamoxifen-treated rats amounting to a total of 19 samples were used for extraction.

Embryos

Normal embryos sired by three different control or tamoxifen-treated male rats were excised from the uterus on an ice pack and the embryonic part and placenta of each implant were processed individually for extraction. Three resorbing embryos sired by three different tamoxifen-treated male rats were excised from the uterus on an ice pack and the entire implant as a whole was used for extraction. The normal and resorbing embryos used for DNA extraction were obtained from the same litter. Therefore, the total of 15 samples processed for DNA extraction comprised control samples, three normal embryos and three normal placentae, and treated samples, three normal embryos, three normal placentae, and three resorbing embryos.

DNA was extracted from individual embryos (~25 mg tissue) using HiPurA Mammalian Genomic DNA Purification Spin Kit (HiMedia) according to the manufacturer's protocol. The DNA was eluted in HPLC grade water and the concentration was determined spectrophotometrically.

Identification of putative DMRs and primer design

For identifying the putative DMRs, BLAST of known mouse DMR sequences was performed with the rat genomic sequence database and the web-based software MethPrimer was employed to determine the CpG island (CGI) location (<http://www.urogene.org/methprimer/>). The details of identification of rat putative DMR sequences are described below.

The paternally expressed genes included are *Dlk1* promoter: a CGI of ~116 bp was present in the mouse *Dlk1* gene. The corresponding region in the rat is conserved to 91% identity and harbors a CGI of ~119 bp (Fig. 1A, left panel). *Dlk1-Meg3* intergenic DMR (IGDMR): a CGI of ~195 bp was identified in the mouse *Dlk1-Meg3* gene cluster (AJ320506). The corresponding region in the rat *Dlk1-Meg3* gene cluster (NW_001084847) is conserved to 77% identity and harbors a CGI of ~396 bp (Fig. 1A, right panel). *Peg5*: a CGI of ~760 bp was identified in the mouse *Peg5* gene (AF303656). The corresponding region in the rat *Peg5* gene (NW_001084817) is conserved to 91% identity and harbors a CGI ~700 bp (Fig. 1B). *Plagl1*: a CGI of ~470 bp was identified in the mouse *Plagl1* gene (AJ308559). The corresponding region in the rat *Plagl1* gene (NW_001084746) is conserved to 84% identity and harbors a CGI of ~600 bp (Fig. 1C). *Peg3*: two CGIs of ~160 and ~190 bp were identified in the mouse *Peg3* gene (AF105262). The corresponding region in the rat *Peg3* gene (NW_001084760) is conserved to 94% identity and harbors two CGIs of ~220 and ~330 bp (Fig. 1D).

The maternally expressed genes included are *Grb10*: a CGI of ~1065 bp was identified in the mouse *Grb10* gene (AL663087). The corresponding region in the rat *Grb10* gene (NW_001084694) is conserved to 88% identity and harbors a CGI of ~1100 bp (Fig. 2A). *Igf2r*: a CGI of ~950 bp was identified in the mouse *Igf2r*, region 1 (L06445). The corresponding region in the rat *Igf2r* gene (NW_001084751) is conserved to 92% identity and harbors a CGI of ~895 bp (Fig. 2B). Four CGIs (nucleotide positions 800–2300) were observed in the mouse *Igf2r*, region 2 (L06446). However, the corresponding region was found to be very poorly conserved in the rat, with a sequence coverage of only 53% and identity of 80%. Hence, we did not select this region for our study. *Cdkn1c*: a CGI of ~1495 bp was identified in the mouse *Cdkn1c-Kcnqot1* domain (AP001293). The corresponding region in the rat sequence is conserved to 95% identity and harbors a CGI of ~1425 bp (Fig. 2C, left panel). *Kcnq1* DMR: a CGI of ~320 bp was identified in the mouse *Cdkn1c-Kcnqot1* domain (AP001295). The corresponding region in the rat *Cdkn1c-Kcnqot1* domain (NW_001084774) is conserved to 78% identity and harbors a CGI of ~290 bp (Fig. 2C, right

Figure 1 Identification of CGIs in paternally expressed imprinted genes in the rat. The CGIs in the mouse sequence (upper panel) and the corresponding CGI identified in the rat sequence (lower panel) using MethPrimer. The CGIs in *Dlk1* IGDMR (A, right panel); *Dlk1* promoter (A, left panel); *Peg5* (B), *Plagl1* (C), and *Peg3* (D) genes were used for the EpiTYPER analysis. Additional details are given in the Materials and Methods section.

Figure 2 Identification of CGIs in maternally expressed imprinted genes in the rat. The CGIs in the mouse sequence (upper panel) and the corresponding CGI identified in the rat sequence (lower panel) using MethPrimer. The CGIs in *Grb10* (A), *Igf2r* (B), *Kcnq1* DMR (C, left panel), *Cdkn1c* (C, right panel), and *Ascl2* (D) genes were used for the EpiTYPER analysis. Additional details are given in the Materials and Methods section.

panel). *Ascl2*: a CGI of ~270 bp was identified in the mouse *Cdkn1c-Kcnq1* domain (AJ251835). The corresponding region in the rat sequence is conserved to 92% identity and harbors a CGI of ~135 bp (Fig. 2D).

Primers were designed using EpiDesigner software (Sequenom, San Diego, CA, USA) to cover the regions with the most CpG sites. A T7-promoter tag (CAGTAATACGACTCACTATAGGGAGAAGGCT) was added to the reverse primer to obtain an appropriate product for *in vitro* transcription and a 10 mer-tag (AGGAAGAGAG) was added to the forward primer to balance the PCR primer length.

Bisulfite modification of DNA

The bisulfite conversion of 1 µg of genomic DNA was done using the EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the manufacture's protocol with minor modifications. After addition of the bisulfite reagent, samples were placed in a thermal cycler for a cycling incubation using Zymo's 'Alternative Cycling Protocol', which involves denaturation at 95 °C for 30 s and conversion at 50 °C for 15 min. This was repeated for 20 cycles followed by incubation at 0–4 °C (on the thermal cycler) for 10 min. The post-incubation processing was done as detailed in the user manual. The modified DNA was eluted in HPLC water and stored at –20 °C for later use.

EpiTYPER procedure

The bisulfite-treated genomic DNA was amplified using Taq DNA polymerase (Roche Diagnostics) in 384-well plates, for the regions described earlier. The thermal cycling conditions involved initial denaturation at 95 °C for 4 min followed by 45 cycles of 95 °C for 20 s, annealing at optimal temperature for 30 s, and extension at 72 °C for 1 min. Final extension took place at 72 °C for 3 min. PCR products were further processed for MassARRAY analysis using MassCLEAVE T7 Kit (Sequenom). Unincorporated dNTPs were dephosphorylated by adding 1.7 µl H₂O and 0.3 U shrimp alkaline phosphatase followed by incubation at 37 °C for 40 min and heat inactivation of shrimp alkaline phosphatase at 85 °C for 5 min. For the *in vitro* transcription, PCR product (2 µl) was used as a template in a 5 µl transcription reaction. T7 RNA and DNA polymerases

(20 U) were used to incorporate dTTP in the transcripts. Ribonucleotides were used at 1 mmol/l and the dNTP substrate at 2.5 mmol/l. RNase A enzyme was added in the same step for base-specific cleavage of the RNA transcripts (T-cleavage assay). The T-cleavage reaction was carried out at 37 °C for 3 h and further diluted with H₂O to a final volume of 27 µl. Sample conditioning of the phosphate backbone was achieved by adding 6 mg Clean Resin from the Clean Resin Kit (Sequenom) and 22 nl cleavage reaction was robotically dispensed onto a 384-well sample chips (SpectroCHIP Arrays, Sequenom) using the MassARRAY Nanodispenser. The SpectroCHIPs were then placed into the Sequenom MassARRAY Compact Analyzer. The data were collected and analyzed using the EpiTYPER software v1.0.2 (Sequenom).

EpiTYPER data analysis

The spectra quality (peak height and confidence score) for each reaction was examined using the EpiTYPER program. Fragments with a mass of 1700 Da or less were removed from analysis in this study as they are more likely to match with other small fragments and lead to inaccurate peak calls (Coolen *et al.* 2007). CpG sites that gave no data in >30% of the samples were discarded. Finally, individual samples were removed if they had no data at 30% or more of the CpG sites within the amplicon. Following filtering to exclude poor-quality data, the methylation percentages at each CpG site were averaged for each tissue within the control/treated groups using Microsoft Excel to generate the percentage methylation for the amplicon. The mean and s.d. of percentage methylation for each group of tissues was calculated using Microsoft Excel. The statistical significance between groups was assessed by the Student's *t*-test ($P \leq 0.05$ was considered significant). The bisulfite modification and EpiTYPER experiment were done in collaboration with Ace Probe, New Delhi (Sequenom service provider in India).

EpiTYPER study

A pilot experiment was done using the primers mentioned in Table 1 in order to confirm their DMR methylation status using DNA from embryo and sperm from a control rat (if the region is a gametic DMR, then the sperm would be either

Table 1 Rat primer sequences used for EpiTYPER analysis.

Gene symbols	Primer sequences ^a (5'-3')	Size of PCR product (bp)
<i>Dlk1-Meg3</i> IGDMR	F-TAGTGTAGGTTTGGTGTGGTATAGA R-CATTATAACACAAACAAAAAATCCA	396
<i>Dlk1</i>	F-GTTTTTTTGTGTTTGTGGTTT R-AAATACCTCCCAATACAATCTCCTT	358
<i>Peg5</i>	F-GAGATTGTGTTAGGGATTGTTTT R-CACCCTCCAAAAATCTACTCTA	211
<i>Peg3</i>	F-GAGAGATTGTTTATTTTGGGTTGGT R-CAACTACAACCTTATCAATCACCC	263
<i>Plagl1</i>	F-GTTATTTTTTGGTTGGTTTGTG R-CAACACCAAACTACTTACTCTAT	304
<i>Igf2r</i>	F-AGGATTGTTTGTGTTAGTTGGTTT R-AACCTACAACCCCAAAATTTAATC	232
<i>Grb10</i>	F-AGATTGTTGGGAGGTAAATTTTTT R-AAATCTATACTAACCACTCCAAA	130
<i>Kcnq1 DMR</i>	F-GAGAGTTTTTTTGTGTTAGTTGGTTTT R-CAATAAATCATCTCTACTTCTAACCA	190
<i>Cdkn1c</i>	F-GAGATTGTGTTAGGGATTGTTTT R-TTCAACTTCCAATAATATACCTCTT	385
<i>Ascl2</i>	F-GGTTTGTGTTAGTTTAAAGTT R-AAAAAACACAAATATACTCTACAAT	394

F, forward primer; R, reverse primer.

^aPlease refer to Materials and Methods section for details of primer design.

hypomethylated or hypermethylated and the embryos would be partially methylated (40–50%).

A total of ten amplicons from nine genes were selected for the final experiment. The ten amplicons were from *Dlk1-Meg3* IGDMR, *Dlk1*, *Peg5*, *Plagl1*, *Peg3*, *Grb10*, *Igf2r*, *Kcnq1 DMR*, *Cdkn1c*, and *Ascl2* genes. These amplicons were assayed in 19 sperm DNA samples and 15 embryonic DNA samples (normal embryo, normal placenta, and resorbing embryo) from control and tamoxifen-treated group, along with water controls. The EpiTYPER procedure and data analysis were performed as described above. The results of the pilot experiment and the final study are described below.

Bisulfite sequencing PCR

DNA (1 µg) obtained from sperm (as mentioned earlier) was modified using CpGenome Fast DNA Modification kit (Chemicon-Millipore, Billerica, MA, USA) according to the manufacturer's recommendations. The modified DNA was resuspended in HPLC grade water and immediately stored at –20 °C until further use. The completion of the bisulfite modification of sperm DNA was confirmed by PCR with wild-type primer 'W', which anneals to any DNA (methylated or unmethylated) that has not undergone bisulfite modification. The PCR was performed in 25 µl reaction volume containing reaction buffers (FastStart PCR Master Kit from Roche Applied Science) and 10 pM each of forward primers 5'-GATATCCTGTGGCGAGGTA-3' and reverse primers 5'-GCCTCAAGGCAGTGGTTTAC-3' for *Dlk1-Meg3* gene cluster and 80 ng of the template. Thermal cycling conditions included initial denaturation at 94 °C for 5 min and then 35 cycles of denaturation at 94 °C for 1 min, annealing at 51.5 °C for 1 min, extension at 72 °C for 1.5 min, and final extension at 72 °C for 15 min. The PCR products were resolved on 1% agarose gel.

The bisulfite-modified DNA, from sperm samples of three controls and three tamoxifen-treated rats, was PCR amplified in 25 µl reaction volume containing reaction buffers (Hot Start PCR Mix from Roche Applied Science) and 25 pM each of forward primer (5'-TAGGTTTGGTGTGGTATAGATATA-3') and reverse primers (5'-AATATATACCTCAAAACAATAATTA-3') for the *Dlk1-Meg3* IGDMR and 80 ng the template. Thermal cycling conditions included initial denaturation at 94 °C for 5 min and then 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1.5 min, and final extension at 72 °C for 15 min. The PCR products were gel purified and cloned into pCR 2.1 TOPO cloning vector (Invitrogen). Eight to ten clones per sample were sequenced on ABI Prism DNA Sequencer version 3.7 (Perkin Elmer, Norwalk, CT, USA). DNA methylation data obtained from sequencing were analyzed by quantitative methylation analysis (QUMA). For individual CpG sites, the percentage of CpG sites methylated to the total number of CpG sites assessed for that site was estimated and expressed as mean ± s.d. The extent of methylation at individual CpG sites at the *Dlk1-Meg3* IGDMR in each group was analyzed by Student's *t*-test ($P \leq 0.05$ was considered significant).

Global DNA methylation status of embryos

The global DNA methylation status of postimplantation embryos was assessed with Methylamp Global DNA Methylation Quantification Kit (Epigentek, Brooklyn, NY, USA). A standard curve containing 6.25, 12.5, 25, 50, and 100 ng methylated DNA control (positive) was prepared from a 100 µg/ml stock solution diluted with DNA binding solution. To eight-well assay strips, standards (concentrations given above) or samples (200 ng genomic DNA isolated from embryos) were added in duplicates, the volume in all wells adjusted to 30 µl with DNA binding solution, and the contents were mixed thoroughly. For the blank, 30 µl DNA binding solution was added to the wells instead of DNA. The strip wells were incubated at 37 °C for 2 h followed by incubation at 60 °C for 30 min to evaporate the solution and dry the wells. The remaining steps were followed as described by the manufacturer, except that after the addition of primary antibody (1 mg/ml) the plate was incubated at 4 °C overnight and after addition of secondary antibody (400 µg/ml) the plate was incubated at room temperature for 1 h. The absorbance was read on a microplate reader at 450 nm. The optical density (OD) values were plotted against the amount of methylated DNA control standards, the slope of the plot was determined, and then the amount of methylated DNA in samples was calculated using the following formula:

$$\text{Methyl DNA (ng)} = \frac{\text{OD (sample - blank)}}{\text{Slope}}$$

$$\text{Methylation (\%)} = \frac{\text{Methyl DNA amount}}{\text{Sample DNA amount added}} \times 100$$

The mean ± s.d. of percentage methylation levels in embryos sired by control and tamoxifen-treated male rats ($n=3$) was analyzed by Student's *t*-test ($P \leq 0.05$ was considered significant).

A

B

Results

Postimplantation embryo loss on paternal tamoxifen treatment

A significant increase in POL was observed in tamoxifen-treated males when compared with controls ($P \leq 0.05$). The mean $\pm$ S.E.M. of percentage POL (per male) in the tamoxifen-treated group was found to be 10 ± 2 , while in the control group, the percentage POL (per male) was found to be 3 ± 0.68 ($n = 38$ male rats and each male was mated with at least two female rats in both the groups). This is in confirmation with our earlier studies (Kedia *et al.* 2004, Pathak *et al.* 2009, 2010, Kedia-Mokashi *et al.* 2011).

Identification of DMRs at paternally expressed imprinted genes and their methylation in embryos sired by tamoxifen-treated rats

Except for *Igf2-H19* DMR, the DMRs for other paternally expressed imprinted genes have not been reported in the rat, so we checked the homology of known mouse DMR sequences with the rat genomic sequence database and also confirmed the presence of CGI in these regions. In addition, a pilot study was conducted to find out whether the regions selected for the study were indeed differentially methylated.

We classified ~ 0 – 10 % methylation as hypomethylation, ~ 40 – 50 % as partial methylation, and 90 – 100 % as hypermethylation. Based on this classification, if the region is a gametic DMR, then it should be either hypomethylated or hypermethylated in sperm and partially methylated in embryos. The details of the CGI and amplicons used in the experiment are described in the Materials and Methods section. The results of the pilot study are described below.

The 8 kb-long IGDMMR located ~ 15 kb upstream of *Meg3* gene and 70 kb downstream of mouse *Dlk1* promoter is hypermethylated in sperm and hypomethylated in oocytes (Takada *et al.* 2002). A total of 24 CpGs located within a CGI of ~ 396 bp in the rat *Dlk1* gene were found to be partially methylated in embryos and hypermethylated in sperm, suggesting it to be a DMR (Fig. 3A-i, right panel). Previously, we had reported downregulation of *Dlk1*

transcripts in the resorbing embryos (Kedia-Mokashi *et al.* 2011); hence, in this study, we also assessed methylation status of *Dlk1* promoter. A total of 20 CpGs located within a CGI of ~ 119 bp in the rat *Dlk1* gene were found to be hypomethylated in both embryo and sperm (Fig. 3A-i, left panel).

John *et al.* (2001) identified a CGI in the mouse *Peg5/Nnat* gene spanning exon 1 and extending into intron 2, which was methylated on the maternal allele. A total of nine CpGs located within a CGI of ~ 700 bp in the rat *Peg5* gene were found to be partially methylated in embryos and hypomethylated in sperm, suggesting it to be a DMR (Fig. 3A-ii). Smith *et al.* (2002) identified an extensive DMR of ~ 1586 bp in the mouse *Plagl1* gene that was hypermethylated on maternal alleles and hypomethylated on the paternal alleles. A total of 31 CpGs located within a CGI of ~ 600 bp in the rat *Plagl1* gene were found to be partially methylated in embryo and hypomethylated in sperm, suggesting it to be a DMR (Fig. 3A-iii). The 5 kb region surrounding first exon of *Peg3* and *Usp29* genes was found to contain a CGI that is hypermethylated maternally and hypomethylated on the paternal allele (Li *et al.* 2000, Lucifero *et al.* 2002). A total of 17 CpGs located within the CGIs of ~ 220 and ~ 330 bp in the rat *Peg3* gene were found to be partially methylated in embryos and hypomethylated in sperm, suggesting it to be a DMR (Fig. 3A-iv).

The methylation status of the above-mentioned amplicons was assessed in embryos sired by tamoxifen-treated male rats (Fig. 4A). A significant increase in methylation was observed at the *Dlk1-Meg3* DMR in the resorbing embryos from the treated group when compared with normal embryos/placentae from both the groups (Fig. 4A-i). Similarly, a significant increase in methylation was also observed at the CGI within the *Dlk1* promoter in the resorbing embryos from the treated group (Fig. 4A-ii). A significant increase in methylation was observed at the *Peg5* DMR in the resorbing embryos from the treated group when compared with normal embryos from both groups (Fig. 4A-iii). A significant decrease in methylation was observed at the *Plagl1* DMR in the normal embryos from treated group when compared with those from the control group (Fig. 4A-iv).

Figure 3 (A) CGIs selected for the pilot study to assess methylation at paternally expressed imprinted genes in the rat. Schematic diagram of the imprinted loci along with CGI/DMR known to regulate their expression, prepared from information available in the mouse. The EpiTYPER analysis, done as a pilot study to validate the *in silico* analysis of CGIs in *Dlk1* promoter (i, left panel), *Dlk1-Meg3* IGDMMR (i, right panel), *Peg5* (ii), *Plagl1* (iii), and *Peg3* (iv) genes in a normal embryo and sperm DNA obtained from a control male rat. The epigram depicting the percentage methylation at each CpG dinucleotide is represented by circles in shades of red (0% methylation) to yellow (100% methylation). The CpG dinucleotide sites that could not be analyzed are depicted as grey circles. Selection of CGIs and primer sequences is described in the Materials and Methods section. (B) CGIs selected for the pilot study to assess methylation at maternally expressed imprinted genes in the rat. Schematic diagram of the imprinted loci along with CGI/DMR known to regulate their expression, prepared from information available in the mouse. The EpiTYPER analysis, done as a pilot study to validate the *in silico* analysis of CGIs in *Grb10* (i), *Igf2r* (ii), *Cdkn1c* (iii, left panel), *Kcnq1* DMR (iii, middle panel), and *Ascl2* (iii, right panel) genes in a normal embryo and sperm DNA obtained from a control male rat. The epigram depicting the percentage methylation at each CpG dinucleotides is represented by circles in shades of red (0% methylation) to yellow (100% methylation). The CpG dinucleotide sites that could not be analyzed are depicted as grey circles. Selection of CGIs and primer sequences is described in the Materials and Methods section.

Figure 4 (A) CpG methylation analysis of paternally expressed imprinted genes in rat embryos. Percentage average CpG methylation per amplicon obtained from EpiTYPER analysis of *Dlk1* IGDMR (i), *Dlk1* (ii), *Peg5* (iii), *Plagl1* (iv), and *Peg3* (v) genes in normal embryo (Emb) and placenta (Pla) at E13 sired by control and tamoxifen-treated male rats ($n=3$) and resorbing (Res) embryos at E13 sired by tamoxifen-treated male rats ($n=3$). Values are mean $\pm$ s.d. *Significant difference after Student's *t*-test at $P \leq 0.05$. (B) CpG methylation analysis of maternally expressed imprinted genes in rat embryos. Percentage average CpG methylation per amplicon obtained from EpiTYPER analysis of *Igf2r* (i), *Grb10* (ii), *Kcnq1* DMR (iii), *Ascl2* (iv), and *Cdkn1c* (v) genes in normal embryo (Emb) and placenta (Pla) at E13 sired by control and tamoxifen-treated male rats ($n=3$) and resorbing (Res) embryos at E13 sired by tamoxifen-treated male rats ($n=3$). Values are mean $\pm$ s.d. *Significant difference after Student's *t*-test at $P \leq 0.05$.

A significant increase in methylation of *Plagl1* DMR was observed in the resorbing embryos when compared with treated normal embryos but was unchanged when compared with normal embryos from control group. A significant decrease in methylation was observed at the *Peg3* DMR in the placenta derived from normal embryos of the treated group when compared with those from the control group (Fig. 4A-v). A significant increase in methylation of *Peg3* DMR was observed in the resorbing embryos when compared with the placenta of treated group but was unaffected when compared with control group.

Identification of DMRs in maternally expressed imprinted genes and their methylation in embryos sired by tamoxifen-treated rats

Three of the maternally expressed imprinted genes selected for the study were *Grb10*, *Igf2r*, and *Kcnq1*. In the case of the DMRs for maternally expressed imprinted genes, identification was done as described for paternally expressed genes. The details of the CGI and amplicons used are described in the Materials and Methods section. The results of the pilot study are described below.

Domain 2 identified within the mouse *Grb10* gene harbors CGI2, which is hypermethylated in the oocyte and unmethylated in the sperm (Arnaud *et al.* 2003). A total of eight CpGs located within a CGI of ~ 1100 bp in the rat *Grb10* gene were found to be partially methylated in embryos and hypomethylated in sperm, suggesting it to be a DMR (Fig. 3B-i). Stoger *et al.* (1993) had identified two regions with the mouse *Igf2r* gene: region 1, which contained the transcription start site;

and region 2, which contained in an intron located 27 kb downstream of the transcription start site. They reported that region 1 acquires methylation after fertilization whereas methylation on region 2 is inherited from the female gamete and hence is the gametic DMR. A total of 11 CpGs located within a CGI of ~ 895 bp of the rat *Igf2r* gene (corresponding to region 1) were found to be partially methylated in embryos and hypomethylated in sperm, suggesting it to be a DMR (Fig. 3B-ii). Four CGIs (nucleotide positions 800–2300) were observed in the mouse *Igf2r*, region 2 (L06446). However, the corresponding region was found to be very poorly conserved in the rat, with a sequence coverage of only 53% and identity of 80%. Hence, we did not select this region for our study.

Yatsuki *et al.* (2002) had identified CGI8b as a gametic DMR of the mouse *Cdkn1c-Kcnq1ot1* sub-domain, which is unmethylated on the paternal allele. This allele expresses a large non-coding antisense transcript called *Kcnq1ot1* (*Lit1*), which silences the *Kcnq1* gene along with neighboring imprinted genes *Phld2*, *Cdkn1c*, *Kcnq1*, and *Ascl2* (Mancini-DiNardo *et al.* 2003, Thakur *et al.* 2004). A total of 12 CpGs located within a CGI of ~ 290 bp in the rat *Cdkn1c-Kcnq1ot1* domain were found to be partially methylated in embryos and hypomethylated in sperm, suggesting it to be a DMR (Fig. 3B-iii, middle panel). Previously, we had reported downregulation of *Cdkn1c* and *Ascl2* transcripts in the resorbing embryos sired by tamoxifen-treated rats (Kedia-Mokashi *et al.* 2011); hence, in this study, we also assessed the methylation status of the *Cdkn1c* and *Ascl2* promoters. Yatsuki *et al.* (2002) had also identified CGI5 within the mouse *Cdkn1c* promoter, which was methylated on the paternal allele (somatic DMR). A total

of 31 CpGs located within a CGI of ~1425 bp in the rat *Cdkn1c* promoter were found to be hypomethylated in both embryos and sperm (Fig. 3B-iii, left panel). A total of 22 CpGs located within a CGI of ~135 bp in the rat *Ascl2* promoter were found to be hypomethylated in both embryos and sperm (Fig. 3B-iii, right panel).

The methylation status of the amplicons given above was assessed in embryos sired by tamoxifen-treated male rats (Fig. 4B). A significant increase in methylation at the *Igf2r* DMR was observed in the resorbing embryos from the treated group when compared with the normal embryos from both groups (Fig. 4B-i). An increase in methylation was also observed at the *Grb10* DMR in the resorbing embryos from the treated group when compared with

normal embryos from both groups; however, the difference was not statistically significant due to variations in the methylation levels (Fig. 4B-ii). The methylation at the *Kcnq1* DMR (Fig. 4B-iii) and the CPI in the promoters of *Ascl2* (Fig. 4B-iv) and *Cdkn1c* (Fig. 4B-v) genes were comparable in embryos from all the groups.

Methylation of imprinted genes in the sperm of tamoxifen-treated rats

The putative DMRs of all the four paternally expressed imprinted genes displayed comparable levels of methylation in the sperm of control and tamoxifen-treated rats (Fig. 5A). The DNA methylation levels in sperm ranged

Figure 5 CpG methylation analysis of imprinted genes in rat sperm. (A) Percentage average CpG methylation per amplicon obtained from EpiTYPER analysis of maternally expressed (*Igf2r*, *Kcnq1* DMR, *Ascl2*, *Cdkn1c*, and *Grb10*) and paternally expressed (*Dlk1* IGDMM, *Dlk1*, *Plagl1*, *Peg3*, and *Peg5*) genes in sperm obtained from control ($n=10$) and tamoxifen-treated ($n=9$) male rats. Values are mean $\pm$ s.d. (B) Representative bisulfite genomic sequencing data obtained from sperm DNA of control and tamoxifen-treated rats. The methylation of 16 CpG sites within the ~396 bp CGI in the rat *Dlk1-Meg3* cluster (which was also used for EpiTYPER studies) is represented by circles. Each line represents a single-sequenced clone and eight to ten clones per animal were sequenced. Black and white circles represent methylated and unmethylated cytosines respectively. (C) Analysis of *Dlk1-Meg3* IGDMM methylation of individual CpG sites in sperm DNA of control and tamoxifen-treated male rats ($n=3$). For individual CpG sites, percentage CpG sites methylated to the total number of CpG sites assessed for that site was estimated. The numbers on the X-axis represent the positions of each individual CpG site. Values are mean $\pm$ s.d. Student's *t*-test was used for statistical analysis.

from 90 to 100% at the *Dlk1-Meg3* DMR and 0 to 10% at the DMRs of the *Plagl1*, *Peg5*, and *Peg3* genes, which is similar to that reported in mice. In the case of the maternally expressed imprinted genes, the sperm from the tamoxifen-treated rats displayed comparable levels of methylation when compared with control rats (Fig. 5A). The DNA methylation levels in sperm ranged from 0 to 10% at the DMRs within the *Igf2r*, *Grb10*, and *Kcnq1* genes, which was similar to what is reported in mice.

The results obtained for one of the DMRs, namely the *Dlk1-Meg3* IGDMR, were reconfirmed using bisulfite sequencing PCR. The methylation of 16 CpG sites within the ~396 bp CGI in the rat *Dlk1-Meg3* cluster (which was also used for EpiTYPER studies) was assessed by bisulfite sequencing in the sperm of three rats each from the control and tamoxifen-treated groups. A representative pattern of methylation detected at the 16 CpG sites in control and treated sperm is depicted in Fig. 5B. The average methylation detected is summarized in Fig. 5C. The majority of CpG sites analyzed were methylated in the control group with a mean of 96.6% methylation. In addition, the tamoxifen-treated group also showed identical levels of methylation with a mean of 94.9% methylation. No change in methylation at the *Dlk1-Meg3* IGDMR was detected in the sperm of the tamoxifen-treated group following sequencing of the bisulfite-modified DNA as was observed with EpiTYPER.

Global DNA methylation status of postimplantation embryos

The 5-methyl cytosine (5mC) levels in normal embryos sired by control and tamoxifen-treated rats were identical (Fig. 6). The resorbing embryos from the treated group had methylation levels identical to those of the normal embryos of both groups.

Discussion

We had previously observed aberrant expression of imprinted genes in embryos sired by tamoxifen-treated male rats (Kedia-Mokashi *et al.* 2011). Therefore, in this study, we assessed the methylation status of several imprinted loci in embryos sired by tamoxifen-treated male rats. Initially, the DMRs of imprinted genes were identified and the selected CGIs were subjected to methylation analysis in the sperm of tamoxifen-treated rats and in the embryos sired by them, using EpiTYPER. However, the sperm of tamoxifen-treated rats did not show any change in methylation at the DMRs studied. Hence, the results obtained from EpiTYPER analysis were reconfirmed by bisulfite sequencing of the IGDMR of the *Dlk1-Meg3* genes, using sperm from control and tamoxifen-treated rats. Bisulfite sequencing gave results identical to the EpiTYPER analysis, thereby validating its use in the study. Similar results were also observed in

Figure 6 5-Methyl cytosine (5mC) levels in rat embryos at E13. The mean $\pm$ s.d. of percentage of 5mC levels in embryo (Emb) and placenta (Pla) at E13 sired by control and tamoxifen-treated male rats ($n=3$) and resorbing (Res) embryos at E13 sired by tamoxifen-treated male rats ($n=3$) using Global DNA Methylation Quantification Kit.

another study recently published by our group, on male partners of women undergoing idiopathic recurrent spontaneous abortions (Anolkar *et al.* 2013). An increase in methylation at the DMRs of several imprinted genes was observed in the resorbing embryos sired by tamoxifen-treated male rats, suggesting that the epimutations were not transmitted through sperm but could have been acquired post-fertilization. Our earlier studies on gene expression profiling by microarray analysis had shown similar expression profiles for resorbing embryos in both control and treatment groups (Kedia-Mokashi *et al.* 2011). This indicated that resorbing embryos from both groups might have a similar methylation status. Therefore, methylation status of resorbing embryos from the control group was not done in this study.

The *Dlk1-Meg3* cluster is similar to the *Igf2-H19* cluster, consisting of a paternally expressed gene and a maternally expressed untranslated RNA in mice and humans. It is also known that the IGDMR, controlling parental-specific expression, is paternally methylated (Wylie *et al.* 2000, Takada *et al.* 2002). In this study, the IGDMR of the *Dlk1-Meg3* gene was found to be hypermethylated in sperm and partially methylated in the embryos of the control group. Increased methylation at the IGDMR observed in the resorbing embryos sired by tamoxifen-treated male rats should have resulted in increased expression of *Dlk1* transcripts. However, downregulation of *Dlk1* transcripts was observed in these embryos. This could have occurred due to the observed increase in methylation at the *Dlk1* promoter and/or due to the downregulation of *Plagl1*, which can affect the expression of many imprinted genes through the imprinted gene network (IGN; Varrault *et al.* 2006). The search for genes frequently co-regulated with *Plagl1* identified a significant number of imprinted genes including *Meg3*, *H19*, *Dlk1*, *Peg3*, *Grb10*, *Igf2*, and

Igf2r (Varrault *et al.* 2006). Over-expression or down-regulation of *Plagl1* results in a subsequent change in the levels of the genes in the network (Varrault *et al.* 2006). The IGN appears to regulate the expression of genes in the network, in the event of alterations in one gene, influencing the overall stability of the system.

The DMR of the mouse *Cdkn1c-Kcnq1ot1* sub-domain is methylated on the maternal allele (Yatsuki *et al.* 2002). We found the DMR within the *Kcnq1* gene to be hypomethylated in sperm and partially methylated in embryos. This suggests that the DMR identified within the rat *Kcnq1* gene is also probably maternally methylated and it might be regulating the expression of genes in the cluster. We had observed downregulation of transcripts of *Ascl2*, *Cdkn1c*, and *Phlda2* genes in the resorbing embryos (Kedia-Mokashi *et al.* 2011). Therefore, we assessed the methylation at the CGIs within the promoters of *Ascl2* and *Cdkn1c* genes in addition to the *Kcnq1* DMR. However, no change in methylation was observed at the *Kcnq1* DMR and/or at the promoters of the *Ascl2* and *Cdkn1c* genes in the resorbing embryos. Decreased expression of genes in this cluster might have also been mediated via abrogation of the IGN in these resorbing embryos.

The DMRs of the remaining imprinted genes investigated in this study are known to be maternally methylated in mice (as stated in the Results section). In this study, the DMRs of *Plagl1*, *Peg5*, *Peg3*, *Igf2r*, and *Grb10* genes were found to be hypomethylated in sperm and partially methylated in embryos, suggesting that they might be maternally methylated in the rat. These genes

are important for embryo development (Kagitani *et al.* 1997, Kikyo *et al.* 1997, Hiby *et al.* 2001, Curley *et al.* 2004, Hitchins & Moore 2004, Varrault *et al.* 2006). In the resorbing embryos obtained at E13 from the treatment group, an increase in methylation at the DMRs of *Plagl1*, *Peg5*, *Peg3*, *Igf2r*, and *Grb10* genes was observed, which concurs with the downregulation of their transcripts reported in our earlier studies (Kedia-Mokashi *et al.* 2011). In addition, the DMRs of *Plagl1* and *Peg3* genes showed a reduction in methylation in the normal embryos sired by tamoxifen-treated male rats. The reason for this observation is presently not clear.

In order to understand whether the observed methylation changes were global and/or locus specific, we studied the global 5mC levels in postimplantation embryos sired by tamoxifen-treated male rats. The 5mC levels were found to be identical in embryos and the placentae of normal embryos sired by control or tamoxifen-treated male rats. In addition, the 5mC levels observed in the resorbing embryos sired by tamoxifen-treated male rats were also comparable to the normal embryos and placentae sired by both groups, suggesting that the methylation changes observed in this study are indeed locus specific. In this study, we have used resorbing embryos as a whole, as it was difficult to distinguish the embryonic and the placental parts in the resorbing embryos. This limits the scope of the study as it cannot be delineated whether aberrant methylation is in the embryo or placenta, or both.

An increase in methylation at DMRs suggests that along with the normally methylated alleles, the

Figure 7 The proposed model of embryo loss involving aberrant DNA methylation at multiple imprinting control regions in embryos sired by tamoxifen-treated male rats. Imprints are established in the gametes. Fertilization is accompanied by the demethylation on the parental genome but imprinted genes resist this demethylation. Subsequently, before implantation, there is *de novo* DNA methylation but imprinted genes resist *de novo* methylation, which is then stably maintained in somatic and extra-embryonic tissues. In resorbing embryos obtained from tamoxifen-treated male rats, increase in methylation of *Dlk1-Meg3* IGDMR was observed. In addition, there was increased methylation of the CGIs identified at other maternally methylated genes (MMG, viz., *Plagl1*, *Peg3*, *Peg5*, *Igf2r*, and *Grb10*) in the resorbing embryos. This caused aberrant expression of imprinted genes in these embryos and placental defects, which significantly increased the postimplantation embryos loss. Methylation at CpG dinucleotides is represented by filled circles.

unmethylated alleles have gained methylation in the resorbing embryos. After fertilization, the preimplantation embryos undergo genome-wide active and passive demethylation. However, the germline imprints appear to be resistant to this methylation reprogramming allowing them to be stably inherited by the offspring (Reik & Walter 2001). A second wave of developmental reprogramming occurs soon after implantation and is characterized by genome-wide *de novo* methylation (Reik & Walter 2001). Recently, several proteins involved in the maintenance of parental-specific imprints have been described, implying that this process might be more precisely regulated than was previously thought (Arnaud 2010). Thus, mechanisms that provide protection to the unmethylated/methylated alleles from getting methylated/demethylated must be active during these periods of genome-wide methylation reprogramming. Thus, deregulation of mechanisms protecting the unmethylated allele from being methylated might have resulted in increased methylation of alleles observed in the resorbing embryos of this study.

Proteins like ZFP57, DPPA3 (STELLA), and MBD3 bind to the DMRs of imprinted genes and assist the DNA methylation machinery in the methylation reprogramming occurring post-fertilization (Nakamura *et al.* 2007, Reese *et al.* 2007, Li *et al.* 2008). Also note that reduced expression of these proteins is associated with hypomethylation of DMRs of imprinted genes (Nakamura *et al.* 2007, Li *et al.* 2008). However, in this study, increased methylation of DMRs was observed, which rules out this possibility.

Recently, the CTCF protein that binds to the unmethylated maternal allele of the *Igf2-H19* DMR has also been implicated in protecting this allele against acquiring DNA methylation (Schoenherr *et al.* 2003, Engel *et al.* 2006). In the absence of CTCF binding, the mouse *H19* DMR is susceptible to methylation at the normally unmethylated maternal allele during postimplantation *de novo* methylation (Engel *et al.* 2006). Interestingly, ZFP42 (REX1; reduced expression gene 1) is another zinc finger protein that has been implicated in the protection of the unmethylated paternal allele of the *Peg3* DMR against DNA methylation during early embryogenesis (Kim *et al.* 2011). ZFP42 is expressed at high levels in the preimplantation embryo and at modest levels in sperm and the placenta. In addition, *Zfp42*-null blastocysts exhibit hypermethylation of the DMR of *Peg3* and *Gnas* imprinted domains (Kim *et al.* 2011). Therefore, downregulation of proteins like ZFP42 could be responsible for the increased methylation at the DMR of imprinted genes in resorbing embryos.

We conclude that the increased methylation at the DMRs of imprinted genes in resorbing embryos sired by tamoxifen-treated male rats could be responsible for their aberrant expression and hence the abrogation of the IGN, collectively causing an increase in postimplantation embryo loss. We hypothesize (Fig. 7)

that the levels of proteins involved in the protection of the unmethylated alleles from being methylated during the wave of *de novo* methylation that occurs post-implantation could have been deregulated in these embryos causing a gain of methylation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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