

# Abnormal methylation of KCNQ1OT1 and differential methylation of H19 imprinting control regions in human ICSI embryos

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## Summary

To evaluate the integrity of genomic imprinting in embryos that failed to develop normally following intracytoplasmic sperm injection (ICSI), we analysed the methylation profile of H19 and KCNQ1OT1 imprinting control regions, H19DMR and KvDMR1 respectively, in high-grade blastocysts and in embryos that exhibited developmental anomalies. Significant hypomethylation of KvDMR1 was specifically observed in 5/5 atypical blastocysts graded BC, which probably reflected the vulnerability of the imprint in the inner cell mass during the methylation remodelling phase in the early embryo. In addition, KvDMR1 was hypermethylated in 2/5 CC graded atypical blastocysts and in 2/8 embryos that exhibited developmental delay. H19DMR appeared differentially methylated in all groups of embryos. DNA methyltransferase 1 (DNMT1) expression was similar in most of the tested embryos and could not account for the abnormal methylation patterns of KvDMR1 observed.

Keywords: ART, Development, Human blastocysts, Imprinting, KCNQ1OT1

## Introduction

While the majority of mammalian genes are expressed from both parental genomes, a set of genes (about 100 have been identified in mammals), are transcribed exclusively either from the maternal or the paternal allele (Reik & Walter, 2001). These imprinted genes are regulated through DNA sequences known as imprinting control regions (ICRs). These regions are methylated differentially according to their parental origin. Imprints are erased in primordial germ cells, early in fetal life and reset in a sex-specific manner during gametogenesis (Reik & Walter, 2001). Following

fertilization, a second important wave of epigenetic remodelling arises: the paternal genome is actively and globally demethylated within a few hours (Mayer *et al.*, 2000) while the maternal genome is demethylated over a few cell cycles. Imprinted genes escape this demethylating process and maintain their sex-specific pattern of DNA methylation, in order to be expressed properly later in development (Delaval & Feil, 2004). The maintenance of the DNA methylation imprint during preimplantation development has been assigned to DNA methyl transferase 1 (DNMT1) (Hirasawa *et al.*, 2008). Imprinted genes are critically involved in the regulation of fetal growth, development and placental functions (Ono *et al.*, 2006) and normal development involves the expression of imprinted genes. Previous studies have established a link between aberrant methylation imprinting and developmental failure (Young *et al.*, 2001; Mann *et al.*, 2003; Liu *et al.*, 2008), particularly during late development. In assisted reproductive technology (ART) centres, some embryos are not transferred because they show abnormal developmental timing or abnormal morphology beyond the blastocyst stage. The causes of this developmental failure are largely

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unknown, and the quality of the imprints in these failing embryos has not yet been investigated in humans. In the present study, we have examined the methylation profile of both a maternally and a paternally imprinted control region, KvDMR1 and H19DMR respectively, that each lie on the human chromosome 11p15.5. H19DMR is methylated on the paternal allele and regulates the expression of H19 and IGF2. IGF2 plays a key role in regulating fetal growth and shows monoallelic expression from the 8-cell stage onwards (Lighten *et al.*, 1997). KvDMR1 is located in the promoter of the non-coding KCNQ1OT1 gene and is maternally methylated. KCNQ1OT1 is paternally expressed and is thought to negatively regulate the expression of several maternally expressed genes, including p57<sup>kip2</sup>, a cell cycle regulator.

## Materials and methods

### Source of human embryos

A total of 50 embryos derived from fertilized ICSI oocytes were donated for research by patients of Laboratoire de Biologie de la Reproduction at Hôpital Femme Mère Enfant (Bron, France), after they have given their informed consent. Protocols were approved by Agence de la Biomédecine, the French legal institution for research on human embryos. Embryos were divided into four groups according to the grading system described by Gardner *et al.* (2000). The embryos included 14 high-grade ICSI embryos, 17 delayed embryos (compacted morula), 11 abnormal BC blastocysts and eight abnormal CC blastocysts. ICSI indications were heterogeneous, but all embryos originated from superovulated oocytes. Zona pellucida and attached cumulus cells were removed by digestion with proteinase K (9 units/ml). Denuded embryos were carefully examined under an inverted microscope with Hoffman Modulation Contrast optics (Leica DM IRB) and only cumulus cell-free embryos were selected for analysis and individually stored at  $-80^{\circ}\text{C}$ .

### DNA methylation analysis

The methylation profiles of KvDMR1 and H19DMR were determined by bisulphite mutagenesis and sequencing as previously described (Borghol *et al.*, 2006), in five control blastocysts, eight delayed embryos, five BC blastocysts, five CC blastocysts,  $2 \times 10^6$  sperm cells,  $1 \times 10^6$  lymphocytes and in five pools of 10 to 20 cumulus cells. After treatment with bisulphite and purification, the DNA was immediately used for duplex polymerase chain reaction (PCR). Five independent duplex PCRs followed by gene-

specific nested PCRs were performed per embryo. We analysed 22 CpG sites in a 265-bp fragment of KvDMR1 (66,536–66,800 bp, GenBank U90095) and 18 CpG sites in a 234-bp fragment of H19DMR (6097–6330 bp, GenBank AF087017) that harboured a single nucleotide polymorphism (SNP) A/C (A/T following bisulphite treatment) at nucleotide 6236, as shown in Fig. 1A. The 234 bp H19DMR fragment contains the sixth CCCTC-binding factor (CTCF) site. The primers specific to bisulfite-converted DNA are listed in Table 1.

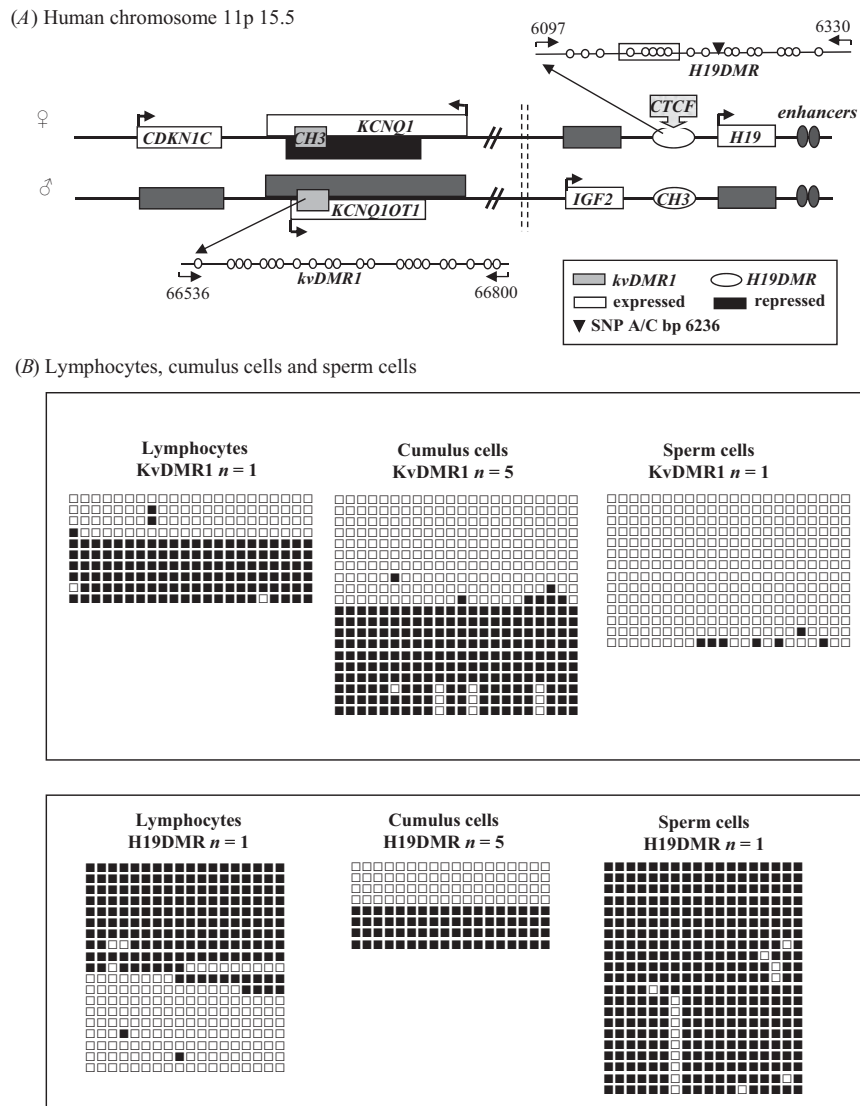
The duplex PCR was less efficient in amplifying H19DMR than in amplifying KvDMR1 and we obtained a signal for both regions in only 13 out of 23 analysed embryos. The PCR products were subcloned into the pGEM-T plasmid (Promega). Five clones were sequenced for each PCR product (Biofidal, Lyon, France). Because of the limiting starting material and because bisulphite treatment is deleterious to DNA, identical sequences from separated PCRs definitely represent distinct chromosomes; identical sequences obtained from the same PCR product were counted only once, as previously discussed (Borghol *et al.*, 2006).

### Real-time quantitative PCR:

The relative mRNA level of DNMT1 was assessed by real-time quantitative PCR, using the mRNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. Twenty-seven embryos (nine normal ICSI embryos, nine delayed embryos, six BC abnormal embryos and three CC abnormal embryos) were subjected individually to a one-tube RT-PCR protocol as described previously (Ziyyat & Lefevre, 2001).

In total, 2.5  $\mu\text{l}$  of RT product was utilized for real-time PCR to study the expression of DNMT1 and GAPDH (reference gene) genes in a single embryo. This procedure was carried out using Light Cycler Fast start DNA master SYBR green I (Roche Diagnostics) and the Light Cycler real-time PCR system (Roche Diagnostics), according to the manufacturers' protocols. The primers used are listed in Table 1; primers were chosen to encompass one intron to avoid amplification of genomic DNA (Girault *et al.*, 2003) and to amplify both the somatic and the oocyte variant of DNMT1 (Hayward *et al.*, 2003).

Standardization was carried out with serial dilutions of RT products of both genes that corresponded to different concentrations of total RNA from fibroblast reference cells. We verified that the amplification efficiencies of DNMT1 and GAPDH were approximately equal. The average Ct (Ct DNMT1 – Ct GAPDH) was determined. The linear regression analysis of the data is shown in Fig. 2. The deviation from zero of the slope



**Figure 1** (A) Schematic representation of human chromosome 11p 15.5. (B) Bisulphite sequencing analysis of KvDMR1 and H19DMR in lymphocytes, cumulus cells and sperm cells. Each line represents a single allele. A black square indicates a methylated CpG and an open square denotes an unmethylated CpG. (C) Bisulphite sequencing analysis of KvDMR1 and H19DMR in control blastocysts. Each line represents a single allele. A black square indicates a methylated CpG and an open square denotes an unmethylated CpG. The percentages of methylated CpG for all embryos are summarized in the graph below the figure. H19 DMR: Genbank AF087017; Kv DMR1: Genbank U90095.

of the line was not significant. Therefore the expression of DNMT1 was calculated using the  $2^{-\Delta\Delta Ct}$  method where  $\Delta\Delta Ct = (Ct\ DNMT1 - Ct\ GAPDH)_{sample} - (Ct\ DNMT1 - Ct\ GAPDH)_{standard}$  (Livak & Schmittgen, 2001).

**Statistics**

Statistical analysis was performed using non-parametric *t*-test or analysis of variance (ANOVA) and a difference was considered significant when the *P*-value was  $\leq 0.05$ .

**Results**

The 50 human embryos studied derived from fertilized ICSI oocytes; they were divided into four groups according to the Gardner *et al.* (2000) grading system. Group1: 14 high-grade ICSI blastocysts suitable for transfer constituted the control group. Thirty-six embryos not suitable for transfer were excluded from the ICSI procedure and distributed in groups 2, 3 and 4. Group 2: 11 blastocysts graded BC (the inner cell mass contained several cells, loosely grouped; the trophectoderm contained very few large cells forming

(C) Control blastocysts

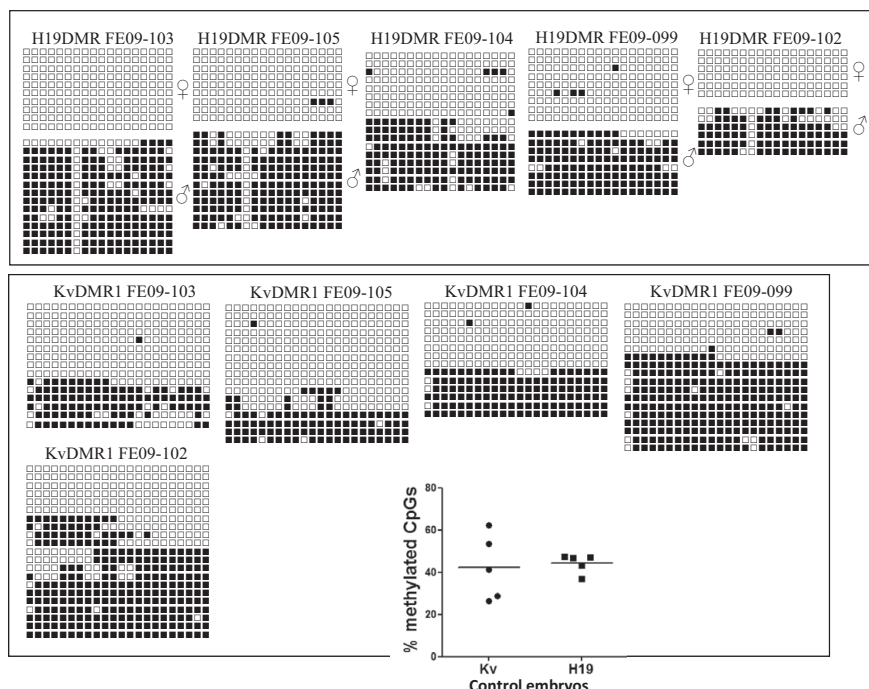


Figure 1 Continued.

**Table 1** Primers for amplification of bisulphite-treated DNA and real-time PCR

Primers	
KvDMR1 F (duplex PCR) bisulphite	5'-TGTTTTTGTAGTTTATATGGAAGGGTTAA-3'
KvDMR1 R (duplex PCR) bisulphite	5'-CTCACCCCTAAAACTTAAACCTC-3'
H19DMR F (duplex PCR) bisulphite	5'-AATAATGAGGTGTTTTAGTTTTATGGATG-3'
H19DMR R (duplex PCR) bisulphite	5'-ACTTAAATCCCAAACCATAACACTAAAAAC-3'
KvDMR1 F (nested PCR) bisulphite	5'-GTTAGGGAAGTTTTAGGGTGTGAAT-3'
KvDMR1 R (nested PCR) bisulphite	5'-AAACATACCAAACCACCCACCTAACAAA-3'
H19DMR F (nested PCR) bisulphite	5'-TTGTATAGTATATGGGTATTTTTGGAGGT-3'
H19DMR R (nested PCR) bisulphite	5'-ACTCCTATAATATCCTATTCCTCCCAAATAACCCC-3'
GAPDH F real-time PCR	5'-GCACCGTCAAGGCTGAGAAC-3'
GAPDH R real-time PCR	5'-TGGTGAAGACGCCAGTGA-3'
DNMT1 F real-time PCR	5'-TACCTGGACGACCCTGACCTC-3'
DNMT1 R real-time PCR	5'-CGTTGGCATCAAAGATGGACA-3'

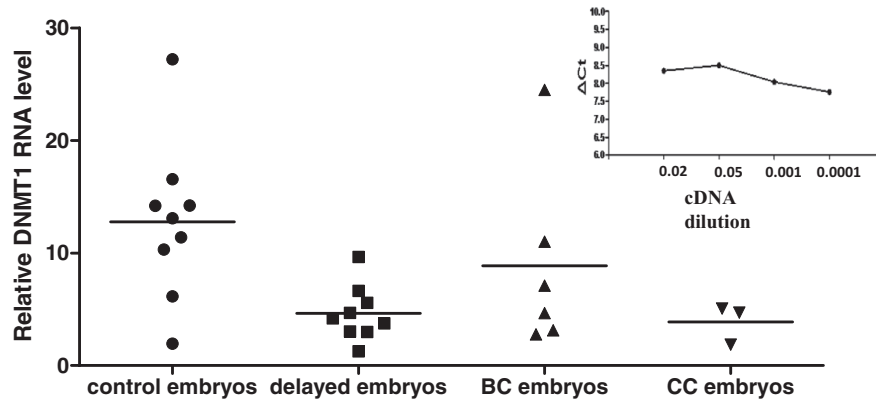
F: forward primer; R: reverse primer

KvDMR1: Genbank U90095, 66536–66800 bp; H19 Genbank: AF087017, 6097–6330 bp; GAPDH

Genbank: NM\_03, 276–412 bp; DNMT1: Genbank FLJ6293, 1303–1406 bp.

a loose epithelium). Group 3: eight blastocysts graded CC (the cavity was larger than the embryo; the inner cell mass and the trophectoderm were formed of very few cells). Group 4 corresponded to 17 embryos that did not reach the blastocyst stage after 6 days in culture (delayed). The study was performed on individual embryos to take into account the variability between embryos. We first verified that sperm was hypermethylated at H19DMR and hypomethylated at KvDMR1 and that the protocol used amplified methylated and hypomethylated strands in DNA

equally from lymphocytes and cumulus cells, despite the relatively limiting starting material (Fig. 1B). Therefore, even though we could not distinguish the paternal from the maternal allele for KvDMR1, because we did not find any polymorphism in the region analysed, we propose that the significant differences observed with the statistical analysis of the results do not reflect an artefact either during PCR or the cloning processes, but do reflect real hypo- or hypermethylation of KvDMR1 in the embryos. This hypothesis has been verified for H19DMR,



**Figure 2** DNA methyl transferase 1 (DNMT1) gene expression patterns in individual blastocysts from control, delayed or atypical groups. DNMT1 expression was determined by real-time quantitative RT-PCR and results were normalised with expression of a housekeeping gene, glyceraldehyde 3-phosphate (GAPDH) and an internal standard sample. The relative DNMT1 RNA level was calculated using the  $2^{-\Delta\Delta Ct}$  method, where  $\Delta\Delta Ct = (Ct\ DNMT1 - Ct\ GAPDH)_{sample} - (Ct\ DNMT1 - Ct\ GAPDH)_{standard}$ . The bars indicate the median expression value. The linear regression analysis of the standardization test is shown inside the frame.

as the amplified region carries a single nucleotide A/C polymorphism (base 6236) that allows parental alleles discrimination in some embryos. In these embryos, approximately half of the total sequences were hypomethylated and likely to be of maternal origin, while the other half were hypermethylated and likely to be of paternal origin.

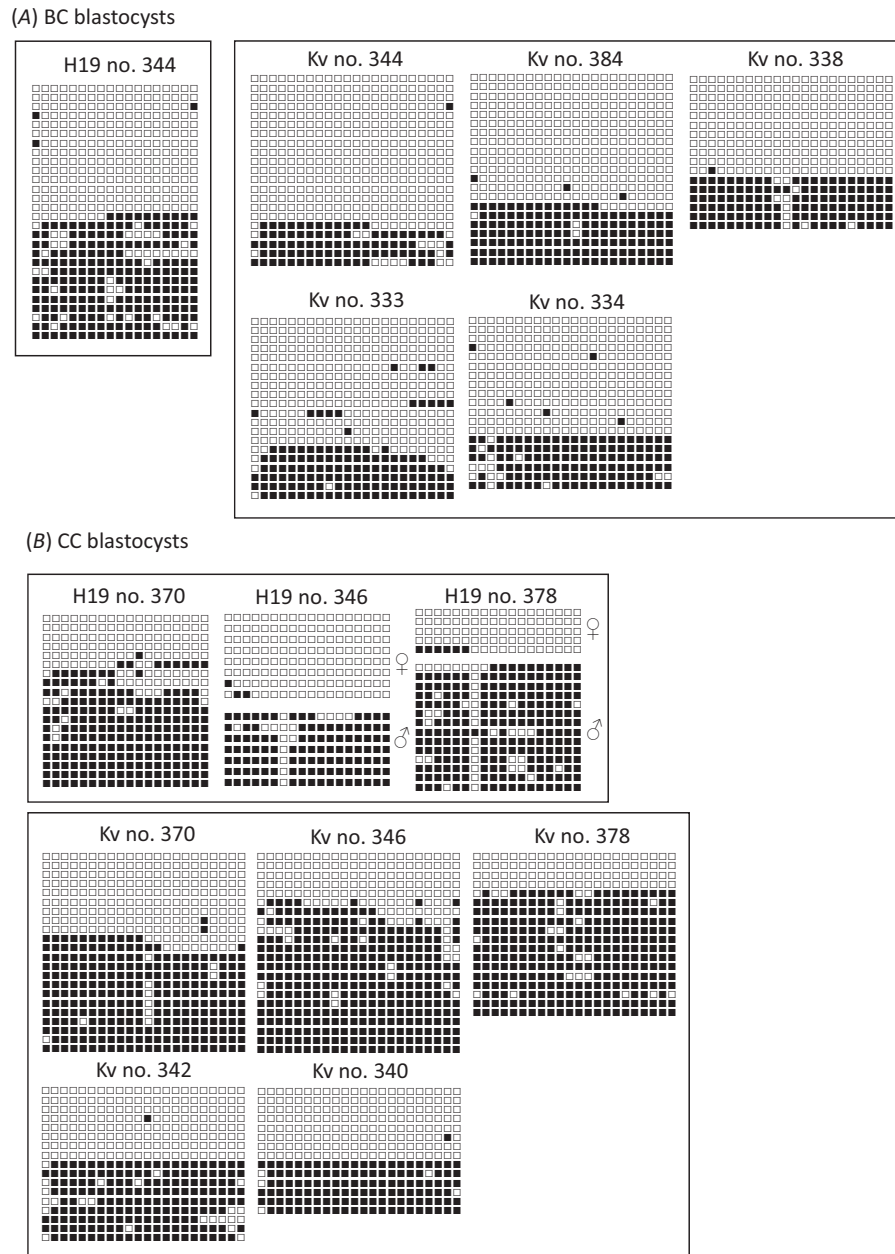
### H19DMR methylation

The amplified differentially methylated region contained 18 CpGs over 234 bp and comprised the sixth CTCF (the insulator protein CCCTC-binding factor) binding site (Bell & Felsenfeld, 2000; Hark *et al.*, 2000). The five blastocysts from the control group exhibited relatively homogeneous methylation profiles at H19DMR with 46 methylated alleles and 45 hypomethylated alleles on 91 alleles analysed (Fig. 1C and Table 2). Only one BC embryo (no. 344) from group 2 could be analysed and exhibited the expected balance between methylated and unmethylated strands (Fig. 3A). The three CC blastocysts in group 3 (no. 370–346–378) exhibited an average of  $55.5 \pm 11.9\%$  methylated CpGs, 32 alleles were methylated out of 53 (Fig. 3B). In the two embryos where parental alleles could be distinguished (no. 346–378), sequences carrying the same polymorphism at base 6236 (A or C) showed similar methylation profiles, either hypomethylated and likely to be of maternal origin, or hypermethylated and likely to be of paternal origin. Another SNP C/T has been observed bp 6194; it cannot be utilized to identify parental alleles as it was located within the seventh CpG. Embryos that showed developmental delay (Fig. 3C, D and Table 2) also exhibited balanced patterns

between methylated and unmethylated strands, with an average of  $47.6 \pm 6\%$  methylated CpGs. Again, in two embryos in which parental alleles could be distinguished (no. 379–355), sequences that carried the same polymorphism at base 6236 showed similar methylation profiles, which demonstrated that there was neither methylation acquisition by the maternal allele, nor methylation lost by the paternal allele, as expected for H19DMR in preimplantation embryos. None of the examined embryos, in any group, exhibited significant hypermethylation.

### KvDMR1 methylation

The amplified KvDMR1 region contained 22 CpGs over 265 bp, and strands that lacked 11 or more methylated CpGs were considered to be hypomethylated. Within the control group (Fig. 1C; Table 2), two blastocysts (no. FE-09–103/105) were significantly hypomethylated (10 and 13 hypomethylated strands out of 15 and 17 respectively, corresponding to an average of  $27.6 \pm 1.7\%$  methylated CpGs, i.e. 194 methylated CpG sites/704 analysed CpGs,  $P \leq 0.001$ ). The other three (no. FE-09–099/102/104) showed an equilibrated distribution between methylated and unmethylated strands that corresponded to an average of  $52.1 \pm 10.2\%$  methylated CpGs (619 methylated CpG sites/1166 analysed CpGs). All BC embryos from group 2 (Fig. 3A; Table 2) exhibited significant deficient methylation with an average of  $28.3 \pm 5.6\%$  methylated CpGs (which corresponded to 606 methylated CpG sites/2156 analysed CpGs compared with 813 methylated CpG sites/1870 analysed CpGs for control embryos,  $P \leq 0.001$ ). CC embryos in group 3 (Fig. 3B and Table 2) exhibited relatively equilibrated



**Figure 3** Bisulphite sequencing analysis of KvDMR1 and H19DMR in atypical BC (A), atypical CC (B) and delayed blastocysts (C). Each line represents a single allele. A black square indicates a methylated CpG and an open square denotes an unmethylated CpG. The percentages of methylated CpG for all embryos are summarized in the graph (D).

distribution of the strands between methylated and unmethylated for three of these (no. 370–342–340) (average of  $48.8 \pm 8.4\%$  of methylated CpGs), while two embryos (no. 346–378) were hypermethylated significantly (14 and 16 methylated strands out of 18 and 22 analysed strands,  $P \leq 0.001$ ). Embryos that showed developmental delay in group 4 (Fig. 3C, D and Table 2) exhibited an average methylation of the CpGs of  $52.2 \pm 13\%$  (which corresponded

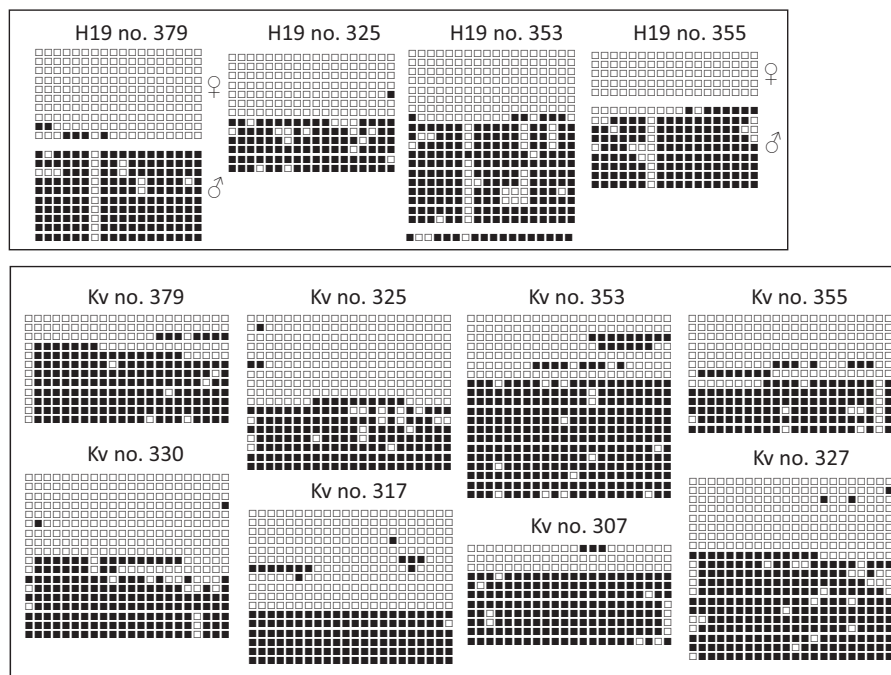
to 1450 methylated CpG sites/2816 analysed CpGs), with relatively homogeneous profiles for six embryos, while two embryos (no. 353–307) showed some hypermethylation ( $P \leq 0.001$ ).

#### DNMT1 expression

As DNMT1 has been shown to be responsible of the maintenance of the imprint during early development,



(C) Delayed blastocysts



(D) Per cent methylated CpGs

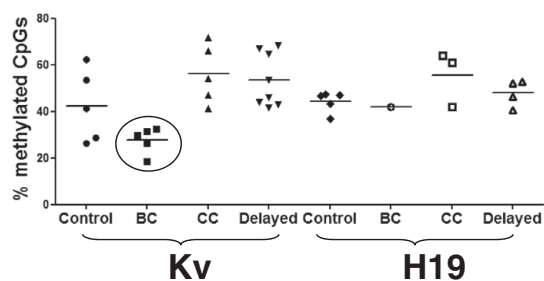


Figure 3 Continued.

a decrease of DNMT1 expression could account for the hypomethylation of imprinted domains. We analysed the expression of DNMT1 in nine cryopreserved control embryos, in nine embryos with developmental delay and in nine embryos with poor quality morphology (six were graded BC and three were graded CC) by real-time-PCR, using primers that amplified both DNMT1s and DNMT1o. Normal blastocysts were formed from approximately 100 cells, while embryos with poor morphology or with developmental delay exhibited a low and variable number of cells. As explained in the Materials and methods section, reverse transcription was done directly on the embryo without extraction and quantification of total RNA contents. Both DNMT1 and GAPDH were amplified from the same cDNA product for each embryo. The relative levels of DNMT1 RNA were unexpectedly variable within the control embryos (Fig. 2). We did not

observe a significant decrease in DNMT1 expression in atypical BC or CC embryos compared with the control group ( $P = 0.165$  and  $P = 0.06$  respectively), contrary to our findings for delayed embryos ( $P = 0.047$ ).

### Discussion

Following the extensive epigenetic remodelling that takes place during gametogenesis, fertilization induces another series of epigenetic modifications. The male pronucleus is actively demethylated before the first replication round (Mayer *et al.*, 2000) while the maternal genome is demethylated over a few cell cycles, most of the genome being demethylated by the morula stage (Santos *et al.*, 2002). Imprinted genes must escape these global changes and maintain the pattern of methylation established in the gametes,

**Table 2** Summarized results of methylation analyses for H19DMR and KvDMR1

Embryos	KvDMR1		H19DMR	
	% of methylated CpG	Methylated CpG/total CpG	% of methylated CpG	Methylated CpG/total CpG
Delayed				
379	64.8	171/264	46.3	167/360
325	40.1	150/374	39.7	93/234
353	<i>67*</i>	<i>295/440</i>	52.2	188/360
355	<i>44</i>	<i>126/286</i>	52.4	132/252
330	41.4	164/396	–	–
317	38.5	144/374	–	–
307	<i>69.4*</i>	<i>168/242</i>	–	–
327	<i>52.7</i>	<i>232/440</i>	–	–
Atypical				
344 BC	<b>18.6*</b>	86/462	41.4	209/504
384 BC	<b>31.6*</b>	146/462	–	–
338 BC	<b>32.9*</b>	123/374	–	–
333 BC	<b>29.1*</b>	128/440	–	–
334 BC	<b>29.4*</b>	123/418	–	–
370 CC	58	281/484	61.1	209/342
346 CC	<i>66.1*</i>	<i>320/484</i>	41.8	113/270
378 CC	<i>71.9*</i>	<i>285/396</i>	63.7	218/342
342 CC	<i>47</i>	<i>176/374</i>	–	–
340 CC	41.5	128/308	–	–
Control				
FE09-103	<b>28.8*</b>	95/330	47	203/432
FE09-105	<b>26.4*</b>	99/374	47.6	180/378
FE09-104	41.2	127/308	43.7	134/306
FE09-099	61.6	244/396	43.5	133/306
FE09-102	53.6	248/462	37.5	81/216

Percentage of methylated CpG at KvDMR1 and H19DMR per embryo and number of methylated CpGs/total CpGs analysed per embryo.

Bold text indicates hypomethylated embryos. Italic underlined text indicates hypermethylated embryos.

\* $p \leq 0.05$ .

in order to be expressed properly later. Very little information is known concerning the maintenance of the imprint in human preimplantation embryos. Geuns *et al.* (2003) found heterogeneous methylation profiles (varying from 0 to 100%) for SNRPN in arrested early embryos that were not suitable for transfer. The same group showed that ICSI embryos with normal morphology and normal developmental timing exhibited some relaxation of the imprint at DLK1-GTL2 by the morula stage (Geuns *et al.*, 2007).

In the present study, we observed that, as it has been described in the mouse, H19DMR and KvDMR1 are both differentially methylated in preimplantation human embryos generated via ART. However, we observed partial relaxation of the maternal KvDMR1 imprint in some embryos, while the paternal imprint at H19DMR appeared stably expressed. All BC embryos showed KvDMR1 hypomethylation, with an average of  $27.8 \pm 5.6\%$  methylated CpGs. Surprisingly, CC embryos which exhibited highly degraded morphology, with a reduced number of cells at both the inner cell

mass (ICM) and the trophectoderm, showed almost normal methylation at KvDMR1. The main difference between BC and CC embryos lies on the number of cells of the ICM that are very few in CC embryos. Thus KvDMR1 hypomethylation is likely to affect specifically the cells from the ICM and to represent a loss of protection of the imprint arising specifically in these cells. Therefore, the significant decrease of methylation at KvDMR1 in BC embryos cannot be inherited from the gametes. Hypomethylation of KvDMR1 in BC embryos was not linked to any known infertility factor of the parents.

Unexpectedly, we observed significant hypomethylation of KvDMR1 in two embryos suitable for transfer, in the control group. However, all embryos utilized in this study, control group included, were issued from ICSI of superovulated oocytes and we previously established that hormonal induction of ovulation led to the release of metaphase (M)II oocytes carrying incomplete imprint at KvDMR1 (Khoueiry *et al.*, 2008). Therefore, the two embryos



that exhibited loss of methylation at KvDMR1 may originate from oocytes that had not acquired a fully and stably established imprint by the MII stage. Hypomethylation at KvDMR1 has been associated with Beckwith-Wiedemann syndrome (BWS), and a loss of methylation on the maternal allele has been observed in more than 90% of BWS children conceived through ART (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003). However, the increased prevalence of BWS in children conceived via ART documented in several reports is controversial (Manipalviratn *et al.*, 2009) and hypomethylation at KvDMR1 has been observed in clinically normal children conceived via ART (Gomes *et al.*, 2009). A gain of methylation was also observed for KvDMR1 in two delayed embryos and in two atypical CC embryos. Hypermethylation of KvDMR1 has been recently depicted in one ICSI child born small for gestational age, and the authors attributed this epimutation to an imprint erasure defect in the paternal germ line (Kanber *et al.*, 2009).

To what extent the imprinting errors observed in some embryos could be attributed to superovulation induced perturbation cannot be evaluated in humans for evident ethical reasons, as control embryos with no hormonal stimulations are not available. Results in the mouse are controversial as one study attributed to superovulation the loss of paternal H19 and maternal KCNQ1OT1 methylation observed in blastocysts (Market-Velker *et al.*, 2009), while another study imputed alteration of H19 methylation to the *in vitro* fertilization process itself and correlated methylation defects with abnormal or failing developmental process (Fauque *et al.*, 2007). These studies were performed on different strains of mice and the high variability of the data obtained highlights the difficulty in extrapolating the results on imprinting studies to other species, particularly to humans.

In the embryos in which a polymorphism allowed parental allele discrimination, we never observed a loss of H19 methylation on the presumably paternal allele or a gain of H19 methylation on the presumably maternal allele, in any blastocyst group. We previously observed a maternal gain of methylation at H19DMR in MII oocytes from stimulated ICSI cycles matured *in vitro*, but essentially associated to oocytes arrested at MI stage following *in vitro* culture (Borghol *et al.*, 2006). The oocytes used to generate the embryos analysed in the present study were matured *in vivo* following hormonal stimulation and retrieved at the MII stage prior to ICSI. Even though they are both located on the human chromosome 11p15.5, H19DMR and KvDMR1 appear independently regulated, KvDMR1 methylation being altered while H19DMR remains differentially methylated.

The maintenance of DNA methylation at imprinted DMRs in preimplantation embryos has been assigned

to the somatic and oocyte forms of DNMT1. Maternal deletion of both isoforms, DNMT1s and DNMT1o, caused a loss of methylation at multiple imprinted loci in mouse blastocysts (Hirasawa *et al.*, 2008) and hypomethylation of satellite I sequences in somatic nuclear transfer bovine blastocysts was associated with lowered Dnmt1 expression (Sawai *et al.*, 2010). In the present study, we did not observe significant up- or down-regulation of DNMT1 expression in any of the blastocysts tested that could explain the loss or the gain of methylation at KvDMR1. Even though DNMT1 appears to be expressed normally in most embryos with developmental failure, this situation does not imply that the protein is present normally in the nucleus of these embryos and this aspect should be further explored. In addition, there are also other *trans*-acting factors critical for the maintenance of methylation at imprinted genes such as ZFP57, which encodes a zinc-finger transcription factor expressed in early development. The loss of both the maternal and the zygotic Zfp57 in the mouse results in complete loss of differential DNA methylation at several imprinted domains and in lethality around mid-gestation (Li *et al.*, 2008). Furthermore, clinical investigations on patients with transient neonatal diabetes have associated hypomethylation at five imprinted domains, including KvDMR1, with mutations in ZFP57, a finding that suggests a role for this factor in the maintenance of DNA imprints during early development (Mackay *et al.*, 2008). The partial loss of methylation of KvDMR1 in BC atypical embryos could be the result of a deficiency in ZFP57, and further investigations on the level of ZFP57 in BC embryos are necessary. The fact that hypomethylation of KvDMR1 happened independently of H19DMR in the embryos is in agreement with the role of different *trans*-acting factors acting to maintain the methylation at different imprinted domain.

Further experiments that focus on the methylation of KvDMR1 in the gametes of the couples are needed to elucidate the origin of the alteration observed in certain arrested embryos and to evaluate the part of inherited epigenetic mutations in embryonic developmental failure. The variability of the results from animal studies highlights the need for studies on humans to evaluate the impact of ART on imprinting, even though they could often be frustrating because of lack of appropriate controls for ethical imperatives.

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