Vitrification at the germinal vesicle stage does not affect the methylation profile of H19 and KCNQ1OT1 imprinting centers in human oocytes subsequently matured in vitro

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Objective: To evaluate the integrity of genomic imprinting in oocytes vitrified at the germinal vesicle (GV) stage and in vitro matured (IVM) after thawing.
Design: Clinical research and application.
Setting: University-based fertility center.
Patient(s): Immature oocytes were donated for research by patients who were included in an intracytoplasmic sperm injection program.
Intervention(s): Immature oocyte retrieval after ovarian stimulation, followed by oocyte vitrification, thawing, and IVM.
Main Outcome Measure(s): Methylation profile of H19 and KCNQ1OT1 imprinting control regions, H19DMR and KvDMR1, respectively.
Result(s): Among 184 vitrified GV oocytes, 102 survived thawing (55.4%), 77 (75.5%) of which reached the meiosis II (MII) stage after IVM. One hundred twenty control GV oocytes were only subjected to IVM; 70.8% reached the MII stage. GV vitrified as well as control oocytes acquired full imprint at KvDMR1 after IVM and generally retained the unmethylated state of H19DMR.
Conclusion(s): For the first time, we show that oocyte vitrification does not affect the methylation profile of H19DMR and KvDMR1: during their IVM, vitrified GV oocytes acquire DNA methylation in the maternally imprinted KCNQ1OT1 gene with the same efficiency as fresh GV oocytes; the vitrification process does not alter the unmethylated state of the paternally imprinted H19 gene. (Fertil Steril® 2011;95:1955–60. ©2011 by American Society for Reproductive Medicine.)

Key Words: Oocytes vitrification, IVM, imprinting, KCNQ1OT1, H19

Cryopreservation of oocytes has gained increased importance in recent years, concomitant with the increased demand to preserve oocytes for future use, partly owing to the improved survival of cancer patients. Vitrification is an attractive alternative to slow freezing standard methods. It requires both ultra rapid cooling rates and very high cryoprotectant concentrations to prevent ice crystal formation and to increase viscosity at low temperatures. A series of successful live births has been recently reported after vitrification of in vitro matured (IVM) meiosis II (MII) human oocytes initially retrieved from stimulated or natural unstimulated cycles (1, 2). In contrast, at least one child was born with the use of slow freezing cryopreserved germinal vesicle (GV) oocytes that were subsequently IVM before the intracytoplasmic sperm injection (ICSI) procedure (3).

Until now, fertility parameters were only examined to assess the efficiency and safety of the vitrification procedure (4, 5). However, to support normal embryonic development, the oocyte must acquire sex-specific information during its growth which, transmitted to the embryo, will result in the monoallelic expression of certain genes. These imprinted genes play important roles in the prenatal growth of the embryo and/or placenta, the regulation of metabolic pathways, and higher brain functions (6–8). These genes are regulated via DNA differentially methylated sequences, according to their parental origin (9). Imprints are erased in primordial germ cells (10) and reestablished according to sex during gametogenesis

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(11, 12). We recently showed that, in humans, the maternal imprint of KCNQ1OT1 was incomplete at the GV stage and that de novo DNA methylation takes place during the maturating process (13). But so far no one has evaluated the impact of the vitrification of GV oocytes on their capacity to both acquire the maternal imprint and protect paternally imprinted genes against de novo DNA methylation during IVM. Thus, for the first time, we report the analysis of the methylation profile of two reporter loci, a maternal (KvDMR1) and a paternal (H19DMR) imprinting center, both located in human chromosome 11p15.5. H19DMR is methylated on the paternal allele and regulates the expression of the H19 and IGF2 genes that play a key role in regulating fetal growth (14); in addition, H19 codes for a nontranslated RNA that acts as a tumor suppressor (15). KvDMR1 is located in the promoter of the noncoding KCNQ1OT1 gene and is maternally methylated. KCNQ1OT1 is paternally expressed and is thought to negatively regulate the expression of several maternally expressed genes (16).

MATERIALS AND METHODS

Source of Human Oocytes

The oocytes were donated for research by patients of Femme Mère Enfant Hospital (Bron, France), after they had given their consent. Protocols were approved by the French legal institution for research on human gametes. The patients included in this study were stimulated for ICSI fertilization with the standard long-term stimulation protocol using recombinant FSH (rFSH) and hCG. Most of the women did not have any known infertility problem, but 10 had polycystic ovary syndrome (PCOS), one dysovulation, one fallopian tube obstruction, six endometriosis, and two endometriosis + PCOs. A total of 304 GV oocytes from 113 patients aged 24–40 years (mean age, 32.2 ± 4.1 years) were included in this study.

Vitrification of GV Oocytes and IVM

After oocyte collection, the cumulus-oocyte complexes were partially denuded of cumulus cells by repeated pipetting in a hyaluronidase solution (150 units, type VII; Sigma, Saint Quentin Fallavier, France). Oocytes were evaluated for maturity; 184 immature partially denuded oocytes were immediately vitrified by using the Medicult vitrification kit (Medicult, Jyllinge, Denmark), according to the manufacturer's protocols. Oocytes in high-security straw (CryoBiosystem, L'Aigle, France) remained stored in liquid nitrogen for 4–6 months. At thawing, the drop containing the oocyte was quickly submerged in a prewarmed solution (Vitrification Thaw, Medicult) containing 1 M sucrose for 3 minutes at 37°C; then the oocytes were transferred and incubated for 3 minutes into two successive room temperature solutions containing, respectively, 0.5 and 0.25 M sucrose. The oocytes were subsequently washed twice in the same medium without sucrose for an additional 6 minutes at room temperature.

Warmed oocytes and control GV oocytes (not subjected to vitrification) were transferred to a maturing solution containing 8 mL of IVM medium (MediCult IVM system) supplemented with 10% patient's serum, hCG, and rFSH and incubated in 5% CO_2 environment at 37°C for 36 hours. MI and MII oocytes were selected for DNA methylation analysis. Zona pellucida and any remaining attached somatic cells were removed by digestion with proteinase K (9 units/mL). After careful examination under an inverted microscope with Hoffman Modulation Contrast optics (Leica DM IRB, Leica Microsystems, Inc, Bannockburn, IL), only cumulus cell–free oocytes were selected for analysis.

DNA Methylation Analysis

The methylation profile of KvDMR1 and H19DMR were determined by bisulphite mutagenesis and sequencing as described elsewhere (17). We analyzed 22 CpG sites in a 265-bp fragment of KvDMR1 (66536–66800 bp, Genbank U90095) and 18 CpG sites in a 222-bp fragment of H19DMR (7875–8096 bp, Genbank AF125183) after nested polymerase chain reaction (PCR). Primers specific for bisulphite-converted DNA and PCR cycling conditions are listed in Table 1. Determination of the KvDMR1 methylation profile was performed at the one-oocyte level, while H19DMR analyses required the pooling of three oocytes per PCR amplification.

The PCR products were subcloned into pGEM-T plasmid (Promega, Charbonnière Les Bains, France). Five clones were sequenced for each PCR product (Biofidal, Lyon, France). Identical bisulfite-modified sequences from separated PCRs are considered to represent distinct chromosomes, but identical sequences from one product are counted only once, as discussed elsewhere (17).

TABLE 1

Primers for amplification of bisulphite-treated DNA.

Primers	
KvDMR1 external F	5'-TGTTTTTGTAGTTTATATGG AAGGGTTAA-3'
KvDMR1 external R	5'-CTCACCCCTAAAAACTT AAAACCTC-3'
KvDMR1 internal F	5'-GTTAGGGAAGTTTTAG GGTGTGAAT-3'
KvDMR1 internal R	5'-AAACATACCAAACCACCC ACCTAACAAA-3'
H19DMR external F	5'-TTYGTAGGGTTTTTGGTAG GTATAGAGTT-3'
H19DMR external R	5'-ATAAATATCCTATTCCCAAA TAACCCC-3'
H19DMR internal F	5'-AGTATATGGGTATTTTTGGA GGTTTTT-3'

Note: F = forward primer; R = reverse primer. KvDMR1: Genbank U90095, 66536-66800 bp; H19 Genbank: AF125183, 7875-8096bp. The PCR conditions used for H19DMR were one cycle of 94°C for 10 minutes, followed by 55 cycles of 94°C for 15 seconds, 56.5°C for 20 seconds, and 72°C for 20 seconds, with final extension of 72°C for 10 minutes, for both the first and the nested round. For KvDMR1, the cycling conditions for the first round of PCR were as follows: one cycle of 94°C for 10 minutes, followed by 55 cycles of 94°C for 15 seconds, 56°C for 20 seconds, and 72°C for 20 seconds, with final extension of 72°C for 10 minutes. For the nested round of PCR, the annealing temperature was 61°C.

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TABLE 2

Vitrification and IVM of GV oocytes.

Variable	Total	%
Vitrification		
No. of patients	63	
No. of vitrified GV oocytes	184	100
No. of warmed oocytes	184	100
No. of surviving oocytes after vitrification	102	55.4 (102/184)
No. of MII oocytes after IVM	77	41.8 (77/184)
No. of MI oocytes after IVM	25	13.6 (25/184)
IVM of GV oocytes		
No. of patients	50	
No. of GV ooccytes	120	100
No. of surviving oocytes after IVM	106	88.3 (106/120)
No. of MII oocytes after IVM	85	70.8 (85/120)
No. of MI oocytes after IVM	16	13.5 (16/120)
No. of GV oocytes after IVM	5	4.1 (5/120)

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Statistical Analysis

Statistical analyses were done using nonparametric analysis of variance, $P \leq .05$ was considered statistically significant. The SEM was calculated for each oocyte group.

RESULTS

IVM of Fresh or Vitrified Human GV Oocytes

Eighty-five out of 120 control GV oocytes matured in vitro, while 21 remained blocked, 16 at the MI stage and five at the GV stage; 14 died. Out of 184 fully grown GV oocytes (120 to 150 μm) that were subjected to vitrification, 102 survived to the warming step and 77 reinitiated their meiosis and matured up to the MII stage after a 36-hour culture period, as shown in Table 2. Most oocytes exhibited normal morphology a few minutes after warming, with the exception of six of them that appeared distorted, while four others were spontaneously activated. Twenty-five oocytes could not achieve completion of the first meiotic division and remained blocked at the MI stage at the end of the IVM period. The failure of certain oocytes to reach the MII state was not linked to any infertility factor.

Analysis of the Methylation Status of KvDMR1

To determine whether the impregnation of oocytes with the very high cryoprotectant concentration that is used to prevent ice crystal formation and to increase viscosity at low temperatures is deleterious for the de novo DNA methylation at KvDMR1 that normally occurs during IVM, as shown elsewhere (13), we examined the methylation pattern of 22 CpGs located within the KvDMR1 sequence. Optimization of the bisulphite sequencing protocol permitted the analysis of oocytes individually for each patient, at the one-oocyte level. Both methylated and unmethylated sequences could be equally obtained from either lymphocytes or pools of five cumulus cells, as previously demonstrated (13), excluding any bias in the PCR amplification between methylated and unmethylated strands. The efficiency of PCR amplification was 77.8% (49 positive PCR amplifications/63). Control MII oocytes obtained after IVM of fresh GV oocytes exhibited highly methylated profiles (763 methylated CpGs out of 814 CpGs analyzed).

After vitrification and IVM, blocked MI oocytes exhibited a heterogenous methylation profile showing 78.4% methylation (corresponding to 328 methylated CpGs over 418 analyzed (Fig. 1). MII oocytes were as highly methylated as control oocytes (P>.99): all sequenced alleles, except one, were methylated in MII oocytes: 27 alleles (96.4%) exhibited 18–22 methylated CpGs out of 22 CpGs analyzed, corresponding to 568 methylated CpG sites/616 analyzed CpGs and a methylation rate of 92.2%. The significantly higher (P<.001) percentage of methylation in MII oocytes compared with MI oocytes means that vitrification at the GV stage does not alter the de novo methylation that occurs in vitro after reinitiation of meiosis. The methylation rate of three oocytes that spontaneously excluded their second polar body appeared significantly lower (P<.001) than that of unactivated MII oocytes (85.7%/92.2% methylated alleles, corresponding to 132 methylated CpG sites/154 analyzed CpGs compared with 568/616). Only one MII oocyte with abnormal morphology could be analyzed, and it exhibited a relatively unmethylated profile. We found no link between unmethylation in MII oocytes and any known maternal infertility factor.

Analysis of the Methylation Status of H19DMR

To evaluate the impact of the vitrification process on the protection of the maternal genome against de novo methylation of paternally imprinted genes, we determined the methylation status of 18 CpGs within H19DMR; this region harbours the sixth CTCF (the insulator protein CCCTC-binding factor) binding site. The efficiency of PCR amplification was lower than that observed for KvDMR1. Consequently, except when specified under the figure, PCR amplification required the pooling of three oocytes. Control MII oocytes obtained after IVM of fresh GV oocytes exhibited almost no methylation at H19DMR (62 methylated CpGs/612 analyzed CpGs).

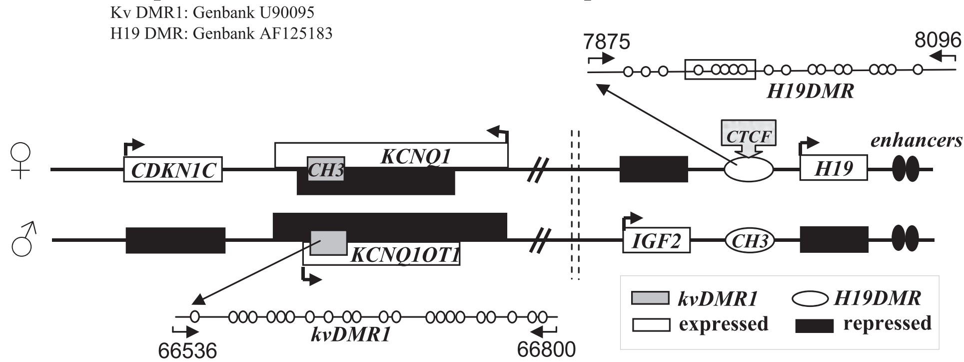
After vitrification and IVM, blocked MI oocytes exhibited a heterogenous methylation profile showing 42.2% methylation (corresponding to 87 methylated CpGs over 206 analyzed); in particular, the sixth CTCF binding site was methylated in seven alleles over 12 alleles analyzed (Fig. 2). Most oocytes that successfully reached the MII state after IVM showed unmethylated CpGs at H19DMR, as shown for controls (P=.18). However, five alleles out of 29 were highly methylated (corresponding to 64 methylated CpGs sites/522 analyzed CpGs), and particularly the sixth CTCF binding site was methylated in six alleles. The level of methylation was significantly higher in blocked MI than in MII oocytes (P<.001). Only one MII oocyte with poor morphology could be analyzed, and it showed high methylation at H19DMR.

DISCUSSION

In this study, we provide evidence that vitrified immature oocytes derived from ovarian stimulation protocols can achieve their maturation in vitro after thawing at an even higher rate (75.5%) than fresh GV oocytes (70.8%). This rate is in the higher range of those published for IVM of fresh GV oocytes: from normal unstimulated ovaries (54.5%–76%; for review see reference 18), from normal ovaries that received gonadotropin stimulation (FSH ± hCG; 71%–77%; 18, 19), and from PCOs oocytes that had benefited (76%–79%; 18) or not from gonadotropin priming (62%–75%; 18, 20). Therefore, the whole vitrification process of GV oocytes has no apparent impact on meiosis resumption as these oocytes can achieve full nuclear maturation up to metaphase II postwarming. The survival rate of vitrified GV oocytes after warming (55.4%) was comparable to that obtained by other groups (59%–63%; 21, 22), as was the rate of MII oocytes obtained through the full procedure, vitrification at the GV stage plus IVM (41.8% in our study compared to 40%–53% in Chung et al. [20] and 38% in Wu et al. [21]). To date, if more than 300 babies have been born worldwide as a result of vitrified oocyte fertilization with no apparent increase in birth anomalies (2, 23), the long-term safety of the technique remains to be confirmed, and any relevant information regarding the integrity of the oocyte is valuable. There is an abundant literature on the more or less controversial link between assisted reproductive techniques (ART) and epigenetic alterations leading to increased incidence of syndromes such as Beckwith-Wiedemann syndrome in ART children (24, 25), but very little research has been devoted to studying the preservation of imprint integrity in oocytes through manipulations in ART centers. Resetting of maternal imprint during human oogenesis is very poorly documented (26, 27, 13). We previously showed that KCNQ1OT1 imprint was not fully established in GV oocytes and that the imprinting process progressed up to metaphase II during meiosis resumption (13). However, in vitro culture as well as the hormon stimulation of the ovary delayed the process (13). The deregulation of imprinted gene expression has important repercussions in many human disorders including cognitive functions and cancers as well as in human ART (28, 29). In addition, initial imprinting errors in the gametes may contribute later in life, even in the next

FIGURE 1

(**A**) Schematic diagram of regions analyzed by bisulfite mutagenesis and sequencing assay. (**B**) Bisulphite sequencing analysis of KvDMR1 in single oocytes after vitrification at the GV stage and IVM, but only IVM for controls. Each line represents a single allele. MIIab = abnormal MII oocyte; MII act = activated MII oocyte; MIIC = control MII oocyte. A black square indicates a methylated CpG, and an open square denotes an unmethylated CpG. n represents the number of independent nested PCRs. The graph summarizes the percentage of unmethylated CpGs ± SEM per oocyte type. *$P<0.001$



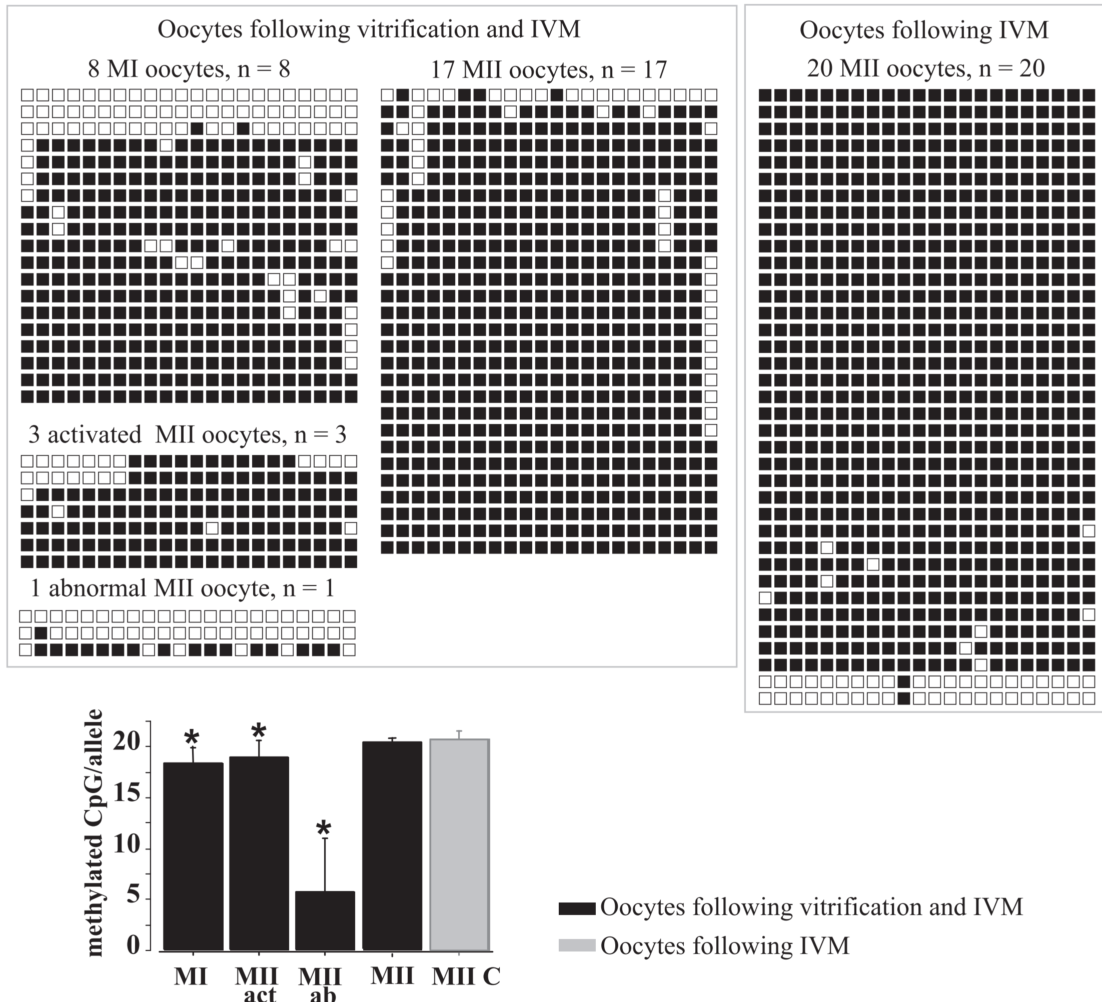


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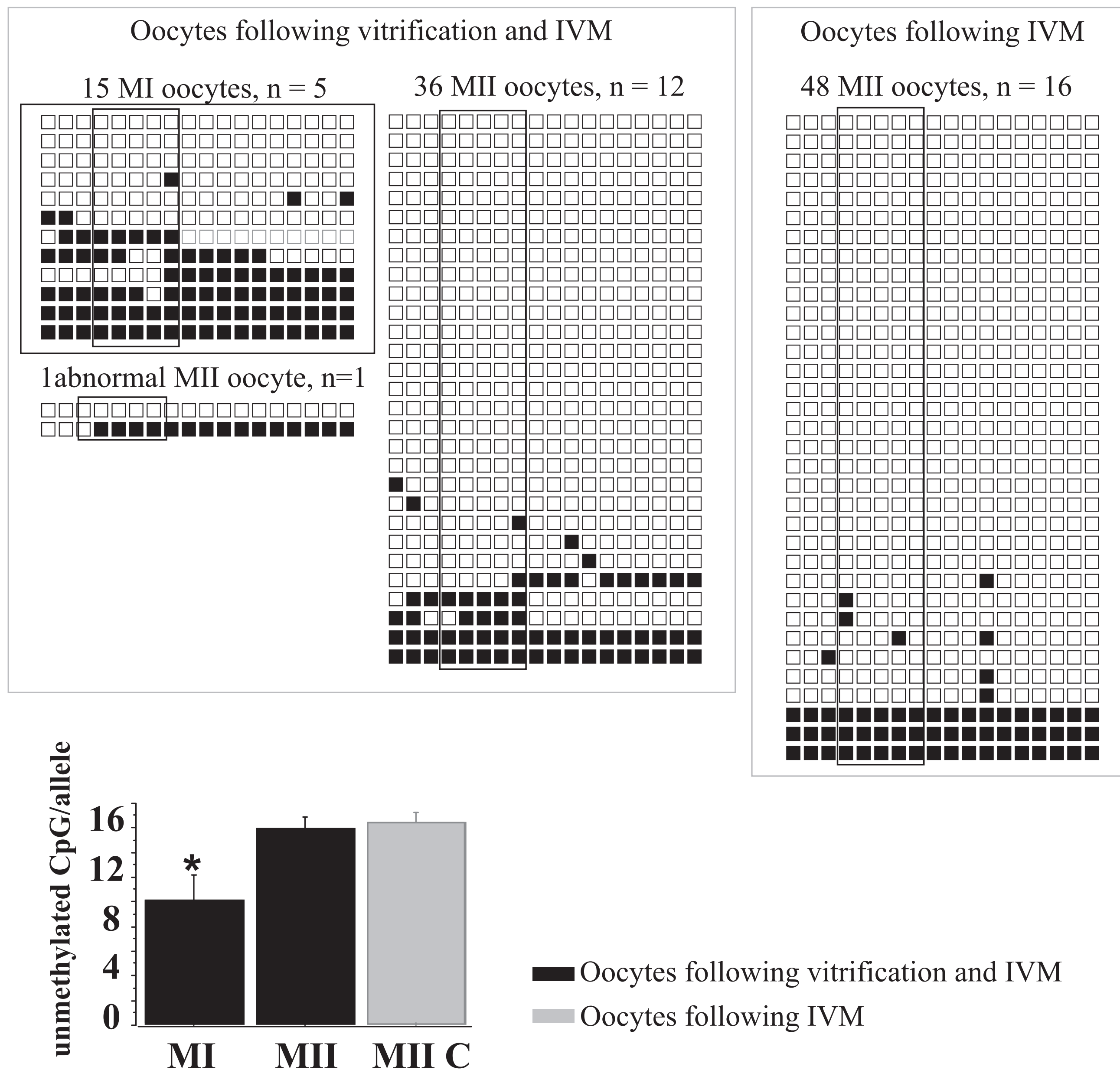
FIGURE 2

Bisulphite sequencing analysis of H19 DMR in pools of oocytes after vitrification at the GV stage and IVM, but only IVM for control. MIIC = control MII oocyte. Each line represents a single allele. A black square indicates a methylated CpG, and an open square denotes an unmethylated CpG. n represents the number of independent nested PCRs. The CpGs within the sixth CTCF binding site are boxed. The graph summarizes the percentage of unmethylated CpGs ± SEM per oocyte type. *$P<0.001$



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generation (30), to gene deregulation. Therefore, it is of foremost importance to evaluate any new technique manipulating the gametes for its capacity to preserve/alter the imprinting process.

We demonstrated for the first time that imprinting can be correctly achieved in oocytes vitrified at the GV stage before their IVM. As well as IVM control oocytes, MII oocytes obtained via a 36-hour IVM period of vitrified GV oocytes carried fully methylated KCNQ1OT1 alleles (96.4%). This 36-hour IVM duration is comparable to that of the in vivo maturing period and permits the oocytes to fully acquire the maternal imprint. As observed elsewhere (13), oocytes with maturing anomalies, that is, early blockage of the meiosis process at the prophase I stage, premature auto activation, or

poor morphology, exhibited partial imprint. The normal maternal imprinting process includes both methylation of maternally imprinted genes and protection against in vivo methylation of paternally imprinted genes. To assess the efficiency of this protecting mechanism through the vitrification process, we examined the methylation pattern of H19DMR, a paternally methylated DMR. If most of the analyzed alleles were unmethylated, as expected, we observed a high degree of methylation in some of them, particularly when meiosis resumption aborted. This inadequate minor methylation is unlikely to result from the vitrification process itself since we already noticed it in control oocytes that were only matured in vitro. It may represent impaired erasure of paternal imprint, however, we cannot exclude that the superovulation process might also have interfered with imprinting establishment (31).

The possibility of vitrifying at the GV stage oocytes obtained from natural cycles or from the ovary after surgery is a decisive advantage in preserving further fertility for women at risk of cancer: it is less demanding in the laboratory than proceeding directly to IVM; it also overcomes the question of the preservation of a normal MII spindle through the vitrification process, which is still controversial (32). From that perspective, the present study provides encouraging yet preliminary data that MII oocytes matured in vitro after vitrification at the GV stage carry a normal maternal imprint.

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