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► **To cite this version:**

Samira Ibala-Romdhane, Mohamed Al-Khtib, Rita Khoueiry, Thierry Blachère, Jean-François Guérin, et al.. Analysis of H19 methylation in control and abnormal human embryos, sperm and oocytes. European Journal of Human Genetics, 2011, 10.1038/ejhg.2011.99 . hal-00646915

HAL Id: hal-00646915

<https://hal.science/hal-00646915>

Submitted on 1 Dec 2011

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1 **Analysis of H19 methylation in control and abnormal human embryos, sperm and**
2 **oocytes**

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19 **Running title:** H19 methylation in human sperm, oocytes and embryos

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21 **Key words:** ART, genomic imprinting , development, H19.

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1 **Abstract**

2 ART is suspected to generate increased imprinting errors in the lineage.
3 Following an ICSI (Intra Cytoplamic Sperm Injection) procedure, a certain number of
4 embryos fail to develop normally and imprinting disorders may be associated to the
5 developmental failure.

6 To evaluate this hypothesis, we analysed the methylation profile of H19DMR, a
7 paternally imprinting control region, in high graded blastocysts, in embryos showing
8 developmental anomalies, in the matching sperm and in oocytes of the concerned
9 couples when they were available.

10 Significant hypomethylation of the paternal allele was observed in half the embryos,
11 independently of the stage at which they were arrested (morula, compacted morula, pre
12 blastocyst or BC graded blastocysts). Conversely, some embryos showed significant
13 methylation on the maternal allele, whereas few others showed both, hypomethylation
14 of the paternal allele and abnormal methylation of the maternal allele. **The matching**
15 **sperm at the origin of the embryos exhibited normal methylated H19 patterns. Thus,**
16 **hypomethylation of the paternal allele in the embryos does not appear inherited from the**
17 **sperm but likely reflects instability of the imprint during the demethylating process**
18 **which occurred in the early embryo. Analysis of a few oocytes suggests that the defect**
19 **in erasure of the paternal imprint in the maternal germ line may be responsible for the**
20 **residual methylation of the maternal allele in some embryos. None of these imprinting**
21 **alterations could be related to a particular stage of developmental arrest; **compared to****
22 **high grade blastocysts, embryos with developmental failure are more likely to have**
23 **abnormal imprinting at H19 (p<0.05).**

24 Key words: ART, genomic imprinting, development, H19.

1 **Introduction**

2 Normal mammalian development requires that both paternal and maternal
3 genome should be expressed properly. Therefore, epigenetic marks acquired in the germ
4 line drive the monoallelic expression, according to parent of origin, of so called
5 “imprinted genes” in the embryo (1). Many imprinted genes are involved in the
6 regulation of fetal and/or placental growth (2). Imprinted genes are regulated through
7 DNA sequences known as imprinting control regions (ICRs) that are differentially
8 methylated, DNA methylation at CpG sequences resulting typically in gene repression.
9 Methylation of ICRs is erased early in life and reset in the germ line, according to sex
10 (2). Another wave of genome-wide demethylation followed later by *de novo*
11 methylation occurs during preimplantation development from which imprinted genes
12 are protected (3).

13 Several reports have supported the idea that artificial reproductive techniques
14 (ARTs) would favour the acquisition of imprinting errors (4-8). Epigenetic
15 abnormalities in ART could be related to parental infertility, corresponding to
16 imprinting errors in the gametes, transmitted at fertilisation, or to *in vitro* manipulation
17 of gametes and embryos (9). Therefore, looking for imprinting defects in embryos that
18 failed to develop normally and in the gametes would provide information on whether
19 unsuitable imprint in the gametes could be transmitted and associated with
20 developmental arrest. Thus, we have analysed the methylation profile of H19DMR (one
21 of the 3 imprinting control region that acquire methylation in the paternal germ line) in
22 control blastocysts, initially suitable for transfer, in preimplantation embryos arrested at
23 different stages of their development: morula, preblastocyst and blastocyst showing

1 poor morphology (graded B C), in the parental sperm and in the oocytes of the couples
2 when they were available.

3 H19DMR regulates the expression of two oppositely imprinted genes (10): H19
4 encodes an untranslated RNA with tumour suppressor activity (11) and it is expressed
5 from the maternal allele ; IGF2 (insulin growth factor 2) encodes a growth factor
6 essential for development (12) and it is expressed from the paternal allele. H19DMR
7 harbours several CTCF (CCCTC-binding factor) binding sites (13). CTCF binds to the
8 maternal unmethylated DMR and prevents IGF2 from access to the common enhancers.
9 Conversely, methylation on the paternal DMR prevents the binding of CTCF,
10 permitting IGF2 expression. Hypermethylation of H19 maternal allele is linked to
11 Beckwith-Wiedemann syndrome (BWS) in some patients (4), while hypomethylation of
12 the paternal allele is associated with Silver-Russell syndrome (14), both syndromes
13 showing opposed growth disorders.

14

1 **Materials and Methods**

2 *Source of human embryos, oocytes and sperm:*

3 A total of 33 embryos from 11 different couples, derived from fertilised ICSI
4 oocytes, were donated for research by patients of Laboratoire de Biologie de la
5 Reproduction at Femme Mère Enfant Hospital (Bron, France), after informed consent.
6 Women included in this study were stimulated prior to ICSI procedure with standard
7 long term stimulation protocol using FSH and HCG. The control group was constituted
8 of 5 high graded ICSI blastocysts; the abnormal embryos were distributed as follows: 5
9 pre-blastocysts, 8 abnormal BC blastocysts (according to Gardner et al. grading system:
10 the inner cell mass contained several cells, loosely grouped and the trophectoderm
11 contained very few large cells forming a loose epithelium), 13 compacted morula and 2
12 morula. Protocols were approved by the French legal institution for research on human
13 embryos, "Agence de la Biomédecine". ICSI indications were heterogeneous as shown
14 in table I, and all embryos originated from super ovulated oocytes. Zona pellucida and
15 attached cumulus cells were removed by digestion with proteinase K (9 units/ml).
16 Denuded embryos were carefully examined under an inverted microscope with Hoffman
17 Modulation Contrast optics (Leica DM IRB) and only cumulus-free embryos were
18 selected for analysis and stored individually at -80°C.

19 After oocyte collection, the cumulus-oocyte complexes were partially denuded
20 of cumulus cells by repeated pipetting in a hyaluronidase solution (150 units, type VII;
21 Sigma) and the oocytes were evaluated for maturity; the immature partially denuded
22 oocytes, either at the germinal vesicle (GV) or at metaphase I (MI) stage, at the time of
23 retrieval, were used for experiments. A total of 15 oocytes from 2 patients were
24 included in this study: 1GV, 12 MI and 2 metaphase II (MII) that were retrieved

1 immature and spontaneously matured in culture medium. Zona pellucida and any
2 remaining somatic cells were removed by digestion with proteinase K (9 units/ml).
3 After careful examination under an inverted microscope with Hoffman Modulation
4 Contrast optics (Leica DM IRB), only cumulus-free oocytes were selected for analysis.

5 The ejaculated sperm samples were collected from the male partner the day
6 when the ICSI procedure was programmed and the methylation analysis was performed
7 on each individual sperm. Control sperm corresponds to sperm of normally fertile men.
8 Routine semen analysis was performed (volume, sperm concentration, motility,
9 morphology) and motile sperm cells were purified on density gradient, to eliminate
10 somatic cell contamination. The sperm was washed repeatedly and placed in phosphate-
11 buffered saline, and DNA was extracted by a standard method.

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14 ***DNA methylation analysis***

15 The methylation profile of H19 DMR was determined by bisulphite mutagenesis
16 and sequencing as previously described (15). After treatment with bisulphite and
17 purification the DNA was immediately used for nested PCR. Five independent nested
18 PCRs were performed per embryo and per sperm sample. We analysed 18 CpG sites in
19 a 234 bp fragment of H19DMR (6097-6330 bp, AF087017) harbouring a single
20 nucleotide polymorphism (SNP) A/C (A/T following bisulphite treatment) at nucleotide
21 6236. This 234 bp fragment contains the 6th CTCF binding site. Primers specific for
22 bisulfite converted DNA were the followings: external forward: 5'-
23 AATAATGAGGTGTTTTAGTTTTATGGATG-3'; external reverse: 5' -
24 ACTTAAATCCCAAACCATAACACTAAAAC- 3'; internal forward: 5'-

1 TTGTATAGTATATGGGTATTTTTGGAGGTT-3'; internal reverse: 5'-
2 ACTCCTATAAATATCCTATTCCCAAATAACCCC-3'. The PCR products were sub
3 cloned into pGEM-T plasmid (Promega, France). Three to six clones were sequenced
4 for each PCR product (Biofidal, Lyon, France).

5

6 **Statistics**

7 Statistical analysis were done using non parametric t-Test or ANOVA and a difference
8 was considered significant when $p \leq 0.05$. To compute the significance of altered
9 methylation in arrested embryos, we used **Fisher's exact test** and a p value ≤ 0.05 was
10 considered significant.

11

12 **Results**

13 Fifteen to 30 clones were sequenced per embryo issued from five independent
14 PCR. Because of the limited starting material and as bisulfite treatment being
15 deleterious for DNA, identical sequences from separated PCRs are certain to represent
16 distinct chromosomes, but identical sequences obtained from the same PCR product are
17 counted only once, as previously discussed (15); twenty to 30 clones could be scored for
18 control embryos and 12 to 15 clones for embryos that failed to develop (see
19 supplementary information figure 1 bis and 2 bis).

20

21 ***Control blastocysts and control sperm***

22 Control blastocysts were cryopreserved for 2 to 9 years before they were donated
23 for research. The SNP localised within the amplified sequence allow parental allele
24 discrimination in 4 out of the 5 analysed blastocysts as shown in figure 1. Thus, even

1 though the corresponding sperms were not available, all methylated sequences carrying
2 the same SNP are most likely from paternal origin while all unmethylated sequences
3 carrying the other SNP are most likely from maternal origin. Therefore, control
4 blastocysts appeared differentially methylated, as expected, with an average of $85.42\% \pm$
5 2.76 methylated CpGs (615 CpGs being methylated out of 720 analysed) for the
6 presumed paternal allele and $1.08\% \pm 0.95$ methylation (8 methylated CpGs out of 738
7 analysed) for the presumed maternal allele. A pool of 5 sperms from fertile men
8 exhibited 93.65% methylation and showed both 6236-C and 6236-A alleles while the
9 sperm from a single fertile man was homozygous 6236-C and exhibited 96.49%
10 methylation. Thus, the highly methylated state of the paternal allele of H19 was
11 conserved in blastocysts, even though with 8.7% significant leakage. According to the
12 SNP depicted, the strands were distributed into two distinct profiles, 6236-C or 6236-A.

13 ***Embryos with developmental failure and matching sperms***

14 Sperms that matched embryos showing developmental failure belonged to
15 various patients, presenting normal or altered sperm parameters, either severe sperm
16 morphology abnormalities (teratozoospermy), mobility defect (asthenozoospermy), or
17 associated sperm morphology, mobility and concentration defects (oligo-astheno-
18 teratozoospermy), as shown in table 1. All exhibited normal high methylation at H19 as
19 shown in figure 1 and 2, with an average of 95.16% methylated CpGs ± 2.61 , which is
20 comparable to that observed in sperms from fertile men. Six were homozygous (6236-C
21 or 6236-A) and five were heterozygous (6236-C and 6236-A) for the SNP described.

22 In 21 embryos out of 28, the observed SNP permitted parental allele
23 discrimination, as shown in figure 1 and 2. Embryos included in this study were
24 blocked at various stages, from morula to compacted morula and pre-blastocyst stage,

1 while 5 embryos reached the blastocyst stage but with poor morphology (graded BC).
2 Embryos from the same couple could be arrested at different stages of development, and
3 there was no correlation between the stage of blockage and any infertility factor (Table
4 1 and 2). We observed significant relaxation of H19 paternal imprint in 8 embryos out
5 of 21 (4.1; 4.3; 5.4; 26.2; 30.2; 30.3; 31.1; 33.3) where parental allele discrimination
6 was possible, the altered strands being partially to totally unmethylated (figure 1 and 2).
7 Moreover, 8 of these embryos (4.1; 30.1; 30.3; 30.4; 33.3; 52.1; 52.2; 52.5) exhibited
8 significant, even minor, methylation of the maternal allele, with 2 embryos, 33.3 and
9 52.2, showing up to 15.87% and 20.14 % methylation respectively. Altered methylation
10 patterns on the paternal and the maternal alleles were not correlated, nor were they
11 associated with a particular stage of developmental arrest (Table 2).

12 The methylation status of the oocytes could be analysed for 2 couples (figure 1).
13 In couple 5, the father was homozygous C while the mother was heterozygous A or C.
14 One GV oocyte, carrying the SNP 6236-C, exhibited hypermethylated alleles while a
15 pool of 2 mature MII oocytes, carrying the SNP 6236-A, showed normal
16 hypomethylation. As hypothesised in figure 3, the hypermethylated 6236-C alleles
17 would be inherited from the grand father and would correspond to alleles that escaped
18 erasure of paternal marks, early during oogenesis. Thus, embryo 5.4 inherited a paternal
19 6236-C allele, the corresponding sperm being homozygous 6236-C and a maternal
20 6236-A allele showing normal hypomethylation that is likely to be inherited from the
21 grand mother. The paternally inherited 6236-C allele exhibited significant
22 hypomethylation ($p < 0.001$). In couple 33, both the father and the mother were
23 heterozygous 6236-C and 6236-A. Twelve immature MI oocytes could be collected and
24 analysed. They showed two different allelic profiles: 6236-A, normally hypomethylated

1 and presumably inherited from the mother or 6236-C, highly methylated (35 methylated
2 CpGs out of 36 analysed) and presumably paternally inherited, corresponding to alleles
3 that escaped erasure of paternal marks early during oogenesis, as previously discussed.
4 Thus, embryo 33.3 is likely to originate from a paternal spermatozoon carrying a 6236-
5 A methylated allele, since the two methylated 6236-A strands carried an unmethylated
6 6194-C nucleotide, as can be seen in all 6236-A sperm alleles, and an oocyte carrying a
7 6236-C methylated allele for H19, likely inherited from the grand father as speculated in
8 figure 3. The presumed paternal allele is significantly hypomethylated (39 methylated
9 CpGs out of 72 analysed $p < 0.001$) while the presumed maternal allele is significantly
10 methylated (20 methylated CpGs out of 126 analysed, $p < 0.001$).

11 Compared to high grade blastocysts, embryos with developmental failure are more
12 likely to have abnormal imprinting at H19 (13/21 versus 0/4, $p < 0.05$).

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1 **Table 1:** ICSI indication *per* couple. PCOs: polycystic ovary syndrome. Mar test +:
 2 presence of sperm antibody.

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N° of couple	♂ infertility factor	♀ infertility factor
4	oligo-astheno-teratozoospermy	
5	asthenozoospermy	PCOs
26	oligo-astheno-teratozoospermy	
30	asthenozoospermy	PCOs
31	asthenozoospermy	PCOs + hydrosalpinx
33	astheno-teratozoospermy	fallopian tube obstruction
45	Mar test +, normozoospermy	
46	astheno-zoospermy, Mar test +	
50		fallopian tube obstruction
52	Mar test +, astheno-teratozoospermy	PCOs
56	normozoospermy	fallopian tube obstruction

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- 1 **Table 2:** Methylation of H19DMR in embryos in which parental allele discrimination
- 2 could be done and in sperms.
- 3

N° of embryo	Embryo type	♂ allele % methylation	♀ allele % methylation	Sperm % methylation
4.1	CM	68.75 (99/144) *	8.34 (3/36) *	97.68 (211/216)
4.3	CM	15.07 (19/145) *	0 (0/36)	97.68 (211/216)
4.5	CM	79.17 (57/72)	1.11 (1/90)	97.68 (211/216)
5.4	P	51.85 (28/54) *	0 (0/36)	96.66 (261/270)
26.2	BC	77.78 (126/162) *	0 (0/18)	100 (36/36)
26.4	BC	94.11 (170/180)	5.56 (1/17)	100 (36/36)
30.1	BC	78.70 (85/108)	2.56 (9/117) *	94.07 (254/270)
30.2	P	75.93 (123/162) *	0.93 (1/108)	94.07 (254/270)
30.3	CM	75.39 (95/126) *	7.40 (4/50) *	94.07 (254/270)
30.4	CM	90.48 (114/126)	5.55 (5/90) *	94.07 (254/270)
31.1	CM	69.44 (75/108) *	0 (0/90)	93.98 (203/216)
33.3	P	54.17 (39/72) *	15.87 (20/126) *	93.70 (253/270)
46	CM	78.70 (85/108)	0 (0/72)	95.55 (258/270)
50.1	M	81.11 (73/90))	1.11 (1/90)	90.87 (229/252)
52.1	CM	84.44 (76/90)	6.35 (8/126) *	98.01 (247/252)
52.2	CM	88.89 (48/54)	20.14 (29/144) *	98.01 (247/252)

52.5	P	80.56 (58/72)	3.96 (5/126) *	98.01 (247/252)
52.6	BC	81.75 (103/126)	1.39 (2/144)	98.01 (247/252)
56.2	BC	93.33 (84/90)	0 (0/90)	94.44 (255/270)
56.3	P	88.89 (80/90)	2.08 (3/144)	94.44 (255/270)
56.4	CM	93.21 (151/162)	2.22 (2/90)	94.44 (255/270)
2	control	89.50 (145/162)	0 (0/198)	
5	control	83.33 (90/108)	0 (0/162)	
6	control	84.92 (214/252)	0 (0/198)	
8	control	85.18 (184/216)	1.66 (3/177)	

1

Control sperm	
a	96.49 (275/285)
b	93.65 (236/252)

2

3 *: $p \leq 0.005$

4 M: morula; CM: compacted morula; BC: abnormal BC blastocysts; P: preblastocysts;

5 control: high graded blastocysts, suitable for transfer. a: sperm from a fertile man; b:

6 pool of sperms from 5 fertile men.

1 **Discussion**

2 Imprinting is both heritable and reversible; in the early embryo, epigenetic
3 information is erased from the genome on a large scale to permit the return of
4 developmental pluripotency to embryonic cells, and this limits the amount of epigenetic
5 information that can be inherited across generation (16). Imprinted genes that carry such
6 heritable epigenetic information must be protected against this powerful remodelling of
7 the epigenome proceeding during preimplantation development. Considering the sensor
8 gene H19, the paternal allele normally escapes the active demethylation of the male
9 genome that occurs in the pronucleus soon after fertilisation and the maternal allele is
10 protected against *de novo* methylation of the zygotic DNA from morula to blastocyst
11 stage (3).

12 Very little is known concerning the differential methylation of imprinted genes
13 in preimplantation human embryos. In the present work, using an SNP within
14 H19DMR, we can determine the differential methylation status of 4 out of 5 control
15 blastocysts and of 21 out of 28 abnormal embryos, the paternally inherited alleles being
16 generally methylated while the maternally inherited alleles were unmethylated. Our
17 results from control embryos confirmed that, as observed in the mouse (17), H19 is
18 differentially methylated in human blastocysts conceived *via* ART. In contrast, 38% of
19 the embryos that failed to develop exhibited significant hypomethylation of the paternal
20 allele, independently of the type of developmental failure: blockage at the compacted
21 morula or pre blastocyst stage, or poor morphology at the blastocyst stage. Since normal
22 methylation of the paternal allele was observed in the other abnormal embryos,
23 imprinting errors at H19/IGF2 are likely not to be primarily involved in the
24 developmental failure of human ICSI embryos. Culture conditions were identical for

1 control and developmentally failing embryos and thus could not be the cause of
2 abnormal methylation.

3 Embryos showing hypomethylated paternal alleles may originate from sperm
4 in which resetting of the imprint has been only partially accomplished. Recently,
5 Kobayashi et al. (18) showed that hypomethylation of H19DMR in aborted ART
6 concepti originated from the parental sperm. A case of Silver-Russel syndrome due to
7 paternal H19DMR hypomethylation in an ICSI patient has been likewise documented
8 (19). In fact, we found no alteration of the normal methylated pattern of H19DMR in all
9 the examined parental sperm, whatever their biological characteristics were, from
10 normozoospermic to oligo-astheno-teratospermic, demonstrating that the alterations in
11 DNA methylation seen in some arrested embryos did not pre-exist in the father's germ
12 line. It is interesting to note that, in sperm carrying the SNP A, the cytosine
13 corresponding to the 4th CpG of the 6th CTCF binding site (nucleotide 6194) commonly
14 appeared as a thymidine (in 84 out of 87 analysed alleles), presumably corresponding to
15 an unmethylated CpG in the original strand. Renda et al. (20) demonstrated that
16 methylation of the first and the second CpGs of the CTCF binding sequence was
17 sufficient to inhibit the binding of CTCF, the methylation of the first CpG being more
18 powerful. On the other hand, in most arrested embryos, hypomethylation of the paternal
19 allele did not particularly target the key CpGs, suggesting normal expression of IFG2.
20 However, the number of samples analysed remained limited, and sperm-transmitted
21 aberrant hypomethylation of the paternal allele of H19 in arrested embryos cannot be
22 totally excluded, since H19 hypomethylation in sperm has been associated with paternal
23 infertility in a number of articles (21-23). Thus, considering that the resetting of
24 methylation during gametogenesis has normally proceeded, it could be hypothesised

1 that the hypomethylation observed is due to an alteration of the methylation
2 maintenance in the early embryo. Maintenance of DNA methylation at imprinting
3 DMRs in preimplantation embryos has recently been assigned to the somatic and the
4 oocyte form of DNMT1, in cooperation. Maternal deletion of both isoforms, DNMT1s
5 and DNMT1o, caused the loss of methylation at multiple imprinted loci in mouse
6 blastocysts (24). Other trans-acting factors critical for the maintenance of methylation at
7 imprinted genes have been identified, such as MBD3 which is required to maintain the
8 methylation of the paternal H19 allele and its silencing (25).

9 Some embryos exhibited significant methylation of the maternal allele. Results
10 from couples 5 and 33 whose oocytes were available allow speculating. In both couples,
11 the paternal allele show some relaxation of the imprint, probably due to alterations in
12 DNA methylation maintenance as previously discussed. In both couples some immature
13 GV and MI oocytes were highly methylated. We previously evidenced such altered
14 pattern of methylation in human oocytes, particularly in oocytes that were immature on
15 the day of retrieval, but also in mature oocytes (15). While the maternally originating
16 H19 strands were not methylated in embryo 5.4, they were significantly methylated in
17 embryo 33.3. Both the father and the mother were heterozygous in couple 33. The allele
18 likely inherited from the father carried the A SNP; it was initially methylated since the
19 sperm appeared normally methylated, and was then partially demethylated in the course
20 of cell divisions. The allele likely inherited from the mother carried the C SNP and
21 showed abnormal methylation. Methylated H19 strands in the oocytes may correspond
22 to the acquisition of abnormal methylation on the maternal allele during oogenesis or
23 lack of erasure of the paternal imprint early in the gonocytes. Because the oocytes from
24 the mother which could be analysed showed either unmethylated A-SNP-H19 strands or

1 methylated C-SNP-H19 strands, methylation apposition during oogenesis is unlikely,
2 otherwise, it would also have randomly mark A-SNP-H19 strands. Lack of erasure of
3 the copies originating from the grand father in the gonocytes of the mother is a more
4 credible scenario. Thus, all methylated copies seen in the oocytes are likely originated
5 from paternal copies, the imprint of which has not been erased, as postulated in figure 3.
6 Such a gain of maternal methylation and a loss of paternal methylation at H19DMR
7 have been recently documented in mouse blastocysts and were attributed to
8 superovulation induced imprinting disorders (17). The authors did not give information
9 on the imprint in the oocytes themselves. On the other hand, we found almost no
10 residual methylation on the maternal allele of control human blastocysts while Market-
11 Velker et al. (17) showed an average methylation of the maternal allele of H19 of up to
12 15% in mouse blastocysts obtained from spontaneously ovulated female, demonstrating
13 one more time the discrepancies existing between species at the level of epigenome
14 regulation.

15 In this work, we show that a significant number of arrested preimplantation
16 embryos have altered H19 imprints on both paternal and maternal alleles, while
17 blastocysts suitable for transfer were normally imprinted. Our results suggested both a
18 failure of erasure of the paternally inherited imprint in the maternal germ line and a lack
19 of methylation maintenance on the paternal allele. The question remains whether there
20 is a link between imprinted errors and parental infertility. No evident link appeared
21 between imprint anomalies and a particular stage of development in failing embryos.
22 However, the data presented show a significant association between developmental
23 failure of embryos and abnormal imprinting of H19.

1 *Acknowledgments*: we are very grateful to all the couples that donated embryos and
2 gametes for research and to the staff of “Service de Biologie de la Reproduction” at
3 FME Hospital, Bron, particularly to Jacqueline Lornage and Astrid Perret. This work
4 was supported by Université Claude Bernard Lyon 1.

5 *Conflict of interest*: The authors declare no conflict of interest.

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12

1

2 **Titles and legends to figures**

3 **Figure1 and 2:** bisulphite sequencing analysis of H19DMR in control blastocysts and
4 control sperm (a: sperm from a fertile man; b: pool of sperm from 5 fertile men), and in
5 arrested embryos and their matching sperm and oocytes (when available, couples 5 and
6 33). Each line represents a single allele. Black squares indicate a methylated CpG and
7 an open square denotes an unmethylated CpG. Blue lines correspond to alleles
8 exhibiting a nucleotide 6236 A, while black lines correspond to alleles carrying a
9 nucleotide 6237 C. In H19DMR, sequence from 6097bp to 6330bp, the SNP A/C, bp
10 6236, is indicated in blue while the 6th CTCF binding site is indicated in red.

11

12 **Figure 3:** Proposed origin of the methylation alterations observed in embryos 5.4 and
13 33.3.

14

Figure 1

H19DMR (6097-6330 bp, Genbank AF087017)

ttgc atagcacatg ggtattctg gaggctctc ctteggctc accgcctgga tggcacggaa ttggtgtag
 ttgtggaatc **ggaagtggcc gcgcgcggc** agtgcaggt cacacatcac agcccagacc cggcccA⁺C⁺act
 ggggtcgc cgtggaacg tcccgggtca cccaagccac gcgtcgcagg gttcacgggg gtcactggg
 aataggacac tcataggac

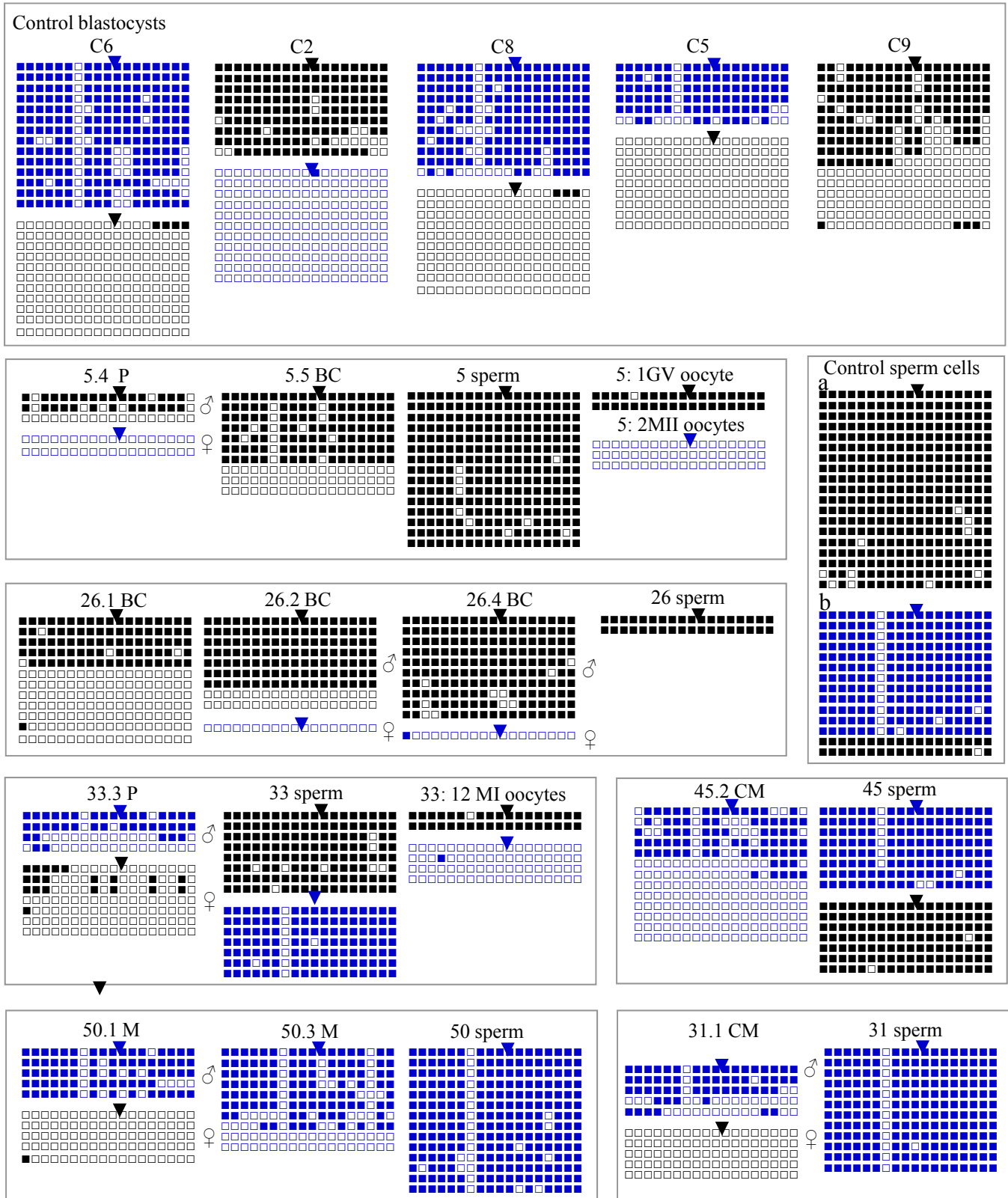


Figure 2

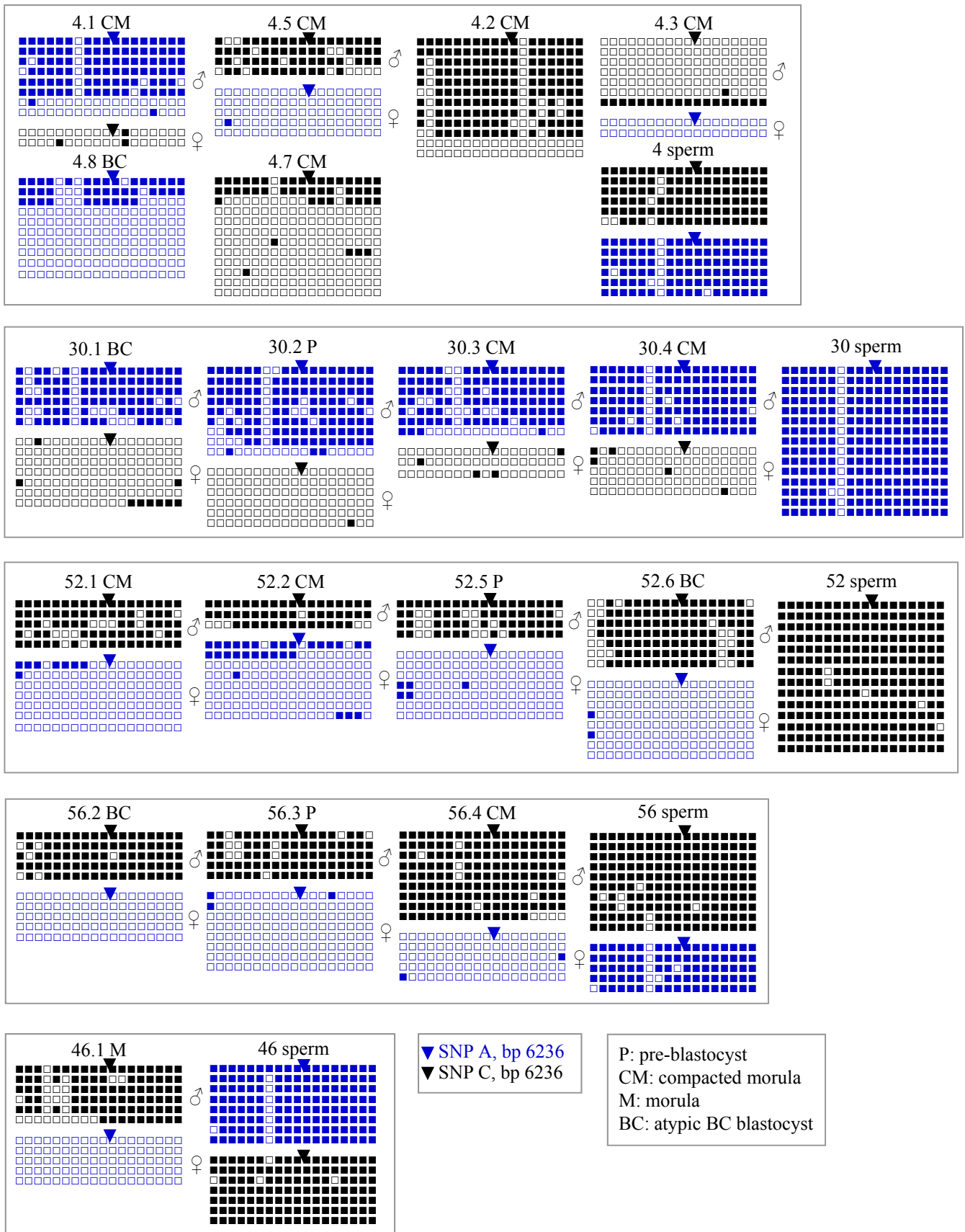
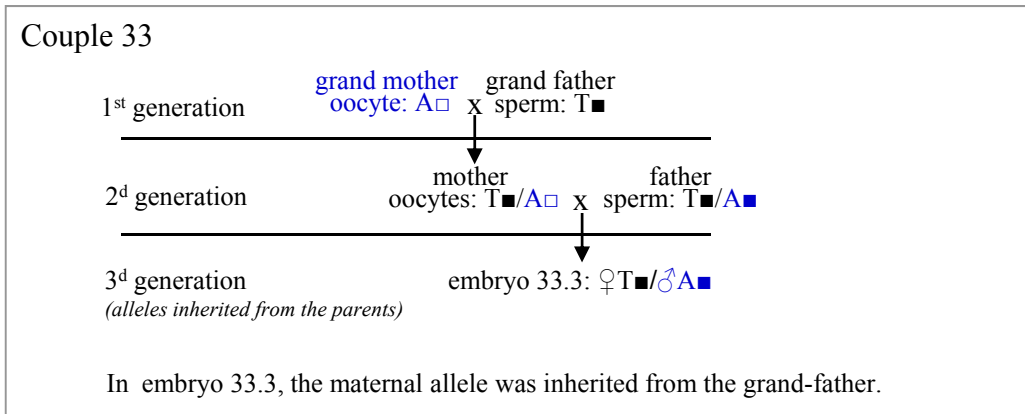
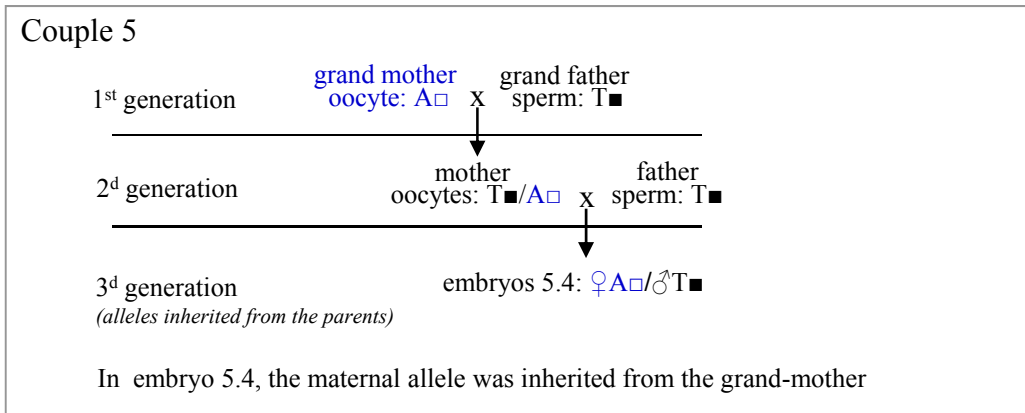


Figure 3



- ■ methylated H19 alleles
- □ unmethylated H19 alleles
- A: snp A base 6236
- C: snp C base 6236

Table 1: ICSI indication *per couple*. PCOs: polycystic ovary syndrome. Mar test +: presence of sperm antibody.

N° of couple	♂ infertility factor	♀ infertility factor
4	oligo-astheno-teratozoospermy	
5	asthenozoospermy	PCOs
26	oligo-astheno-teratozoospermy	
30	asthenozoospermy	PCOs
31	asthenozoospermy	PCOs + hydrosalpinx
33	astheno-teratozoospermy	fallopian tube obstruction
45	Mar test +, normozoospermy	
46	Mar test +, astheno-zoospermy	
50	normozoospermy	fallopian tube obstruction
52	Mar test +, astheno-teratozoospermy	PCOs
56	normozoospermy	fallopian tube obstruction

Table 2: Methylation of H19DMR in embryos in which parental allele discrimination could be done and in sperms.

N° of embryo	Embryo type	♂ allele % methylation	♀ allele % methylation	Sperm % methylation
4.1	CM	68.75 (99/144) *	8.34 (3/36) *	97.68 (211/216)
4.3	CM	15.07 (19/145) *	0 (0/36)	97.68 (211/216)
4.5	CM	79.17 (57/72)	1.11 (1/90)	97.68 (211/216)
5.4	P	51.85 (28/54) *	0 (0/36)	96.66 (261/270)
26.2	BC	77.78 (126/162) *	0 (0/18)	100 (36/36)
26.4	BC	94.11 (170/180)	5.56 (1/17)	100 (36/36)
30.1	BC	78.70 (85/108)	2.56 (9/117) *	94.07 (254/270)
30.2	P	75.93 (123/162) *	0.93 (1/108)	94.07 (254/270)
30.3	CM	75.39 (95/126) *	7.40 (4/50) *	94.07 (254/270)
30.4	CM	90.48 (114/126)	5.55 (5/90) *	94.07 (254/270)
31.1	CM	69.44 (75/108) *	0 (0/90)	93.98 (203/216)
33.3	P	54.17 (39/72) *	15.87 (20/126) *	93.70 (253/270)
46	CM	78.70 (85/108)	0 (0/72)	95.55 (258/270)
50.1	M	81.11 (73/90)	1.11 (1/90)	90.87 (229/252)
52.1	CM	84.44 (76/90)	6.35 (8/126) *	98.01 (247/252)
52.2	CM	88.89 (48/54)	20.14 (29/144) *	98.01 (247/252)
52.5	P	80.56 (58/72)	3.96 (5/126) *	98.01 (247/252)
52.6	BC	81.75 (103/126)	1.39 (2/144)	98.01 (247/252)
56.2	BC	93.33 (84/90)	0 (0/90)	94.44 (255/270)
56.3	P	88.89 (80/90)	2.08 (3/144)	94.44 (255/270)
56.4	CM	93.21 (151/162)	2.22 (2/90)	94.44 (255/270)
2	control	89.50 (145/162)	0 (0/198)	
5	control	83.33 (90/108)	0 (0/162)	
6	control	84.92 (214/252)	0 (0/198)	
8	control	85.18 (184/216)	1.66 (3/177)	

Control sperm	
a	96.49 (275/285)
b	93.65 (236/252)

*: $p \leq 0.005$

M: morula; CM: compacted morula; BC: abnormal BC blastocysts; P: preblastocysts; control: high graded blastocysts, suitable for transfer. a: sperm from a fertile man; b: pool of sperms from 5 fertile men.