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Methylation imprints of the imprint control region of the *SNRPN*-gene in human gametes and preimplantation embryos

Elke Geuns[1], Martine De Rycke[1,*], André Van Steirteghem[2] and Inge Liebaers[1]

[1]Centre for Medical Genetics and [2]Centre for Reproductive Medicine, University Hospital and Medical School, Dutch-speaking Brussels Free University, Laarbeeklaan 101, 1090 Brussels, Belgium

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Imprinting is an epigenetic mechanism leading to mono-allelic expression of imprinted genes. In order to inherit the differential epigenetic imprints from one generation to the next, these imprints have to be erased in the primordial germ cells and re-established in a sex-specific manner during gametogenesis. The exact timing of the imprint resetting is not yet known and the use of immature gametes in assisted reproductive technologies may therefore lead to abnormal imprinting and related diseases. Imprinting is associated with differential allelic methylation in a CpG-context. We studied the methylation patterns of the imprint control (IC) region of the human *SNRPN*-gene in human spermatozoa, oocytes in different developmental stages [germinal vesicle (GV), metaphase I and metaphase II oocytes] and in preimplantation embryos using the bisulphite sequencing technique. In the spermatozoa, almost all potential methylation sites were unmethylated whereas mainly methylated patterns were found in the oocytes at different developmental stages. In the embryos, an average methylation pattern of 53% was found indicating that the imprints, which have been set during gametogenesis, are stably maintained in the preimplantation embryo. Our results indicate that the maternal imprints for the IC-region of the human *SNRPN*-gene are already re-established at the GV stage and that they are not re-established in a late oocyte stage or after fertilization as previously reported. Recent advances in assisted reproductive technologies raise questions concerning safety and the epigenetic risks involved. Our study was the first to check the methylation imprints in human pre-implantation embryos and oocytes at different developmental stages.

INTRODUCTION

Although the maternal and paternal genomes are nearly equivalent in their genetic contribution to embryos, they carry different epigenetic information. Experiments with mouse embryos with two maternal or two paternal genomes showed that these embryos did not complete normal embryogenesis, indicating that the maternal and paternal genomes are not equivalent and that both are needed for complete embryogenesis (1–3). Imprinting is a non-Mendelian form of inheritance where only one of the two alleles is expressed in a sex-specific way. Differential epigenetic marking of the two parental alleles underlies the mono-allelic expression. Methylation of CpG-dinucleotides could serve as such an epigenetic mark, since methylation can differentially mark the two parental chromosomes. The CpGs in differentially methylated regions of imprinted genes are methylated on one parental allele and unmethylated on the other. In order to inherit this epigenetic mark from one generation to the next, these imprints have to be erased in the primordial germ cells (PGC) (4,5) and re-established during gametogenesis in a sex-specific manner. The aim of this study was to gain a better insight into the timing of imprint re-establishment in human oocytes and the maintenance of the imprints in human preimplantation embryos. Therefore oocytes at the germinal vesicle stage (GV), metaphase I (MI) oocytes, metaphase II (MII) oocytes and pre-implantation embryos were analysed for their methylation status at the imprint control (IC) region of the small nuclear ribonucleoprotein polypeptide N (*SNRPN*) gene. The *SNRPN*-gene is a paternally expressed imprinted gene that is located on chromosome 15q11–13, a region involved in Prader–Willi and Angelman syndromes (AS). The IC-region of the *SNRPN*-gene counts 23 CpG-sites that are methylated on the maternal chromosome and unmethylated on the paternal

*To whom correspondence should be addressed. Tel: +32 24776073; Fax: +32 24776860; E-mail: lgendrem@az.vub.ac.be

chromosome (6). In mice, it was reported that the methylation patterns of the *Snrpn*-gene are established in growing oocytes that are arrested in the diplotene stage of meiosis (7). Today safety questions have been raised when using assisted reproductive technologies. Embryo manipulation, *in vitro* maturation of gametes, *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) may disturb the process of genomic imprinting and lead to abnormal imprinting and related diseases (8–13). A clear view on the timing of imprint re-establishment in oocytes and on how they are maintained in preimplantation embryos will help to estimate the safety of these techniques. The methylation status at the IC-region of the *SNRPN*-gene was analysed in human gametes and preimplantation embryos using the bisulphite sequencing technique. This technique is a highly sensitive approach to analyse every potential methylation site on a target sequence. The bisulphite sequencing technique was first adapted to the single-cell level because of the limited availability of human study material.

RESULTS

Single-cell PCR

The bisulphite sequencing protocol as previously described (14) was adapted to the single-cell level. A modified protocol consisting of a bisulphite treatment of single-cells embedded in low melting point (LMP) agarose beads followed by a single-cell PCR was developed. The human IC-region of the *SNRPN*-gene counts 23 CpG-sites (named A to W) that are methylated on the maternal allele and unmethylated on the paternal allele (Fig. 1A) (6). Our bisulphite sequencing protocol was validated on genomic DNA and an average methylation pattern of 48.27% (711/1473) was obtained after several independent experiments (Fig. 1B), indicating no bias towards methylated or unmethylated alleles. Our single-cell PCR allowed the determination of the methylation status of 20 of the 23 CpG-sites. The efficiency of amplification of the IC-region of the *SNRPN*-gene at the single-cell level, which was obtained with single blastomeres and oocytes, was 28.7% (58/202). This figure includes some single cells that were not analysed after PCR amplification. A lower efficiency was obtained with single sperm cells, which was probably due to a difficult cell lysis. Blanks were included in each bisulphite sequencing experiment. In total, four out of 146 blanks gave amplification after PCR, resulting in a contamination percentage of 2.7%.

Oocytes

When collecting the oocytes, special attention was paid to the complete removal of the zona pellucida (ZP) and attached cumulus cells. Ten to 15 clones were sequenced for each of the seven single GVs analysed and 99.8% (1736/1738) of the potential methylation sites were found to be methylated (Fig. 1C). A methylation pattern of 98% (676/690) was found in the three MI oocytes whereas for the three mature MII oocytes all of the sites were found to be methylated (580/580). The first polar bodies (PB) had been removed from two of the three MII oocytes. Bisulphite sequencing of single PB was not successful in our hands.

Sperm

To verify if indeed the spermatozoa had the opposite methylation status on the IC-region of the *SNRPN*-gene, single spermatozoa and pools of four and five spermatozoa were analysed. Of the five samples analysed, two samples were directly sequenced after PCR while the other three samples were first cloned prior to sequencing. All CpGs that could be analysed were unmethylated except one CpG-site in one clone of the sample with one single spermatozoon (Fig. 1D). These results clearly show that the spermatozoa had the opposite methylation pattern of the oocytes.

Embryos

The imprints that are set during gametogenesis in a sex-specific way, have to be maintained after fertilization. To study the maintenance of methylation during the preimplantation period, 30 human embryos conceived after ICSI (day 2–6) were analysed (Table 1) (15,16). Ten embryos were analysed as whole embryos, while 20 embryos were first dissociated into single blastomeres. The efficiency of amplification of the IC-region of the *SNRPN*-gene at the single-cell level which was obtained with single blastomeres and oocytes, was 28.7%. The calculation of the average methylation percentage in the embryos took into account that the contribution of each amplifying cell/embryo to the average result should be independent of the number of clones analysed. A factor F ($F = 1$ equals 20 CpG-sites) (column 8, Table 1) was introduced to recalculate the ratio of the methylated CpGs (column 6, Table 1) thereby taking into account the amplification efficiency of the PCR after bisulphite treatment as well as the number of cells in each embryo. For embryos analysed as single blastomeres, the F factor equals the number of blastomeres amplified from the embryo (column 4, Table 1). For whole embryo samples with more than three cells, the value of F equals the number of blastomeres of the embryo amplified with 0.287. For whole embryo samples with one to three cells, F was given the value of one as on average one cell is amplified in these embryos. In the 30 embryos analysed an average methylation pattern of 53% (460.6/869.4) was detected. Thirteen embryos had a mainly methylated pattern (79–100%) while 11 embryos showed a mainly unmethylated pattern (0–19%). In all the single blastomeres and in some embryos only one epi-allele with either a methylated or unmethylated pattern could be detected. In the embryos with an intermediate methylation pattern, a mixture of mainly methylated and unmethylated clones was found (Fig. 2).

DISCUSSION

In this study we adapted the bisulphite sequencing technique to the single-cell level to determine the methylation status of the IC-region of the *SNRPN*-gene in human oocytes, spermatozoa and preimplantation embryos in order to clarify the process of imprint establishment and estimate the risks of imprinting alterations after assisted reproductive technologies.

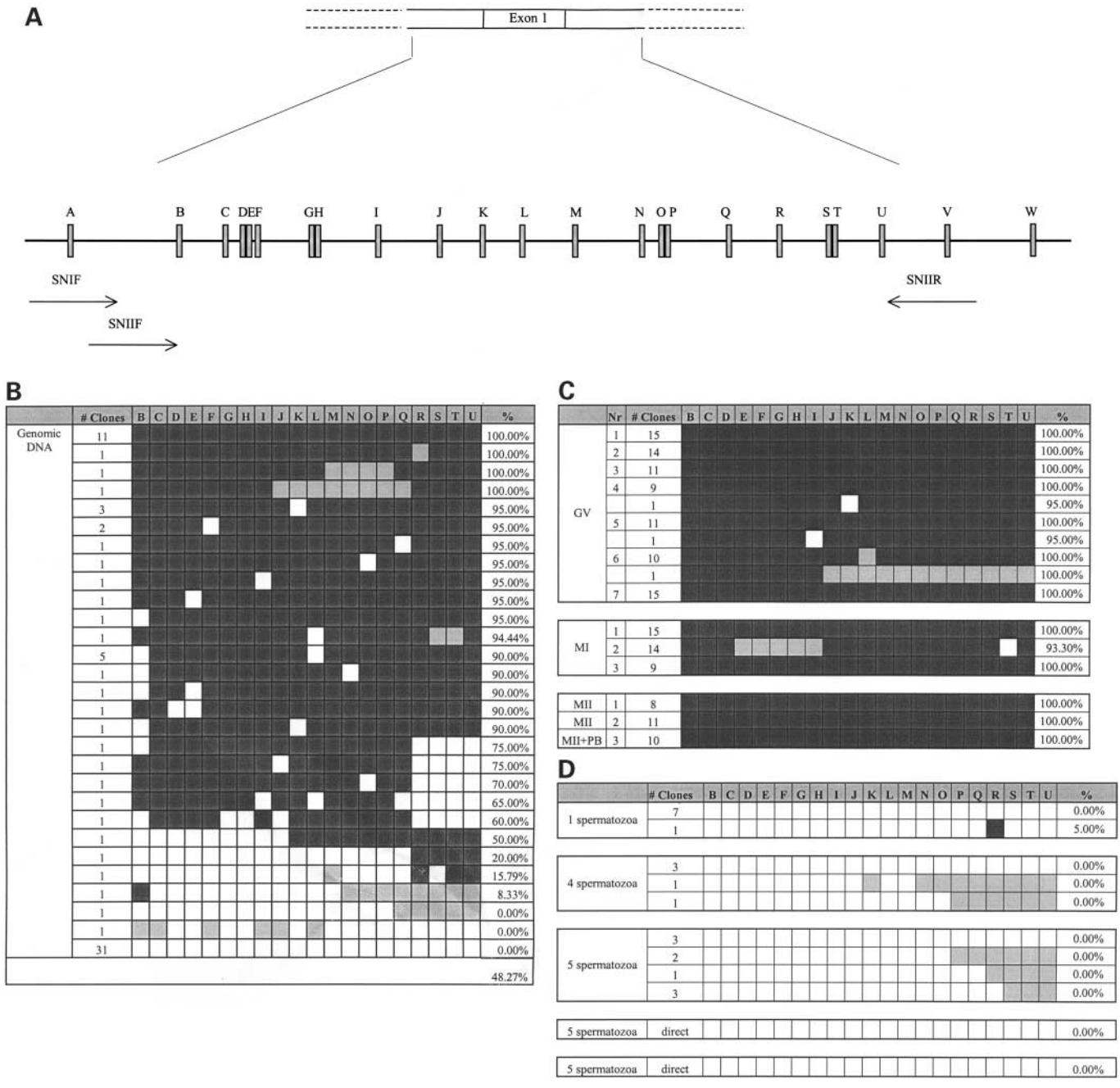

Figure 1. Methylation analysis of the IC-region of the *SNRPN*-gene. (**A**) Schematic representation of the analysed IC-region of the *SNRPN*-gene. The vertical bars (A–W) each represent a single CpG-site. The primers used in the hemi-nested single-cell PCR are shown. (**B**) Methylation profile of the human IC-region of the *SNRPN*-gene in genomic DNA. (**C**) Methylation profile of the human IC-region of the *SNRPN*-gene in germinal vesicles (GV), metaphase I (MI) oocytes, metaphase II (MII) oocytes without polar body (PB) and one MII oocyte with PB. (**D**) Methylation profile of the human IC-region of the *SNRPN*-gene in spermatozoa. Samples were directly sequenced (direct) after PCR or cloned prior to sequencing. The analysed CpG-dinucleotides are indicated (B–U). The total number of clones that were sequenced for each bisulphite treated sample is mentioned. For each sample, clones with similar methylation patterns have been grouped. Dark grey boxes represent methylated CpG-sites, white boxes are unmethylated CpG-sites and light grey boxes are sites that could not be analysed.

In several independent experiments, a mainly methylated pattern was found in all the GVs, MI and MII oocytes analysed. In the spermatozoa on the other hand, the same region showed a completely unmethylated pattern. Our experiments further indicate that these differential methylated patterns in the gametes were stably maintained in the preimplantation embryos.

Very little is known about the mechanisms involved in the cycle of imprint erasure, resetting and maintenance. Most of the currently available studies have been performed in mice. Several groups have analysed the methylation patterns of imprinted genes in mouse germ cells in order to elucidate the exact timing of imprint re-establishment. For *H19* it was

Table 1. Methylation analysis of 30 ICSI embryos

Embryo	No. of cells	Whole embryo	Single blastomeres (no. of blastomeres analysed)	No. of clones	No. of CpGs methylated/no. of CpGs analysed	%Methylated	Factor (F)	No. of CpGs methylated ($=20\times F\times$ %methylated)	Total no. of CpGs analysed ($=20\times$ F)
1	4c	X		6	0/120	0.0%	1.15	0.0	23.0
2	1c	X		1	3/20	15.0%	1.00	3.0	20.0
3	3c	X		8	143/160	89.4%	1.00	17.9	20.0
4	7c	X		12	157/229	68.6%	2.00	27.4	40.0
5	9c	X		11	88/217	40.6%	2.58	20.9	51.6
6	3c		X (1)	4	80/80	100.0%	1.00	20.0	20.0
7	8c		X (2)	7	129/140	92.1%	2.00	36.8	40.0
8	4c		X (1)	4	0/67	0.0%	1.00	0.0	20.0
9	2c		X (1)	direct	0/20	0.0%	1.00	0.0	20.0
10	5c		X (1)	direct	20/20	100.0%	1.00	20.0	20.0
11	6c		X (1)	direct	2/20	10.0%	1.00	2.0	20.0
12	6c		X (1)	direct	0/20	0.0%	1.00	0.0	20.0
13	3c		X (1)	direct	1/20	5.0%	1.00	1.0	20.0
14	5c		X (2)	direct	20/40	50.0%	2.00	20.0	40.0
15	7c		X (1)	direct	0/20	0.0%	1.00	0.0	20.0
16	3c		X (1)	direct	20/20	100.0%	1.00	20.0	20.0
17	15c		X (5)	direct	19/100	19.0%	5.00	19.0	100.0
18	7c		X (2)	direct	40/40	100.0%	2.00	40.0	40.0
19	4c	X		10	0/153	0.0%	1.15	0.0	23.0
20	2c	X		10	185/197	93.9%	1.00	18.8	20.0
21	4c		X (1)	direct	19/20	95.0%	1.00	19.0	20.0
22	8c		X (2)	direct	19/36	52.8%	2.00	21.1	40.0
23	5c	X		12	0/236	0.0%	1.44	0.0	28.8
24	4c	X		12	240/240	100.0%	1.15	23.0	23.0
25	4c	X		12	190/239	79.5%	1.15	18.3	23.0
26	7c		X (2)	direct	20/40	50.0%	2.00	20.0	40.0
27	6c		X (2)	direct	40/40	100.0%	2.00	40.0	40.0
28	5c		X (1)	direct	19/20	95.0%	1.00	19.0	20.0
29	4c		X (1)	direct	20/20	100.0%	1.00	20.0	20.0
30	7c		X (2)	direct	10/30	33.3%	2.00	13.3	40.0
								460.6	869.4
								53.0%	

Calculation of the average methylation percentage of 30 ICSI embryos at the IC-region of the SNRPN-gene. Whole embryos or single blastomeres were analysed. Samples were directly sequenced (direct) after PCR or cloned prior to sequencing (number of clones are mentioned). Sequencing data are represented by the ratio of number of CpGs methylated/number of CpGs analysed. Corrections were made in the calculation of the average methylation percentage so that the contribution of each amplified blastomere/embryo is independent of the number of clones analysed. A factor F was introduced taking into account the amplification efficiency after PCR and the number of cells in each embryo.

demonstrated that the methylation imprints on the paternal alleles appear during the fetal stages whereas methylation of the maternal alleles in the mouse male germ cells started later during perinatal stages (17,18). In case of the human *H19* gene, it was shown that the methylation patterns first appear in adult spermatogonia (19). Several reports in mice, based on expression studies (20,21) and on methylation studies (7,22), established the time of maternal imprint re-establishment at the stage of oocyte growth while there is only one report on this issue in humans (23). The data of this report indicate that the maternal methylation imprints of the *SNRPN*-gene are established during or after fertilization. This is in contrast with our findings of complete methylation of the IC-region of the *SNRPN*-gene in all the GVs, MI and MII oocytes analysed. This may be due to technical differences. The bisulphite genomic sequencing method is a powerful technique allowing analysis of every potential methylation site within the region under study. However, the technique is clearly affected by the quality and the quantity of the starting material (24–26). The purity of the cells is an important parameter. Most methylation studies are carried out on pools of oocytes with an intact ZP and therefore contamination with DNA from cumulus cells cannot be excluded. In our experiments, special attention was paid to the complete removal of follicular cells and the ZP. Bisulphite treatment of oocytes carefully washed and denuded of cumulus cells but retaining an intact, but thinned, zona, gave methylated and unmethylated clones in our hands (data not shown). These differentially methylated patterns were probably derived from adhering (lysed) cumulus cells on the ZP. Contamination of oocytes has also been observed in our laboratory when performing single-cell PCR on polar bodies. If oocyte pools were contaminated with diploid cumulus cells, the question remains how to explain the findings of only unmethylated patterns? This could be due to stochastic amplification and/or preferential amplification. It was demonstrated that multiple PCRs are necessary to ensure accurate methylation analysis especially when the DNA input is limited (25). Bisulphite conversion yields T-rich (unmethylated) and C-rich (methylated) molecules. One type of molecules may amplify preferentially. Such a PCR bias may result in an inaccurate estimate of methylation. Our bisulphite sequencing protocol was validated on genomic DNA and an average

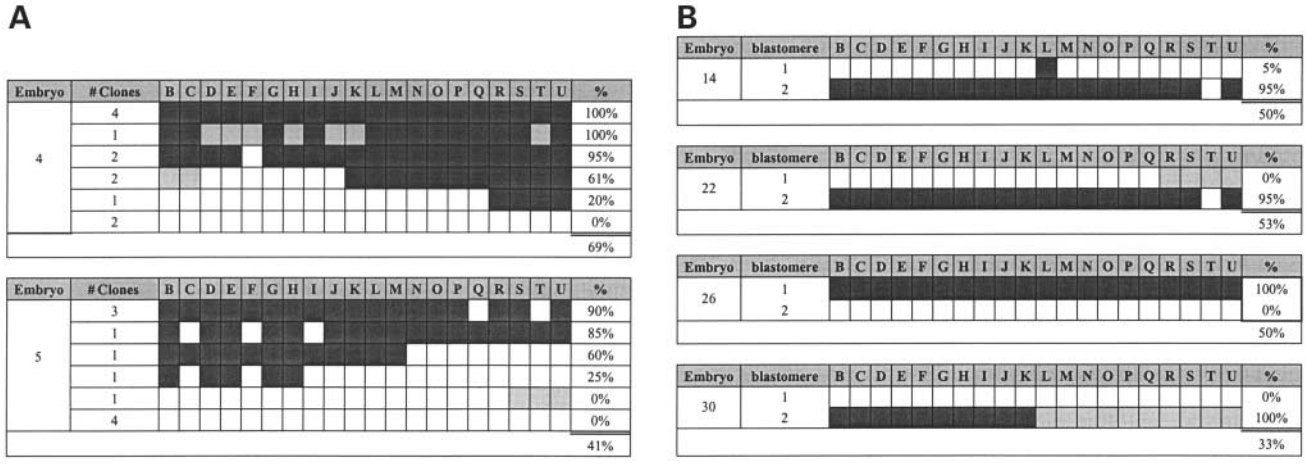

Figure 2. Methylation analysis of the IC-region of the *SNRPN*-gene in preimplantation embryos with an intermediate methylation pattern. (**A**) Embryos analysed as whole embryos. The number of clones analysed with a given methylation pattern is indicated. (**B**) Embryos analysed as single blastomeres. The methylation pattern of each blastomere is shown after direct sequencing. The analysed CpG-dinucleotides are indicated (B–U). Dark grey boxes represent methylated CpG-sites, white boxes are unmethylated CpG-sites and light grey boxes are sites that could not be analysed.

methylation pattern of 48.27% was obtained after several independent experiments, indicating no bias towards methylated or unmethylated alleles. Our data on human oocytes, sperm cells and preimplantation embryos have also been gathered from multiple PCRs. Only one epi-allele, either methylated or unmethylated, has been detected in some embryos and single blastomeres. This reflects the absence of a PCR bias in our experiments on one hand, and highlights the need for repeated experiments to circumvent stochastic amplification on the other hand. In other words, the methylation analysis will be more accurate when relying on several independent PCRs of single (diploid) cells than when relying on one PCR of a small number of cells as stochastic amplification also occurs in this type of PCR.

Our results indicate that the maternal imprints for the human IC-region of the *SNRPN*-gene are already re-established in the GV oocyte stage. These results correspond to the data reported in mice. It was reported that in mice mid-size growing oocytes had a mosaic pattern of allelic methylation at the *Snrpn*-gene and full acquisition of the methylation imprints was completed by the MII stage (7). Based on expression studies in mice, it was shown that the imprint signals appeared throughout a period from primary oocytes to antral follicle stage oocytes with a specific time window for each imprinted gene (21). The mouse *Snrpn*-gene is imprinted in the primordial to primary follicle stage concurrently with *Znf 127* and *Ndn*, which are controlled by the same imprint control element. It remains to be elucidated at which specific time the imprints appear in human oocytes. Similar to mice, it is possible that each imprinted gene or cluster of imprinted genes has its own time window for setting the methylation imprints.

For the spermatozoa, a methylation pattern opposite to the oocytes was found. These results are similar with those found in mice.

The imprints that are erased in the PGC and re-established during gametogenesis are then stably maintained in the preimplantation period. This period is characterized in mice by global waves of demethylation and methylation (27–30). In the human embryos of 2–6 days old that were analysed, an average methylation pattern of 53% was seen. A mainly methylated (>79% methylation) pattern was found in 13 preimplantation embryos while a mainly unmethylated (<19%) pattern was found in 11 embryos. Intermediate methylation patterns (33–69%) were obtained in six embryos. These patterns were derived from a mixture of mainly methylated and mainly unmethylated epi-alleles and not from semimethylated alleles (Fig. 2). For four embryos, one blastomere with a methylated epi-allele and one blastomere with an unmethylated epi-allele was detected. In the two embryos analysed as whole embryos, six epi-alleles representing both methylated and unmethylated alleles were detected. The results of an average methylation pattern of 53% together with the finding that the majority of the clones had a mainly methylated or mainly unmethylated methylation pattern, suggest that the differential methylation patterns acquired in the gametes are maintained in the preimplantation embryos. Moreover, the average methylation pattern of the embryos is very similar to the average methylation pattern of the IC-region in somatic cells. Our results are in agreement with another molecular study of the IC-region of the *SNRPN*-gene that reported no aberration of the global methylation patterns in 83 children born after ICSI (31). An expression study reported that *SNRPN*, *PEG1* and *UBE3A* are expressed in human oocytes and preimplantation embryos (32). *In vitro* culture systems and embryo manipulations cause imprinting defects in animal models (9,33). It was demonstrated in sheep that large offspring syndrome (LOS) after *in vitro* culture was associated with reduced levels of fetal methylation and expression of *IGF2R*. So far, LOS has not been reported in humans as a result of assisted reproduction. On the contrary, neonatal data have indicated a lower birthweight in the IVF and ICSI singletons as compared with naturally conceived children (34). In humans, there is less evidence that embryo manipulation and culture may influence the imprinting mechanism, although recent reports suggest that assisted reproductive technologies might cause imprinting disorders. Two children conceived by ICSI were reported with AS (10), whereas an association between assisted reproductive

technologies (IVF and ICSI) and Beckwith–Wiedeman syndrome was found (13). Molecular studies performed in both reports showed sporadic imprint defects at the maternal allele. It has been suggested that *in vitro* embryo culture which is a common element of IVF and ICSI procedures might interfere with the maintenance of maternal methylation patterns at imprinted loci. Further molecular studies are required in order to better understand the process of genomic imprinting and assess the risks linked to assisted reproductive techniques.

MATERIALS AND METHODS

Samples

The human embryos were research embryos from our ICSI programme and were unsuitable for freezing or transfer because of fragmentation or arrested cleavage. The embryos were donated by the patients after they gave informed consent and with approval of the institutional ethical committee. All the embryos used were obtained after ICSI with ejaculated sperm. The oocytes used were immature oocytes from our IVF/ICSI programme (GVs and MI oocytes) or were *in vitro* matured (MII oocytes). The oocytes were denuded from cumulus cells using a combination of enzymatic (40 IU/ml hyaluronidase; Sigma Chemical Company, St Louis, MO, USA) and mechanical (pipetting) methods (35). The ZP was removed from the oocytes and embryos using acidic Tyrode's solution (14 mM NaCl, 0.2 mM KCl, 0.2 mM $CaCl_2 \cdot 2H_2O$, 0.05 mM $MgCl_2 \cdot 6H_2O$, 5.5 mM glucose) adjusted to pH 2.4 with HCl. Special attention was paid to the complete removal of the ZP and follicular cells, which was harder for oocytes than for cleavage stage embryos. Single oocytes and embryos were transferred to droplets of Ca^{2+}- and Mg^{2+}-free medium [14 mM NaCl, 0.2 mM KCl, 0.04 mM $NaH_2PO_4 \cdot H_2O$, 5.5 mM glucose, 1.2 mM $NaHCO_3$, 0.02 mM EDTA, 0.01% (w/v) phenol red] supplemented with 4 mg/ml bovine serum albumine (Sigma Aldrich, Bornem, Belgium) for prevention of cell adhesion using a mouth-controlled pipette. The embryos and oocytes were washed three times in this medium. Some embryos were first dissociated into single blastomeres using trypsin (1 mg/ml, Sigma Aldrich) in the medium prior to the washing steps. Single-cells were transferred to a 1.5 ml tube containing 2 µl alkaline lysis buffer (ALB; 50 mM DTT, 200 mM KOH) and stored at −80°C until use. An aliquot from the last washing droplet was taken to serve as a blank. Semen samples were obtained from the male partners of couples of the fertility centre. Ejaculated sperm samples with normal semen parameters (36) were purified by Percoll treatment during 20 min at 300*g* in two layers of 45 and 90%. The single sperm cells were picked up in droplets of 10% polyvinylpyrolidone (MW 40,000) (PVP; Sigma Aldrich) using a micromanipulator (Nikon Narishige, Tokyo, Japan) at 400× magnification. The sperm cells (one single spermatozoon or pools of four or five spermatozoa) were washed three times in PVP and transferred to a 0.2 ml PCR tube containing 2.5 µl ALB and stored at −80°C until use.

Bisulphite treatment

The bisulphite treatment was performed according to a published protocol (14) with specific adaptations for the single-cell level. After removal from the −80°C ultrafreezer, oocytes and embryos were incubated at 80°C for 10 min in a warm water bath, while the spermatozoa were incubated for 15 min. After lysis, one volume of neutralization buffer (0.9 M Tris–HCl pH 8.3, 0.3 M KCl, 0.2 M HCl) and 1 µl rRNA (0.8 µg/µl) (Sigma Aldrich) as carrier were added. At this point the samples containing spermatozoa were transferred to 1.5 ml tubes. The samples were then embedded in LMP agarose (Sigma Aldrich) at a final concentration of 1.6% by adding 7 µl melted LMP agarose at 80°C. Bead formation was induced by overlaying the samples with 100 µl cold mineral oil. DNA was denatured by incubating the bead samples for 15 min at 80°C. Subsequently, tubes were put on ice to re-solidify the beads and 100 µl of sodium bisulphite solution [2.5 M sodium metabisulphite (Sigma Aldrich), 125 mM hydroquinone (Sigma Aldrich), 266 mM NaOH] at pH 5 was added. The samples were incubated at 50°C for 4 h. Mineral oil and bisulphite were then removed and the beads were equilibrated against TE (10 mM Tris/1 mM EDTA) pH 8 (4×10 min) followed by desulfonation in 500 µl 0.2 M NaOH (2×10 min) and neutralization with 100 µl 1 M HCL (1×10 min). Finally, the beads were washed in 1 ml TE (1×10 min) and 1 ml H_2O (2×10 min). The beads were immediately used in a PCR reaction or kept at 4°C until use.

PCR

A hemi-nested single-cell PCR was developed to amplify the bisulphite converted DNA using the forward primer (Eurogentec, Seraing, Belgium) SNIF (5′-TTAGGTTATTTYGGTGAGGGAGGG-3′) (nucleotide position 15489–15515 in GenBank accession number U41384) labelled with 5′ indocarbocyanine (Cy5) and the reversed primer SNIIR (5′-ACCACCRACACT$_x$AG$_y$TAACCTTACC-3′) (15280–15304) in the first round. Both primers contain a wobble (Y and R) on a potential methylation site. In the second PCR round, the Cy5-labelled SNIIF primer (5′-AGGGAA$_x$TTC$_y$GGATTTTTGTATTG-3′) (15473–15495) together with SNIIR was used. Mismatches were introduced at positions *x* and *y* in SNIIF and SNIIR to generate a restriction site that could be used in a possible later cloning step. A PCR reaction mix containing 0.4 µM of each primer, 0.2 mM dNTPs (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), 1× PCR buffer (Applied Biosystems, Nieuwerkerk a/d IJsel, The Netherlands), 2 mM $MgCl_2$ (Applied Biosystems), 1.25 U AmpliTaq DNA Polymerase (Applied Biosystems) in a total volume of 25 µl was used. The reaction mix of the first round was decontaminated with restriction enzyme *Mnl* I (New England Biolabs, Leusden, The Netherlands) for 3 h at 37°C followed by an inactivation of the enzyme by heating at 65°C for 20 min. The following PCR programme was used: 5 min denaturation at 96°C followed by 22 cycles of 30 s at 96°C, 30 s at 61°C and 30 s at 72°C and a final extension for 5 min at 72°C. Three microlitres of the first round was used as DNA input for amplification in the second round with the following programme: 5 min denaturation at 94°C followed by 38 cycles of 30 s at 96°C, 30 s at 52°C and 30 s at 72°C and a final extension for 5 min at 72°C. The PCR fragments were analysed on an ALFExpress automated sequencer (Amersham Pharmacia Biotech). Positive samples were directly sequenced or first cloned and then sequenced.

Cloning and sequencing

The PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Merelbeke, Belgium). Purified clones were automatically sequenced on the ABI 310 (Applied Biosystems).

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REFERENCES

1. McGrath, J. and Solter, D. (1984) Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell*, **37**, 179–183.
2. Surani, M.A.H., Barton, S.C. and Norris, M.L. (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature*, **308**, 548–550.
3. Azim, M. and Surani, M.A.H. (1984) Differential roles of paternal and maternal genomes during embryogenesis in the mouse. *Bioessays*, **1**, 224–227.
4. Lee, J., Inoue, K., Ono, R., Ogonuki, N., Kohda, T., Kaneko-Ishino, T., Ogura, A. and Ishino, F. (2002) Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. *Development*, **129**, 1807–1817.
5. Hajkova, P., Erhardt, S., Lane, N., Haaf, T., El-Maarri, O., Reik, W., Walter, J. and Surani, M.A. (2002) Epigenetic reprogramming in mouse primordial germ cells. *Mech. Dev.*, **117**, 15–23.
6. Zeschnigk, M., Schmitz, B., Dittrich, B., Buiting, K., Horsthemke, B. and Doerfler, W. (1997) Imprinted segments in the human genome: different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. *Hum. Mol. Genet.*, **6**, 387–395.
7. Lucefero, D., Mertineit, C., Clarke, H.J., Bestor, T.H. and Trasler, J.M. (2002) Methylation dynamics of imprinted genes in mouse germ cells. *Genomics*, **79**, 530–538.
8. Tesarik, J. and Mendoza, C. (1996) Genomic imprinting abnormalities: a new potential risk of assisted reproduction. *Mol. Hum. Reprod.*, **5**, 295–298.
9. Young, L.E. and Fairburn, H.R. (1999) Improving the safety of embryo technologies: possible role of genomic imprinting. *Theriogenology*, **53**, 627–648.
10. Cox, G.F., Bürger, J., Lip, V., Mau, U.A., Sperling, K., Wu, B.L. and Horsthemke, B. Intracytoplasmic sperm injection may increase the risk of imprinting defects. *Am. J. Hum. Genet.*, **71**, 162–164.
11. De Rycke, M., Liebaers, I. and Van Steirteghem, A. (2002) Epigenetic risks related to assisted reproductive technologies—risk analysis and epigenetic inheritance. *Hum. Reprod.*, **17**, 2487–2494.
12. Thompson, J.G., Kind, K.L., Roberts, C.T., Robertson, S.A. and Robinson, J.S. (2002) Epigenetic risks related to assisted reproductive technologies—short- and long-term consequences for the health of children conceived through assisted reproduction technology: more reason for caution? *Hum. Reprod.*, **17**, 2783–2786.
13. DeBaun, M.R., Niemitz, E.L. and Feinberg, A.P. (2003) Association of *in vitro* fertilization with Beckwith–Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am. J. Hum. Genet.*, **72**, 156–160.
14. Olek, A., Oswald, J. and Walter, J. (1996) A modified and improved method for bisulphite based cytosine methylation analysis. *Nucl. Acids Res.*, **24**, 5064–5066.
15. Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A.C. (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*, **340**, 17–18.
16. Van Steirteghem, A.C., Nagy, Z., Joris, H., Liu, J., Staessen, C., Smitz, J., Wisanto, A. and Devroey, P. (1993) High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum. Reprod.*, **8**, 1061–1066.
17. Davis, T.L., Trasler, J.M., Moss, S.B., Yang, G.L. and Bartolomei, M.S. (1999) Acquisition of the H19 methylation imprint occurs differentially on the parental alleles during spermatogenesis. *Genomics*, **58**, 18–28.
18. Davis, T.L., Yang, G.J., McCarrey, J.R. and Bartolomei, M.S. (2000) The H19 methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development. *Hum. Mol. Genet.*, **9**, 2885–2894.
19. Kerjean, A., Dupont, J.M., Vasseur, C., Le Tessier, D., Cuisset, L., Paldi, A., Jouannet, P. and Jeanpierre, M. (2000) Establishment of the paternal methylation imprints of the human H19 and MEST/PEG1 genes during spermatogenesis. *Hum. Mol. Genet.*, **9**, 2183–2187.
20. Obata, Y., Kaneko-Ishino, T., Koide, T., Takai, Y., Ueda, T., Domeki, I., Shiroishi, T., Ishino, F. and Kono, T. (1998) Disruption of primary imprinting during oocyte growth leads to the modified expression of imprinted genes during embryogenesis. *Development*, **125**, 1553–1560.
21. Obata, Y. and Kono, T. (2002) Maternal primary imprinting is established at a specific time for each gene throughout oocyte growth. *J. Biol. Chem.*, **277**, 5285–5289.
22. Kono, T., Obata, Y., Yoshimzu, T., Nakahara, T. and Carroll, J. (1996) Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse. *Nat. Genet.*, **13**, 91–94.
23. El-Maarri, O., Builting, K., Peery, E.G., Kroisel, M.P., Balaban, B., Wagner, K., Urman, B., Heyd, J., Lich, C., Brannan, C.I. *et al.* (2001) Maternal methylation imprints in human chromosome 15 are established during or after fertilization. *Nat. Genet.*, **27**, 341–344.
24. Warnecke, P.M., Stirzaker, C., Melki, J.R., Millar, D.S., Paul, C.L. and Clark, S.J. (1997) Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA. *Nucl. Acids Res.*, **25**, 4422–4426.
25. Warnecke, P.M., Mann, J.R., Frommer, M. and Clark, S.J. (1998) Bisulfite sequencing in preimplantation embryos: DNA methylation profile of the upstream region of the mouse imprinted H19 gene. *Genomics*, **51**, 182–190.
26. Kerjean, A., Vieillefond, A., Thiounn, N., Sibony, M., Jeanpierre, M. and Jouannet, P. (2001) Bisulfite genomic sequencing of microdissected cells. *Nucl. Acids Res.*, **29**, E106–106.
27. Mayer, W., Niveleau, A., Walter, J., Fundele, R. and Haaf, T. (2000) Demethylation of the zygotic paternal genome. *Nature*, **403**, 501–502.
28. Reik, W., Dean, W. and Walter J. (2001) Epigenetic reprogramming in mammalian development. *Science*, **293**, 1089–1093.
29. Oswald, J., Engemann, S., Lane, N., Mayer, W., Olek, A., Fundele, R., Dean, W., Reik, W. and Walter, J. (2002) Active demethylation of the paternal genome in the mouse zygote. *Curr. Biol.*, **10**, 475–478.
30. Santos, F., Hendrich, B., Reik, W. and Dean, W. (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev. Biol.*, **241**, 172–182.
31. Manning, M., Lissens, W., Bonduelle, M., Camus, M., De Rycke, M., Liebaers, I. and Van Steirteghem, A. (2000) Study of DNA-methylation patterns at chromosome 15q11–q13 in children born after ICSI reveals no imprinting defects. *Mol. Hum. Reprod.*, **6**, 1049–1053.
32. Salpekar, A., Huntriss, J., Bolton, V. and Monk, M. (2001) The use of amplified cDNA to investigate the expression of seven imprinted genes in human oocytes and preimplantation embryos. *Mol. Hum. Reprod.*, **7**, 839–844.
33. Young, L.E., Fernandes, K., McEvoy, T.G., Butterwith, S.C., Gutierrez, C.G., Carolan, C., Broadbent, P.J., Robinson, J.J., Wilmut, I. and Sinclair, K.D. (2001) Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat. Genet.*, **27**, 153–154.
34. Bonduelle, M., Liebaers, I., Deketelaere, V., Derde, M.P., Camus, M., Devroey, P. and Van Steirteghem, A. (2002) Neonatal data on a cohort of 2889 infants born after ICSI (1991–1999) and of 2995 infants born after IVF (1983–1999). *Hum. Reprod.*, **17**, 671–694.
35. Van de Velde, H., Nagy, Z.P., Joris, H., De Vos, A. and Van Steirteghem, A.C. (1997) Effects of different hyaluronidase concentrations and mechanical procedures for cumulus cell removal on the outcome of intracytoplasmic sperm injection. *Hum. Reprod.*, **12**, 2246–2250.
36. WHO (1999) *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction*, 4th edn. Cambridge University Press, Cambridge, UK.