Dynamic regulation of DNA methyltransferases in human oocytes and preimplantation embryos after assisted reproductive technologies

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Submitted on October 2, 2013; resubmitted on June 16, 2014; accepted on June 27, 2014

ABSTRACT: DNA methylation is a key epigenetic modification which is essential for normal embryonic development. Major epigenetic reprogramming takes place during gametogenesis and in the early embryo; the complex DNA methylation patterns are established and maintained by DNA methyltransferases (DNMTs). However, the influence of assisted reproductive technologies (ART) on DNA methylation reprogramming enzymes has predominantly been studied in mice and less so in human oocytes and embryos. The expression and localization patterns of the four known DNMTs were analysed in human oocytes and IVF/ICSI embryos by immunocytochemistry and compared between a reference group of good quality fresh embryos and groups of abnormally developing embryos or embryo groups after cryopreservation. In humans, DNMT1o rather than DNMT1s seems to be the key player for maintaining methylation in early embryos. DNMT3b, rather than DNMT3a and DNMT3L, appears to ensure global DNA remethylation in the blastocysts before implantation. DNMT3L, an important regulator of maternal imprint methylation in mouse, was not detected in human oocytes (GV, MI and MII stage). Our study confirms the existence of species differences for mammalian DNA methylation enzymes. In poor quality fresh embryos, the switch towards nuclear DNMT3b expression was delayed and nuclear DNMT1, DNMT1s and DNMT3b expression was less common. Compared with the reference embryos, a smaller number of cryopreserved embryos showed nuclear DNMT1, while a delayed switch to nuclear DNMT3b and an extended DNMT1s temporal expression pattern were also observed. The spatial and temporal expression patterns of DNMTs seem to be disturbed in abnormally developing embryos and in embryos that have been cryopreserved. Further research must be performed in order to understand whether the potentially disturbed embryonic DNMT expression after cryopreservation has any long-term developmental consequences.

Key words: epigenetics / DNA methylation / DNA methyltransferases / human preimplantation embryo development / cryopreservation

Introduction

DNA methylation at cytosine residues of CpG dinucleotides is a major epigenetic modification involved in the regulation of gene expression. It is well established that mammalian DNA methylation is essential for embryonic development, genome stability, X chromosome inactivation and genomic imprinting (Robertson and Jones, 2000; Paulsen and Ferguson-Smith, 2001). In the mouse, the global DNA methylation patterns are dynamically reprogrammed during gametogenesis and early embryogenesis (Reik, 2007). The era of DNA methylation patterns in the primordial germ cells migrating to the genital ridges during gametogenesis is later on followed by genome-wide de novo methylation and establishment of allele-specific marks at imprinted loci. After fertilization, the paternal genomic DNA is actively demethylated before replication starts, while the maternal genomic DNA is passively demethylated during every cell cleavage between the 2-cell and morula stage. Methylation levels in the murine preimplantation embryo are typically at their lowest by the morula stage, and as the blastocyst is formed; they are restored after implantation (Dean and Ferguson-Smith, 2001; Santos et al., 2002, Seisenberger et al., 2013). This global epigenetic reprogramming allows the nucleus to take on the task of supporting embryonic growth and differentiation. The allele-specific methylation patterns of imprinted genes, inherited from the gametes, are resistant to the reprogramming process in the early embryo (Reik, 2007).

Eukaryotic DNA methylation enzymes have been considerably conserved (Jurkowski and Jeltsch, 2011). Four major DNA methyltransferase enzymes (DNMTs) have been described: the maintenance DNMT protein, Dnmt1, two de novo DNMTs, Dnmt3a and Dnmt3b, and their regulator, Dnmt3L.
It has been shown in the mouse that Dnmt1 is responsible for the maintenance of the methylation patterns during replication (Branco et al., 2008; Kurhara et al., 2008). As Dnmt1 is mainly excluded from the nucleus during preimplantation development, the passive DNA demethylation is presumably due to the lack of maintenance methylation activity (Howell et al., 2001). Two isoforms of Dnmt1 have been identified in the mouse (Carlson et al., 1992) and homologues have been identified in humans (Hayward et al., 2003): an oocyte-specific isoform (DNMT1o) found only in oocytes and cleavage stage preimplantation embryos and a somatic form (DNMT1s). Dnmt1o is distinct from Dnmt1s in that it uses the oocyte-specific S′ exon, resulting in the formation of an N-terminally truncated protein.

Two DNA methyltransferases, Dnmt3a and Dnmt3b, contribute to the creation of de novo DNA methylation patterns in embryos and germ cells. When murine Dnmt3a or Dnmt3b is inactivated, residual Dnmt3b or Dnmt3a, respectively, partly compensates for the lack of the gene (Okano et al., 1999). This suggests that Dnmt3a and Dnmt3b possess similar functional specificities. On the other hand, they show distinct sequence preferences and deficiencies of either protein result in a different phenotype. The de novo DNMTs act in close relationship with the Dnmt3-like protein Dnmt3L. Dnmt3L is essential for maternal imprint establishment and setting of DNA methylation patterns at retrotransposons in male germ cells (Bourc’his and Bestor, 2004), but does not possess enzymatic activity. Rather, in vitro analysis indicates that Dnmt3L stimulates DNA methylation through direct binding to either Dnmt3a or Dnmt3b and potentiating their activity (Nimura et al., 2006).

It has been suggested that procedures associated with assisted reproductive technologies (ARTs) may affect the epigenetic make-up of gametes and preimplantation embryos (Le Bouc et al., 2010; El Haj and Haf, 2013). ART bypasses a number of biological selection filters and exposes gametes and preimplantation embryos to hormonal stimulation, culture media and physical stress.

Previous evidence that ART can influence the epigenetic make-up of embryos has been found in animal studies (Young, 2001; Hou et al., 2007). In the mouse, a good correlation was found between aberrant genome-wide DNA methylation patterns and abnormal embryonic development and preimplantation embryonic loss (Shi and Haf, 2002). In humans, several papers have reported on the association of ART and imprinting disorders (Cox et al., 2002; Maher et al., 2003; Halliday et al., 2004; Ludwig et al., 2005) and errors in DNMT1o trafficking have been proposed to underlie the mosaic DNA hypomethylation at multiple imprinted loci (Cirio et al., 2008; Bliek et al., 2009; Lim et al., 2009). Reprogramming of DNA methylation during development has also been suggested as an important mechanism of ‘fetal origin’ diseases (Tarry-Adkins and Ozanne, 2011).

Although epigenetic reprogramming has been studied extensively in mice, few data are available about this process in human embryos due to legal and ethical restrictions, and because of limitations on the quality and quantity of human oocytes and embryos available for research. As DNA methylation dynamics are not well conserved in mammals (Ikeda et al., 2010; Ma et al., 2012), the primary goal of this study was to investigate the spatial and temporal expression of DNMTs in individual fresh human oocytes and preimplantation embryos of good quality. Secondary, we evaluated the influence of embryo cryopreservation and abnormal development on DNMT expression and localization patterns. The DNMTs represent only a subset of proteins and factors involved in DNA methylation reprogramming. Still, the study represents a step towards a better understanding of early human embryonic processes and the establishment of epigenotypes.

Materials and Methods

Ethics statement

Spare human oocytes and preimplantation embryos were donated for research after informed consent of patients treated at our Centre for Reproductive Medicine and with the approval of the Institutional Ethical Committee and the Federal Ethical Committee for Scientific Research on Human Embryos In Vitro.

Human oocytes and preimplantation embryo collection

Human research oocytes (n = 159) and preimplantation embryos (n = 697), fresh and cryopreserved, were collected over several years. Some stages of embryos are more difficult to acquire and therefore the number of embryos in the different groups is heterogeneous.

Oocytes used were immature oocytes at the germinal vesicle (GV) stage (n = 47) or the metaphase I (MI) stage (n = 31) and oocytes at metaphase II (MII) (n = 18) stage fixed ~2 h after oocyte pick-up (Day 0), or in vitro matured overnight in Quinns Advantage Fertilization Medium (Sage, Passadena, USA) (Day 1) (n = 63) and fixed ~20 h after oocyte pick-up. Embryo scoring was done according to internal and international guidelines (Alpha Scientists, 2011). Fresh embryos included in this study (n = 689) were obtained from patient treatment cycles: MII oocytes that were inseminated using IVF or ICSI ~2 h after oocyte pick-up and that were normally fertilized (2PN) were cultured in vitro in sequential medium (Quinns Advantage Protein Plus Medium, Sage, USA); embryos became available for research because they were affected after preimplantation genetic diagnosis (PGD) or did not fulfil the stringent criteria for transfer or cryopreservation on Day 3 or 5. These embryos became available for research between 66 and 70 h after fertilization (Day 3 embryos) or between 114 and 118 h after fertilization (Day 5 embryos). They were evaluated and fixed immediately or cultured for another 24 h (Day 4 and 6 embryos) or another 48 h (Day 7 embryos) before evaluation and fixation. Embryos on Days 1 and 2 of development were obtained by applying ICSI on MII oocytes donated for research (n = 8). These embryos were evaluated and fixed at 20 and 44 h after fertilization, respectively. Fresh cleavage stage embryos and blastocysts were classified into three groups according to their developmental timing and their morphological and nuclear quality: the first group of embryos were of good morphological quality with a correct developmental timing (A, good quality fresh embryo group or reference group). These embryos were scored as good quality and top quality embryos according to international guidelines (Alpha Scientists, 2011). The second group had a good morphology as well, but showed delayed embryonic development (B, delayed fresh embryo group); the third group consisted of poor quality embryos with an inferior morphology (fragmentation), developmental arrest or multinucleation (C, poor quality fresh embryo group) (Fig. 1a and Table I).

Frozen-thawed embryos (2PN) had been cryopreserved for the patients after IVF or ICSI on Day 3 using a slow-freezing DMSO protocol (Van den Abbeel et al., 1997); they became available for research after the legally determined period of 5 years. All cryopreserved embryos had a normal morphology and developmental timing until cryopreservation on Day 3. After thawing, they were cultured up to Day 7 in sequential medium (Quinns Advantage Protein Plus Medium, Sage, USA). They were classified into two groups and compared with the reference group of good quality fresh embryos (Fig. 1b and Table II): one group contained embryos with good morphology which developed normally beyond Day 3 (D, good quality
cryopreserved embryo group); the other group contained all embryos with abnormalities in morphology and developmental timing beyond Day 3 (E, delayed and poor quality cryopreserved embryo group).

A comparison with a true reference group of naturally conceived embryos is lacking for obvious reasons.

Antibodies

DNMT1 was detected with two antibodies directed against different epitopes of the DNMT1 proteins to distinguish both isoforms. Goat anti-human DNMT1 (o + s) (C-17: SC-10222 Santa Cruz Biotechnology, Boechout, Belgium) recognizes the C-terminus of both isoforms and mouse anti-human DNMT1s (M01: H00001786 Abnova, Tebu-bio) only recognizes the N-terminus of DNMT1s. The DNMT3 proteins were detected with rabbit anti-human DNMT3L (H-85: SC-20705, Santa Cruz Biotechnology), rabbit anti-human DNMT3a (SC-20703, Santa Cruz Biotechnology, recognizing both isoforms DNMT3a1 and DNMT3a2) and goat anti-human DNMT3b (SC-10235 Santa Cruz Biotechnology). Global DNA methylation was detected with mouse anti-5-methylcytosine (BI-MECY, Eurogentec, Seraing, Belgium). Negative controls were stained with normal goat IgG (SC-2028, Santa Cruz Biotechnology), normal mouse IgG (SC-2025, Santa Cruz Biotechnology) or normal rabbit IgG (SC-2027, Santa Cruz Biotechnology) depending on the host species of the primary antibody. Donkey anti-goat IgG whole Ab conjugated to Alexa Fluor 633 (A-21082, Invitrogen, Merelbeke, Belgium), goat anti-rabbit IgGF(Ab′)2 conjugated to Alexa Fluor 488 (A-21204, Invitrogen) and rabbit anti-mouse IgGF(Ab′)2 conjugated to Alexa Fluor 488 (A-21093, Invitrogen) were used as secondary antibodies.

**Figure 1** Schematic representation of the study design used for DNMT immunostainings of oocytes and preimplantation embryos and quantitative image analysis. (a) DNMT immunostainings of oocytes and preimplantation embryos. Fresh and cryopreserved embryos, classified according to their developmental timing and their morphological and nuclear quality were analysed at different developmental stages. Good quality refers to good morphology and correct developmental timing (reference group), delayed embryos show good morphology but delayed developmental timing while poor quality refers to abnormal morphology and aberrant developmental timing. (b) Quantitative image analysis. Fluorescent signals for DNMT3a, DNMT3b or 5-methylcytosine from fresh and cryopreserved embryos at Day 6, classified according to their developmental timing and their morphological and nuclear quality, were relatively quantified.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Number of embryos (and oocytes) used per group for DNMT immunostainings.</th>
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<tbody>
<tr>
<td></td>
<td>DNMT1</td>
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<tr>
<td>A</td>
<td>40 (25)</td>
</tr>
<tr>
<td>B</td>
<td>25 (5)</td>
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<tr>
<td>C</td>
<td>34</td>
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<tr>
<td>D</td>
<td>12</td>
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<tr>
<td>E</td>
<td>10</td>
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Indirect immunocytochemistry for DNA methyltransferases

Oocytes and embryos were washed in phosphate-buffered saline (PBS; Sigma-Aldrich, Diegem, Belgium) supplemented with 2% bovine serum albumin (BSA; Sigma-Aldrich), fixed with 3.7% formaldehyde (Merck; VWR International, Leuven, Belgium) in PBS for 10 min at room temperature and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 20 min at room temperature. Oocytes and embryos were then incubated with 2 μg/ml primary antibody (10 μg/ml secondary antibody conjugated with Alexa Fluor probes in PBS with 2% BSA for 2 h at 4°C overnight. This was done in single or double immunocytochemistry experiments, using the same concentration of antibody. Next, samples were incubated with 10 μg/ml secondary antibody conjugated with Alexa Fluor probes in PBS with 2% BSA for 2 h at 4°C in the dark. Between every step, samples were washed three times for 5 min in PBS with 2% BSA on a shaking plate at room temperature. For each group of embryos (A–E), at least two independent staining experiments were carried out.

Indirect immunocytochemistry for global DNA methylation

Embryos were washed and fixed as described above and permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 30 min at room temperature. Samples were then incubated with 6 M HCl (Merck) for 10 min at room temperature for DNA denaturation, before washing in PBS with 2% BSA for 20 min. Samples were then incubated with 3 μg/ml mouse anti-5-methylcytosine (methyltransferases in oocytes and preimplantation embryos) in PBS with 2% BSA at 4°C overnight. Next, samples were incubated with 10 μg/ml secondary antibody conjugated with Alexa Fluor probes in PBS with 2% BSA for 2 h at 4°C in the dark. Between every step, samples were washed three times for 5 min in PBS with 2% BSA on a shaking plate at room temperature.

Controls and confocal microscopy

Embryos of similar developmental stages were stained with normal serum of the same host species to serve as an internal negative control for the primary antibody. Control reactions for the secondary antibody were carried out by omitting the primary antibody. After staining, embryos were put between two glass coverslips (24 × 60 mm) in SlowFade Gold Antifade reagent with DAPI (Invitrogen). To prevent squeezing of the embryos, round glass cover slips (10 mm diameter) were used as spacers. Samples were visualized with a fluorescence camera (FviewII, Olympus, Aartselaar, Belgium) and confocal scanning microscopy with a UV laser (350 nm), Aron laser (488 nm) and HeNe laser (633 nm) (IX71 Fluoview 300; Olympus) was performed to record the fluorescent images.

The number of embryos (and oocytes) used per group for DNMT immunostainings is listed in Table I. Expression and localization were reported per embryo, taking into account the fluorescent immunostaining signals from the majority (>90%) of blastomeres within an embryo; a negative embryo means that no significant signals were detected in that embryo. When strong expression was observed mainly in cell nuclei with weak or no cytoplasmic signals, expression was considered nuclear. Embryos for which most cells showed a more abundant protein expression in the cytoplasm than in the nuclei were reported as showing cytoplasmic expression.

Quantitative image analysis

Relative quantitative analysis of a subset of fresh and cryopreserved embryos (Group A versus D and E) was carried out at a single day (Day 6) for DNMT3a/DNMT3b co-immunostaining and for immunostaining with an antibody to 5-methylcytosine. The number of embryos used per group is listed in Table II.

Fluorescent images obtained by confocal laser scanning were analysed with the Volocity imaging software (PerkinElmer, Inc.). A region of interest was drawn around embryonic cells and the fluorescence intensity of 5-methylcytosine, DNMT3a or DNMT3b was measured and expressed over the designated volume. The total fluorescence intensity (FI) per embryo was calculated as the sum of intensities in all embryonic cells. Embryos of the same group were taken together to calculate the mean FI. The mean FI was arbitrarily set at 1 for the good quality fresh embryo group. Calculation of the mean FI per embryo group allowed relative quantification but no statistical analysis was done due to the limited number of embryos tested.

Results

In general, immunostainings revealed a high heterogeneity between embryos, while the variation of fluorescent signals between blastomeres within an embryo was limited, even at later stages. No difference in subcellular localization or intensity of any DNMT immunostaining was observed between inner and outer cells from embryos at the compaction stage. Similarly in blastocysts, inner cell mass (ICM) and trophectoderm (TE) cells showed analogous expression and localization patterns (Fig. 2).

Expression and localization of human DNA methyltransferases in oocytes and preimplantation embryos

As embryos at Days 1–3 are very scarce research materials, only expression of DNMT I and DNMT I s was investigated at these particular stages.

DNMT I s

DNMT I s protein was detected in 63% of the oocytes at Days 0 and 1; signals were mainly present in the cytoplasm with most GV oocytes showing nuclear DNMT I s signals (Fig. 3).

In the reference embryo group (Group A), DNMT I s was restricted to the cytoplasm in embryos at Day 1 and became predominantly located in the nuclei between Days 2 and 5. Embryos from Group B had a similar localization and expression pattern as the reference embryos; for Group C, the number of embryos with nuclear localization between Days 3 and 5 was reduced (15% compared with 53 and 66% in Groups A and B, respectively). From Day 6 onwards no nuclear DNMT I s was detected in any of the fresh embryo groups. In contrast, cryopreserved embryos, analysed between Days 5 and 7, revealed nuclear DNMT I s expression, irrespective of their morphological quality and developmental timing (Fig. 4 and Supplementary data, Figs S1 and S2).

DNMT I

DNMT I (+ s) protein expression was found in all oocytes and in nearly all embryos; only a minority (15%) of embryos from Group C were

<table>
<thead>
<tr>
<th>Group</th>
<th>DNMT3a</th>
<th>DNMT3b</th>
<th>Global methylation</th>
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<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>13</td>
<td>6</td>
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DNMT1 negative (Figs 3 and 4). MI and MII oocytes displayed DNMT1 in the cytoplasm, whereas nuclear localization was observed in most GV oocytes. In zygotes from embryo Group A, DNMT1 was present in the cytoplasm and in the pronuclei as well. DNMT1 protein was found in the nuclei during the first two cleavage divisions and nuclear expression remained present at the later stages of embryonic development (Fig. 2a). Since DNMT1s nuclear expression was only observed between Days 2 and 5, nuclear DNMT1 expression at other stages can be attributed to solely DNMT1o. The percentage of embryos with nuclear DNMT1 expression between Days 3 and 7 was comparable in Groups A and B (90 and 84%, respectively) but was 2-fold lower in Group C (40%). For the cryopreserved embryos, nuclear DNMT1 immunostaining was present in 58% of good quality embryos (Group D) but only in 20% of delayed and poor quality embryos (Group E) (Fig. 4).

DNMT3a
Cytoplasmic DNMT3a was present in 50% of GV, MI and MII oocytes at Day 0. For the reference embryo group, the number of embryos expressing DNMT3a increased from 67% on Day 4 to 100% on Day 6 (Fig. 5). In the majority of cases, DNMT3a was located only in the cytoplasm except for a small subset (20%) of Day 6 embryos showing nuclear DNMT3a expression (Fig. 2b). A minority of Day 6 embryos from Groups C, D and E also displayed nuclear staining (33, 23 and 18%, respectively). Remarkably, embryos with nuclear staining of the delayed fresh Group B were spread out between Days 4 and 6.

DNMT3b
DNMT3b protein was found in the cytoplasm of all oocytes (Days 0 and 1). During preimplantation development, DNMT3b expression was found from Day 4 onwards in most embryos, irrespective of the group, except for Group C in which only half (47%) of the embryos showed DNMT3b expression (Fig. 5). In Group A, DNMT3b protein localization was cytoplasmic at Day 4 and became predominantly nuclear from Day 5 onwards. A similar transition pattern was found in the other groups, although the timing of the transition seemed delayed to Day 6.

Figure 2 Immunocytochemistry results for human maintenance DNA methyltransferases and human de novo DNA methyltransferases in the good quality fresh embryo group (reference group A). (a) Human maintenance DNA methyltransferases. From top to bottom: DNMT1 (o + s) and DNMT1s. From left to right; all used stages of human preimplantation embryos from the day of fertilization to Day 7 of development. Each embryo is shown as a stack of confocal images. (b) Human de novo DNA methyltransferases. From top to bottom: DNMT3a, DNMT3b and DNMT3L. From left to right; all used stages of human preimplantation embryos from Days 4 to 7 of development. Each embryo is shown as a stack of confocal images. Scale bars indicate 50 μm.
No significant DNMT3L signal was observed in human oocytes (Fig. 3) but the antibody could detect murine Dnmt3l in the nuclei of mouse GV oocytes during control experiments (data not shown), in accordance with published results (Lucifero et al., 2007). In embryo Group A, 50% of embryos at Day 4 and almost every embryo from Day 5 onwards (Fig. 5) expressed DNMT3L in the cytoplasm; a transition to the nucleus was not observed. For Group C, a smaller proportion of embryos at Days 5–7 showed DNMT3L cytoplasmic expression. Cryopreserved embryos (Groups D and E) analysed on Days 6 and 7 had similar DNMT3L patterns as the reference group.

**Effect of cryopreservation on embryonic levels of DNMT3a, DNMT3b and global DNA methylation**

We investigated if the process of embryo cryopreservation on Day 3 (using a slow-freezing DMSO protocol) affects the DNA remethylation
**Figure 4** Expression and localization patterns of maintenance DNMTs: DNMT1 (o + s) and DNMT1s in human preimplantation embryos. Patterns were either nuclear, cytoplasmic or negative (no or few immunostaining signals present). Embryos were classified per group (A = reference group, B = delayed fresh group, C = poor quality fresh group, D = good quality cryopreserved group, E = poor quality cryopreserved group) and per day after oocyte pick-up. The number of embryos observed per day is indicated below the bars.

**Figure 5** Expression and localization patterns of the de novo DNMTs: DNMT3a, DNMT3b and DNMT3L in human preimplantation embryos. Patterns were either nuclear, cytoplasmic or negative (no or few immunostaining signals present). Embryos were classified per group (A = reference group, B = delayed fresh group, C = poor quality fresh group, D = good quality cryopreserved group, E = poor quality cryopreserved group) and per day after oocyte pick-up. The number of embryos observed per day is indicated below the bars.
process around the time of implantation. Therefore, protein expression levels of DNMT3a and DNMT3b as well as global DNA methylation levels in embryos at Day 6 from Groups A, D and E were relatively quantified.

The mean FI was arbitrarily set at 1 for the good quality fresh embryo group (Group A). No difference was observed between the mean FI of DNA methylation levels (0.85 ± 0.12) in good quality cryopreserved embryos (Group D) compared with levels in Group A. Similar DNMT3a (1.04 ± 0.32) and DNMT3b (1.30 ± 0.25) levels were also found in Group D compared with Group A (Fig. 6).

The mean FI of DNA methylation levels (0.92 ± 0.37) in Group E was also similar to the levels of Group A, as were the mean FI of DNMT3a (1.00 ± 0.30) and DNMT3b (1.10 ± 0.38) (Figs 7 and 8).

**Discussion**

We found that DNMT1 was constitutively present in the nuclei of human oocytes and embryos at all stages of preimplantation development until Day 7 after fertilization. Nuclear DNMT1s was only observed within a limited time frame (from Days 2 to 5) and in 50–70% of normally developing embryos, therefore we suggest DNMT1 is the major maintenance DNA methyltransferase during preimplantation development. Because of the lack of nuclear localization of DNMT3a and DNMT3L in preimplantation stages, we suggest that only DNMT3b plays a role in remethylation during blastocyst formation (starting at Day 5) before implantation. The expression of DNMT1, DNMT1s and DNMT3b seems to be disturbed in poor quality (fresh and cryopreserved) embryos, and to a lesser extent in good quality cryopreserved embryos and delayed fresh embryos.

The subcellular localization of proteins is crucial for regulation of their activity. Nuclear import of the DNMT enzymes allows the interaction with DNA molecules, whereas confinement to the cytoplasm may help in the regulation of (de)methylation waves. Probably DNMTs have no biological function when they are located in the cytoplasm. DNMT immunostaining of individual oocytes and embryos at different developmental stages allowed examination of temporal and spatial expression patterns. Comparative quantification of fluorescent signals was not feasible and the study therefore remains largely descriptive.

**Human oocytes**

Immunofluorescent signals for DNMT1 (o + s) and DNMT1s were detected in the nuclei of GV oocytes. It may be that the DNMT1 proteins are stored in the oocyte nucleus to assist in methylation maintenance during meiosis (oocyte maturation GV to MII) and preimplantation development. Most de novo methylation reactions must have occurred during earlier stages of oogenesis; nuclear DNMT3a, DNMT3b and DNMT3L were not detected and only DNMT3b was consistently present in the ooplasm of GV, MI and MII oocytes. This is in agreement with a report of Huntriss indicating expression of DNMT1 transcripts only late during oogenesis (starting from secondary follicle and GV stage), whereas DNMT3a and DNMT3b transcripts were present much earlier, from the primordial follicle stage onwards. The report also indicated no detectable DNMT3L transcripts in human ovarian follicles and oocytes (Huntriss et al., 2004). The absence of nuclear DNMT3 proteins in GV oocytes is important with respect to the time window of imprint resetting in the female germline and may suggest that resetting has been completed at the GV stage. Nuclear DNMT3A and DNMT3L, which are indispensable for de novo methylation of maternal imprinting patterns in the mouse, were not detected in human oocytes. The majority of human imprinting studies also suggest that imprinting acquisition is completed in fully grown GV oocytes (Anckaert et al., 2013), implying that DNMT3 proteins probably function during earlier stages of oogenesis. In mouse, de novo methylation occurs asynchronously at imprinted genes between the primary and secondary follicle stage and requires both Dnmt3a and Dnmt3l with Dnmt3l acting as a de novo methylation regulator (Obata and Kono, 2002; Lucifero et al., 2004; Hiura et al., 2006). Given that no DNMT3L transcripts were detected in human follicles and oocytes and the protein could not be detected in GV, MI and MII oocytes either, it will be necessary to analyse whether DNMT3L is dispensable or has a different role in human oogenesis.
<table>
<thead>
<tr>
<th>DNMT3a</th>
<th>DNMT3b</th>
<th>Nuclei</th>
<th>Transmission light microscopy</th>
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<tr>
<td>![Image of DNMT3a stained embryos]</td>
<td>![Image of DNMT3b stained embryos]</td>
<td>![Image of nuclei stained embryos]</td>
<td>![Immunocytochemistry result]</td>
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**Figure 7** Immunocytochemistry results for double staining experiments for DNMT3a and DNMT3b on embryos at Day 6 of preimplantation development.

<table>
<thead>
<tr>
<th>Methylcytosine</th>
<th>Transmission light microscopy</th>
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<tr>
<td>![Image of methylcytosine stained embryos]</td>
<td>![Immunocytochemistry result]</td>
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**Figure 8** Immunocytochemistry results for global methylation in the good quality fresh embryo group and cryopreserved embryo group (reference groups A and D) on Day 6 of development. Each embryo is shown as a stack of confocal images. Scale bars indicate 50 μm.
Human preimplantation embryos

The grouping of the fresh cleavage stage embryos per day allowed the comparison between the good quality embryos (Group A) and the delayed embryos (Group B) that have a less advanced developmental stage on the same day after fertilization.

Of fresh embryos between Days 6 and 7, 29% had 1-cell or 2-cell biopsy on Day 3 for the purpose of PGD. These embryos were distributed evenly over the 2 days and the different embryo groups (A, B and C) and could not be distinguished from non-biopsied embryos based on developmental stage, morphology or expression pattern. It was shown by Goossens et al. (2008) that the removal of two cells decreases the likelihood of blastocyst formation, compared with removal of one cell; however, developmental stage on Day 3 represents a stronger predictor for developmental stage on Day 5 when compared with the removal of one or two cells.

DNMT1 and DNMT1s

The DNMT1 results provide further evidence for species differences in mammalian DNA methylation enzymes. A genome-wide DNA demethylation occurs immediately after fertilization and during subsequent cleavages, but some specific sequences, in particular imprinted genes and repetitive sequences, retain their methylation status. DNMT1 is thought to play a major role in both DNA demethylation and DNA methylation maintenance. In mouse, methylation maintenance at specific sequences is attributed to Dnmt1o at the 8-cell stage and to Dnmt1s for the other stages. Dnmt1o, which is only synthesized in the oocyte, is found in the cytoplasm of all cleavage stages and just traffics to the nuclei during the 8-cell stage, although this latter observation has not been confirmed during later studies (Hirasawa et al., 2008; Kurihara et al., 2008). The depletion of the highly concentrated Dnmt1o from the nucleus has been regarded as a contributing factor to the passive DNA demethylation in the first cleavages. Dnmt1s is detectable by Dnmt1s-specific antibodies in the nuclei at most murine embryonic stages; it is present at much lower concentrations than Dnmt1o and is held responsible for DNA methylation maintenance of imprinted regions (Branco et al., 2008; Kurihara et al., 2008). We found that ~50–70% of the normally developing human embryos also show Dnmt1s nuclear expression, but within a restricted time frame, between Days 2 and 5. The onset may coincide with the major wave of embryonic genomic activation (Braude et al., 1998; Vassena et al., 2011) but it is unclear why Dnmt1s become restrained to the cytoplasm after Day 5. Since DNMT1s nuclear expression was only observed between Days 2 and 5, nuclear DNMT1 expression at other stages can be attributed to solely Dnmt1o. The finding of Dnmt1o protein until Day 7 after fertilization suggests that maternally inherited Dnmt1o transcripts and proteins are very stable, or alternatively, that expression from the oocyte-specific exon 1 of Dnmt1o continues in the early embryo, suggesting that it is no longer oocyte specific. Human Dnmt1s mRNA was found in GV, MI and MII oocytes (Supplementary data, Fig. S3) and during preimplantation development (Huntriss et al., 2004). Murine Dnmt1o mRNA is detected in oocytes and zygotes. The concentration of the murine protein gradually diminishes towards the blastocyst stage (Ratnam et al., 2002) but no information is available on the concentration of the human DNMT1 proteins. No functional difference between the human DNMT1 isoforms has been reported (Ding and Chaillat, 2002) and we speculate that, unlike in mouse, DNMT1o has the lowest concentration. We postulate that the weakly concentrated isform (human Dnmt1o or murine Dnmt1s) is responsible for DNA methylation maintenance at specific sequences, whereas exclusion of the highly concentrated form (human Dnmt1s or murine Dnmt1o) assists in genome-wide DNA demethylation after fertilization. In bovine, only one DNMT1 isoform has been reported (Lodde et al., 2009) and in sheep, transcripts with oocyte-specific 5’ exons are not detected, but an oocyte-specific splice variant has been shown to have an essential role in early development (Taylor et al., 2009). Therefore, it seems that species-specific differences in concentration, stability, source (oocyte or embryo) and subcellular localization of various DNMT1 isoforms contribute to the concurrent regulation of genome-wide DNA demethylation and DNA methylation maintenance at specific sequences during the earliest stages of preimplantation development.

Nuclear expression of DNMT1 was less common in abnormally developing embryo groups (C and E) and in cryopreserved embryos (Groups D and E). The cryopreserved embryo groups also had a different temporal expression pattern for DNMT1s with nuclear proteins detectable beyond Day 5. Extending the nuclear DNMT1 expression window may compensate for the absence or shortage of nuclear DNMT1o. Cryopreservation could affect the ability of DNMT1 to translocate to the nucleus due to changes in the post-translational modifications regulating the nuclear transfer. Particularly, the phosphorylation of the nuclear localization signal is an essential post-translational modification of DNMT1s (Hodge et al., 2007); this phosphorylation status can be altered by cryopreservation (Zilli et al., 2008). Zhao et al. (2013) demonstrated that vitrification decreased the mRNA expression level of Dnmt1o in mouse oocytes, probably as an effect of altered epigenetic marks. Changes to the availability of DNMT1o in the nucleus might require DNMT1s to substitute for DNMT1o to ensure a proper DNA methylation maintenance.

DNMT3a and DNMT3b

In human embryos, a rise of global DNA methylation is seen from the early blastocyst stage onwards (Fulka et al., 2004). DNMT3a and DNMT3b are held responsible for the acquisition of de novo DNA methylation, and mRNA transcripts for both DNMT3a and DNMT3b are present at every embryonic stage (Huntriss et al., 2004).

At Day 4, both DNMT3a and DNMT3b were present in the cytoplasm but only DNMT3b underwent a transition to the nucleus in the majority of Day 5 blastocysts, which corresponds with the timing of global DNA remethylation (Fulka et al., 2004). Nuclear DNMT3a seems only transiently present in a small subset of Day 6 embryos. The absence of any nuclear DNMT3a in blastocysts after Day 6 is noteworthy as it suggests that DNMT3a is actively retained in the cytoplasm after a certain time point, irrespective of the embryo’s developmental stage (Groups A, B and C). It seems that DNMT3b is the major de novo methylation enzyme at the blastocyst stage. This is in accordance with the finding that DNMT3b is a stemness marker in human embryonic stem cells (Richards et al., 2004). The nuclear DNMT3b expression was similar in ICM and TE cells. In contrast, murine Dnmt3b is preferentially expressed in the TE of blastocysts and gradually changes to the embryonic lineage around or after implantation (Hirasawa and Sasaki, 2009).

The finding of DNMT3a nuclear expression over a broader time interval together with a delay in DNMT3b nuclear transition for Group B
raised the question whether DNMT3a may compensate for the absence of DNMT3b. Prior studies have indeed pointed to the interaction and interdependence of DNMT enzymes, implying that DNMT3a and DNMT3b may perform both independent and redundant functions (Okano et al., 1999; James et al., 2006). The switch to nuclear DNMT3b expression is delayed for Groups B, C, D and E but the finding of nuclear DNMT3a expression over a broader time interval is only observed for Group B. For the cryopreserved embryos, the double immunocytochemistry experiments at Day 6 showed DNMT3a and DNMT3b colocalization in the nuclei or in the cytoplasm (Fig. 7) indicating that DNMT3a does not substitute for DNMT3b at this stage.

Taken together, this suggests that DNMT3a and DNMT3b have a distinct biological role in the human blastocyst (and a different stage specificity). This corresponds with the early mouse embryo, where Dnmt3b is more highly expressed than Dnmt3a and plays an important role in the creation of lineage-specific DNA methylation patterns, whereas the role of Dnmt3a is globally dispensable (Watanabe et al., 2002; Hirasawa and Sasaki, 2009). Dnmt3a is strictly required for de novo methylation in the gametes and in post-natal cell lineage specification (Guenatri et al., 2013).

**DNMT3L**

In human embryos, cytoplasmic expression of DNMT3L, probably without biological function, was observed from Day 4 onwards. These findings are in accordance with the report of Huntriss et al. (2004) where embryonic mRNA was detected from the 4-cell until the blastocyst stage. It has also been found that rhesus monkey oocytes expressed little or no DNMT3L mRNA, compared with a high expression in mouse oocytes, but DNMT3L expression increased during primate preimplantation development (Vassena et al., 2005). These findings also fit with recent data from the mouse indicating that the requirement for Dmnt3l as regulator of de novo methylation in embryos is not strict. Low or even absent Dmnt3l enzyme levels may be balanced by higher Dnmt3a levels and still lead to normal embryonic methylation (Guenatri et al., 2013). Our subcellular localization results for DNMT3L suggest that the protein is not functional in the human preimplantation embryo and that de novo methylation occurs independently of DNMT3L. These results provide further evidence for species differences in DNMT3L expression and function.

**Abnormally developing fresh embryos and DNMT expression pattern**

For fresh embryos, the good quality embryos with delayed development (Group C) were separated from the poor quality embryos (Group C). The results showed that the DNMT expression patterns were more severely disturbed in Group C. For the group of delayed embryos, only differences in temporal patterns were observed in comparison with the reference group (a broader DNMT3a expression window and a delayed DNMT3b nuclear transition). The proportion of embryos with nuclear signals for DNMT1, DNMT1s or DNMT3b was not different between Groups A and B while for Group C, the percentage of embryos expressing DNMT1, DNMT1s or DNMT3b was reduced 2-fold, in addition to a delayed nuclear switch for DNMT3b.

**Effect of cryopreservation on embryonic levels of DNMT3a, DNMT3b and global DNA methylation**

From comparisons between the reference Group A and cryopreserved embryos from Groups D and E, no difference was observed for DNMT3a patterns, but the switch to nuclear DNMT3b expression was delayed from Days 5 to 6–7. As DNMT3b seems to be the major de novo methylation enzyme, there was concern that global methylation levels might be affected at Day 6, which is around the time of embryo implantation in humans. However, a relative quantification of global DNA methylation levels and protein expression levels of DNMT3a and DNMT3b showed no differences, indicating that by Day 6, global DNA methylation levels and enzyme levels are reinstated in a timely manner in human blastocysts that have developed from embryos cryopreserved on Day 3 using a slow-freezing DMSO protocol.

As discussed above, nuclear DNMT1 expression was less common after cryopreservation and extended DNMT1s expression may assist DNMT1o in methylation maintenance. Taken together, our results suggest that DNMT expression patterns are not rigidly fixed; they can be disturbed after cryopreservation, but they seem plastic and recovery is possible. Still, IVF clinics should use such embryos with caution.

**Conclusions**

The aim of our study was to assess the expression and localization patterns of the known human DNA methyltransferases in oocytes and during early embryo development and to compare the patterns of good quality fresh embryos with those of abnormally developing embryos and embryos after cryopreservation.

Our study confirms the existence of species differences for mammalian DNA methylation enzymes. Most of the knowledge of DNA methyltransferase function is based on studies in mice. Our results are summarized and compared with Dnmt localization data in mouse preimplantation embryos in Fig. 9. Mammalian DNA methylation enzymes have been considerably conserved. However, studies in other mammals have revealed that methylation reprogramming events occurring in mouse early development do not necessarily reflect those of other species as the extent and timing of the epigenetic events throughout preimplantation development can be different between mammals (Beaujean et al., 2004; Vassena et al., 2005). Nuclear Dnmt3a and Dnmt3l, which are indispensable for de novo methylation of maternal imprinting segments in the mouse, were not detected in human GV, MII and MII oocytes. This is in agreement with earlier studies showing that Dnmt3l transcripts are not detected across human oogenesis (Huntriss et al., 2004), which indicates that there are species-specific differences in the imprinting process.

It must be considered that mouse and human preimplantation embryos are different with respect to the timing of major genomic activation [2-cell stage for the mouse (Bolton et al., 1984) and 4- to 8-cell stage for humans (Braude et al., 1998; Vassena et al., 2011)]. The timing for de novo methylation is different as well: new methylation patterns are observed at the expanded blastocyst stage in mouse (Reik et al., 2001), while they start at the early blastocyst stage in human embryos (Fulka et al., 2004). This may account for the observed differences in the intracellular trafficking of DNMTs between mouse and humans.
Figure 9 Immunocytochemistry results in human and mouse oocytes and preimplantation embryos for maintenance (DNMT1, from Days 1 to 7) and de novo (DNMT3, from Days 4 to 7) DNA methyltransferases. (a) Summary of results from human oocytes and preimplantation embryos and (b) immunocytochemistry data from the mouse: Dnmt1 from Cirio et al. (2008); Dnmt3a and Dnmt3b from Hirassawa et al. (2008); Dnmt3l from Guenatri et al. (2013).
In poor quality fresh embryos, nuclear DNMT1, DNMT1s and DNMT3b expression was less common and the switch towards nuclear DNMT3b expression was delayed. In cryopreserved embryos, a smaller number of embryos showed nuclear DNMT1 in comparison with the reference group embryos; a delayed switch to nuclear DNMT3b and an extended DNMT1s temporal expression pattern were also observed. Cryopreservation might have an effect on the DNMT proteins and reduce for instance the amount of functional enzyme, or on the post-translational modifications regulating intracellular localization of the DNMTs. Abnormal development and cryopreservation can result in disturbed expression patterns of DNMTs in human preimplantation embryos. Further research, particularly on cryopreservation might have an effect on the DNMT proteins and reduce for instance the amount of functional enzyme, or on the post-translational modifications regulating intracellular localization of the DNMTs. Abnormal development and cryopreservation can result in disturbed expression patterns of DNMTs in human preimplantation embryos. Further research, particularly on cryopreserved embryos, is needed to assess whether disturbed embryonic DNMT expression patterns have any long-term developmental consequences.

Supplementary data
Supplementary data are available at http://molehr.oxfordjournals.org/.

Acknowledgements
We thank the couples donating oocytes and embryos for research. We thank everybody for their contributions, in particular the staff at the IVF lab for providing the research samples.

Authors’ roles
L.P. collected the study material, did the experimental work and contributed to the data analysis and interpretation and the writing and final approval of the manuscript; H.V.d.V. contributed to study design, provision of study material, the data analysis and interpretation and the revision and final approval of the manuscript; M.D.R. designed the study, contributed to the data analysis and interpretation and the revision and final approval of the manuscript.

Funding
This work was supported by the Scientific Research Foundation – Flanders (FWO-Vlaanderen) [grant number G020110N].

Conflict of interest
None of the authors have declared a conflict of interest.

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