Chromosome hydroxymethylation patterns in human zygotes and cleavage-stage embryos

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Abstract

We report the sequential changes in 5-hydroxymethylcytosine (5hmC) patterns in the genome of human preimplantation embryos during DNA methylation reprogramming. We have studied chromosome hydroxymethylation and methylation patterns in triploid zygotes and blastomeres of cleavage-stage embryos. Using indirect immunofluorescence, we have analyzed the localization of 5hmC and its co-distribution with 5-methylcytosine (5mC) on the QFH-banded metaphase chromosomes. In zygotes, 5hmC accumulates in both parental chromosome sets, but hydroxymethylation is more intensive in the poorly methylated paternal set. In the maternal set, chromosomes are highly methylated, but contain little 5hmC. Hydroxymethylation is highly region specific in both parental chromosome sets: hydroxymethylated loci correspond to R-bands, but not G-bands, and have well-defined borders, which coincide with the R/G-band boundaries. The centromeric regions and heterochromatin at 1q12, 9q12, 16q11.2, and Yq12 contain little 5mC and no 5hmC. We hypothesize that 5hmC may mark structural/functional genome 'units' corresponding to chromosome bands in the newly formed zygotic genome. In addition, we suggest that the hydroxymethylation of R-bands in zygotes can be treated as a new characteristic distinguishing them from G-bands. At cleavages, chromosomes with asymmetrical hydroxymethylation of sister chromatids appear. They decrease in number during cleavages, whereas totally non-hydroxymethylated chromosomes become numerous. Taken together, our findings suggest that, in the zygotic genome, 5hmC is distributed selectively and its pattern is determined by both parental origin of chromosomes and type of chromosome bands – R, G, or C. At cleavages, chromosome hydroxymethylation pattern is dynamically changed due to passive and non-selective overall loss of 5hmC, which coincides with that of 5mC.

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Introduction

Genome-wide epigenetic reprogramming is a crucial process in mammalian preimplantation development. A major aspect of this process is the establishment of specific DNA methylation patterns (5-methylcytosine, 5mC) through demethylation – decrease in DNA methylation level – and subsequent remethylation (Monk *et al.* 1987, Kafri *et al.* 1992, Santos *et al.* 2002). The reprogramming of DNA methylation is required to obtain developmental competence, including the onset of pluripotency and correct lineage commitment (Reik *et al.* 2001, Patkin 2002, Surani *et al.* 2007).

In postimplantation development, the patterns of 5mC are established in a tissue-specific manner and are characterized by a relative stability and high heritability (Bird 2002, Byun *et al.* 2009, Tolmacheva *et al.* 2011, Bonder *et al.* 2014). The correct DNA methylation patterns provide regulation of cell type-specific gene expression by affecting the capacity of DNA to interact with transcription factors and methyl-CpG-binding proteins (Doerfler 1981, Razin & Cedar 1991, Zou *et al.* 2012). Numerous studies discuss the possible role of the altered DNA methylation patterns in the initiation and progression of various pathologies, including developmental failures (Shi & Haaf 2002,

Pliushch *et al.* 2010, Ehrlich & Lacey 2013, Shubina *et al.* 2013, Skryabin *et al.* 2013, Zheleznyakova *et al.* 2013, Luo *et al.* 2014, Pendina *et al.* 2014).

In mammalian preimplantation development, DNA demethylation involves two mechanisms: replication-independent (active) and replication-dependent (passive) loss of 5mC (Kafri *et al.* 1993, Mayer *et al.* 2000). Until recently, the mechanisms of the active DNA demethylation remained unclear. The discovery of TET-mediated oxidation of 5mC suggested that the oxidation product – 5-hydroxymethylcytosine (5hmC) – is an intermediate in the active DNA demethylation (Tahiliani *et al.* 2009, Ito *et al.* 2010). The involvement of 5hmC in the DNA methylation reprogramming thus far has been demonstrated in mouse, rabbit, bovine, and porcine preimplantation embryos (Inoue & Zhang 2011, Iqbal *et al.* 2011, Wossidlo *et al.* 2011, Salvaing *et al.* 2012, Zhang *et al.* 2012, Cao *et al.* 2014, Wang *et al.* 2014). Relevant data in human have not been obtained yet. It is known that the dynamic changes in 5mC patterns during embryonic cleavages are species specific (for a review see Abdalla *et al.* (2009)). Therefore, it could be expected that patterns of 5hmC – an oxidative derivative of 5mC – in the genome of human preimplantation embryos differ from those described in other mammalian species.

5hmC is present in adult tissues at tissue-specific and quite constant levels (Li & Liu 2011, Nestor *et al.* 2012, Ivanov *et al.* 2013) and shows association with gene regulatory elements and gene bodies (Stroud *et al.* 2011, Wu *et al.* 2011). 5hmC is selectively recognized by transcription factors (Yildirim *et al.* 2011, Zhenilo *et al.* 2013, Teif *et al.* 2014) and its level correlates with gene expression (Wu *et al.* 2011). These data advocate for the own function of 5hmC in the regulation of gene activity (Branco *et al.* 2011). Thus, during human preimplantation development – in the period of genome reorganization for totipotency with the subsequent lineage commitment – the changes in 5hmC patterns can be associated with regulation of the embryonic genome activity and alternatively, or complementarily, with the involvement of 5hmC in global genome reprogramming through oxidation of the 5mC pathway.

In this study, we address the following questions concerning the epigenetic reprogramming in human preimplantation development: i) whether 5hmC patterns undergo changes during DNA methylation reprogramming; ii) whether 5hmC patterns differ between parental genomes; iii) whether 5hmC patterns differ between euchromatic and heterochromatic genome regions; and iv) whether 5hmC is maintained throughout cleavage divisions. A promising experimental approach is the immunofluorescent analysis of 5hmC patterns on metaphase chromosomes. In contrast to interphase studies, it provides information on how whole and individual parental chromosomes and chromosome regions are hydroxymethylated. This seems to be of substantial scientific value, as genome loci possess

different functions and are not uniformly structured and regulated (Craig & Bickmore 1994, Gilbert *et al.* 2004, Zhu *et al.* 2013). The obtained data provide new insights into both the timing and the genome patterning of 5hmC in human preimplantation embryos.

Materials and methods

Collection of human zygotes, cleavage-stage embryos, sperm, and oocytes

The study included only triploid human zygotes and embryos, unsuitable for transfer or for cryopreservation, as normal ones were not available. Zygotes were produced by conventional IVF. Cleavage-stage embryos were produced by the cultivation of trippronucleate zygotes in the ISM1 medium (Origio, 10500060, Måløv, Denmark) until 4–8-cell stage and then in the BlastAssist medium (Origio, 12160010) until blastocyst stage under standard conditions. A total of 81 preimplantation embryos were included in the study: 20 zygotes, four embryos at the 3-cell stage, five embryos at the 4–5-cell stage, 15 embryos at the 7–cell–morula stage, and 37 blastocysts.

Oocytes were aspirated from patients' follicles after hormone stimulation in the IVF cycles. Oocytes were rinsed in Flushing Medium (Origio, 10845060) prewarmed to 37 °C. Then, oocytes were incubated in the ISM1 medium (Origio, 10500060) at 37 °C in an atmosphere of 5% CO₂. After 3 h, routinely prepared sperm was added for IVF. To identify fertilization status, the presence of pronuclei was examined after 20 h. Unfertilized oocytes were selected for the study. A total of 19 oocytes were included in the study.

Semen samples were obtained from sperm donors by masturbation after a 3 to 5 day abstinence period. A regular semen analysis (volume, pH, concentration, motility, vitality, and morphology) was performed. The semen samples were split into aliquots and one 1 ml aliquot from each sample was selected for the study. A total of five samples were included in the study with 100 spermatozoa analyzed in each sample.

All the biological samples were obtained at the Department of Assisted Reproductive Technologies, D.O. Ott Research Institute of Obstetrics and Gynecology (St Petersburg, Russia). This study was approved by the Institutional Review Board of D.O. Ott Research Institute of Obstetrics and Gynecology. All the samples were donated for research with the written informed consent obtained from patients. The study was conducted in accordance with the Declaration of Helsinki.

Slide preparation and immunodetection of 5hmC and 5mC

For sperm preparations, 1 ml aliquots of native semen samples were placed in 10 ml of hypotonic solution (0.9% sodium citrate). After 20 min of incubation at room temperature, hypotonic solution was replaced with 10 ml of freshly prepared fixative (ethanol:glacial acetic acid, 3:1). The semen samples were fixed at 4 °C and the fixative was changed at least twice during a 60 min fixation period. The suspension was centrifuged for 10 min at 190 *g* and the supernatant was discharged leaving 0.5–1 ml over the precipitate. The

suspension was then dropped onto slides. The slides were air dried at room temperature.

Chromosome preparations from oocytes, zygotes, and cleaving embryos were made as described previously (Pendina *et al.* 2011). To obtain chromosome banding, the slides were stained with Hoechst 33258 and counterstained with actinomycin D (QFH/AcD technique), according to the protocol used previously (Grigorian *et al.* 2010). After photoimaging of QFH/AcD-stained metaphases, the slides were washed in distilled water, dehydrated in an ethanol series (70, 80, and 96%), and air dried.

The immunodetection of 5hmC and 5mC was performed according to the protocol used previously (Pendina *et al.* 2011). For DNA denaturation, the preparations of sperm were exposed to 2 M HCl for 20–40 min and the preparations of oocytes, zygotes, and cleaving embryos for 15–20 min at room temperature. Then, the slides were washed thoroughly in ice-cold PBS and distilled water. The slides were incubated with blocking solution (1% BSA, 0.1% Tween 20 in PBS) for 30–40 min at 37 °C in a humidified chamber. Then, the mixture of antibodies against 5hmC (rabbit polyclonal, Active Motif, 39769, Carlsbad, CA, USA) and 5mC (mouse monoclonal, Eurogentec, BI-MECY-0100, Seraing, Belgium) diluted in blocking solution (1:500) was applied to the slides for 90 min at room temperature in the humidified chamber. The slides were washed thrice with PBS for 15 min each, supplemented with 0.5% Tween 20 at 43 °C in a shaking bath. The primary antibodies were detected by goat anti-rabbit Alexa Fluor 488 (Life Technologies, A-11008) and goat anti-mouse Alexa Fluor 555 (Life Technologies, A-21424)/goat anti-mouse Cy3 (GE Healthcare Life Sciences, PA43002, Uppsala, Sweden) antibodies, diluted in blocking solution (1:200) and simultaneously applied to the slides for 60 min at 37 °C in the humidified chamber. The slides were then washed thrice with PBS for 15 min each supplemented with 0.5% Tween 20 at 43 °C in the shaking bath, rinsed in PBS and distilled water, dehydrated in the ethanol series (70, 80, and 96%), and mounted in DAPI-containing Vectashield antifade (Vector Laboratories, H-1200, Burlingame, CA, USA).

Image acquisition and analysis

Fluorescence images were acquired using a Leica DMLS microscope with a Leica DFC320 camera and with the Leica DFC Twain software (for QFH/AcD banded chromosomes), and using a Leica DM 2500 microscope with a Leica DFC345 FX camera and with the Leica Application SuiteV.3.8.0 software (for immunostained and DAPI-stained chromosomes and spermatozoa). The individual images were processed with Adobe Photoshop CS3. The evaluation of 5hmC and 5mC fluorescence intensity of parental chromosome sets at the zygote stage was performed by the Image J 1.48v software. The intensity of fluorescent signal was measured for each chromosome in a set. Then, the total fluorescence intensity in relation to the total chromosome area was calculated for each chromosome set. For the quantification of 5hmC and 5mC fluorescence intensity, the calculation of the relative values was performed: in each zygote, the value from the chromosome set with the darkest signal was set at 100%, and the fluorescence

intensity of the other sets was compared to it. The comparison of 5hmC and 5mC relative fluorescence intensity between the parental chromosome sets was performed in GraphPad Prism 6.01 using the Mann–Whitney *U* test. For the assessment of the specificity of 5hmC and 5mC distribution across metaphase chromosome arms, the plot profiles of QFH, 5hmC, and 5mC fluorescence intensity were built using measurements obtained in Image J 1.48v by the 'segmented line' instrument.

Results

We have investigated the presence of 5hmC in the sets of parental chromosomes, individual chromosomes, and chromosome bands in human preimplantation development. Using indirect immunofluorescence with specific antibodies, we analyzed 5hmC localization and its co-distribution with 5mC on the QFH-banded metaphase chromosomes of zygotes and cleavage-stage embryos. As normal diploid zygotes and embryos were not available, we performed our study on triploid ones. The developmental potential of triploid human embryos is confirmed by their capacity for implantation and even a full-term development (Gardner *et al.* 2011). Chen *et al.* (2010) have reported no difference in the DNA methylation patterns between trippronuclear and

Figure 1 Immunostaining for 5-hydroxymethylcytosine (5hmC) (A), 5-methylcytosine (5mC) (B), merge image (C), and QFH banding (D) of a maternal (♀) and two paternal (♂) chromosome sets of a triploid human zygote. Two paternal chromosome sets identified by the presence of Y chromosomes are highly hydroxymethylated and contain little 5mC. The maternal chromosome set has a low level of 5hmC and is heavily methylated. QFH/AcD staining was performed before the immunodetection of 5hmC and 5mC for chromosome banding and identification.

Figure 2 Box-and-whisker plots of relative fluorescence intensity for 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) in the maternal and paternal chromosome sets of triploid human zygotes. The relative 5hmC fluorescence intensity is significantly higher in paternal chromosome sets than in maternal ones (* $P < 0.0001$, the Mann–Whitney U test), whereas the relative 5mC fluorescence intensity is significantly higher in maternal chromosome sets than in paternal ones (** $P < 0.0001$, the Mann–Whitney U test).

microsurgically corrected bipronuclear human zygotes. A case of a normal birth after microsurgical enucleation of the extrapaternal pronucleus from trippronucleate human zygote (Kattera & Chen 2003) provides further evidence that the genome-wide steps of epigenetic reprogramming in triploid embryos could be similar or identical to those in normal ones.

To detect whether 5hmC patterns differ between the parental genomes in trippronucleate zygotes, we compared chromosomes of different parental origins. In seven out of 20 fixed triploid zygotes, parental chromosomes were visible as separate haploid sets (Fig. 1). In six of these zygotes, two sets demonstrated equally high hydroxymethylation in contrast to the other one, which was poorly hydroxymethylated (Fig. 1). In one zygote, one set was highly hydroxymethylated, while the two others had equally low hydroxymethylation.

The detection of chromosome Y is the most reliable criterion to identify the origin of parental chromosome set in human zygotes, although it allows identification of only approximately half of the genomes of male origin. It can be achieved by FISH, when studying zygotes at the pronuclear stage (Xu *et al.* 2005) or by chromosome banding techniques, when studying zygotic parental chromosome sets. Other criteria reported by some authors, including slightly larger size of the human paternal pronuclei and their location further away from the polar body than the maternal pronuclei (Rawlins *et al.* 1988, Palermo *et al.* 1994), have not been proved to be highly reliable (Kattera & Chen 2003).

We analyzed the number and structure of metaphase chromosomes in seven triploid zygotes with three maternally and paternally derived separate chromosome sets in each zygote (totally 21 chromosome sets). Among 21 parental chromosome sets, eight sets were poorly hydroxymethylated and 13 sets were highly

hydroxymethylated. In all eight poorly hydroxymethylated sets, X chromosome was detected. In four out of 13 highly hydroxymethylated sets, X chromosome was detected, while in the remaining nine, Y chromosome was detected, confirming that the chromosomes with a high level of 5hmC are of paternal origin (Fig. 1).

In 13 out of 20 fixed triploid zygotes, chromosomes were close to each other, making the identification of the whole parental chromosome sets impossible. Homologous chromosomes in all the triads differed in hydroxymethylation intensity: two homologues had equal anti-5hmC fluorescence, while the third one had higher fluorescence in three zygotes and lower fluorescence in ten zygotes. The 5mC patterns were inverse to those of 5hmC: highly hydroxymethylated chromosomes contained little 5mC, while chromosomes with low levels of 5hmC were heavily methylated. The Mann–Whitney U test showed statistically significant difference between the parental chromosome sets in both anti-5hmC ($P < 0.0001$) and anti-5mC ($P < 0.0001$) relative fluorescence intensity (Fig. 2). Low DNA methylation level of the highly hydroxymethylated chromosome sets also indicates that these sets are of paternal origin, as hypomethylation of the paternal genome in human zygotes has been repeatedly shown by other authors using immunofluorescence approach (Beaujean *et al.* 2004, Fulka *et al.* 2004, Xu *et al.* 2005, Pendina *et al.* 2011, Guo *et al.* 2014b). Thus, at the zygote stage, the chromosomes of both parental genomes contain 5hmC,

Figure 3 Immunostaining for 5-hydroxymethylcytosine (5hmC) (A), 5-methylcytosine (5mC) (B), merge image (C), and QFH banding (D) of the chromosomes from a human oocyte. Chromosomes have readily detectable 5hmC and 5mC staining across euchromatic arms, but not in pericentric regions. QFH/AcD staining was performed before the immunodetection of 5hmC and 5mC for chromosome banding and identification.

Figure 4 Immunostaining for 5-hydroxymethylcytosine (5hmC) (A), 5-methylcytosine (5mC) (B), merge image (C), and phase-contrast image (D) of human spermatozoa. Spermatozoa demonstrate weak hydroxymethylation and strong DNA methylation.

but the paternal genome is more hydroxymethylated than the maternal one.

We assumed that the zygotic 5hmC and 5mC patterns can be either acquired from gametes or formed *de novo* after fertilization. We examined DNA hydroxymethylation and methylation patterns of parental

genomes in gametes. In oocytes, chromosomes had readily detectable 5hmC and 5mC staining across euchromatic arms. Pericentric regions of all chromosomes contained neither 5hmC nor 5mC (Fig. 3). In sperm, hydroxymethylation was weakly pronounced, whereas anti-5mC fluorescent signal was strong (Fig. 4). Thus, parental gamete-specific 5hmC and 5mC patterns differ from those of maternal and paternal chromosome sets in zygote. This advocates for *de novo* onset of hydroxymethylation and methylation patterns in the newly formed zygotic genome.

To determine where major hydroxymethylation events occur in the zygotic genome, we analyzed the 5hmC patterns at the level of chromosome bands. 5hmC accumulated in the QFH/AcD-negative chromosome regions (R-bands), avoiding the QFH/AcD-positive regions (G- and C-bands), which were poorly or non-hydroxymethylated (Fig. 5). The strongest hydroxymethylation was detected in T-bands – the subset of R-bands, which is characterized by the highest GC enrichment and the highest gene density. In both parental chromosome sets, 5hmC pattern featured distinct borders that corresponded to the R/G-band boundaries, making each chromosome easily recognizable by its hydroxymethylation pattern. However, in the poorly hydroxymethylated chromosome sets, some 5hmC bands were less pronounced due to the overall decrease in anti-5hmC fluorescence. The pericentric regions of all chromosomes including the largest heterochromatic blocks 1q12, 9q12, 16q11.2, and Yq12 contained no 5hmC, irrespective of their size (Fig. 5). When analyzing DNA methylation in the same chromosomes, in highly hydroxymethylated (paternal) sets, we detected almost homogeneous distribution

Figure 5 QFH banding (QFH) and immunostaining for 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) of human zygotic metaphase chromosomes 1, 9, and 16 from paternal and maternal chromosome sets. The distribution of hydroxymethylated DNA across the euchromatic chromosome arms is highly band specific in both parental chromosome sets: hydroxymethylated loci correspond to R-bands (indicated by arrows), but not G-bands, and have well-defined borders, which coincide with the R/G-band boundaries. The distribution of methylated DNA across the euchromatic chromosome arms is less band specific: the tendency for R-band-specific accumulation of 5mC is more pronounced only in maternal chromosomes, while 5mC is almost homogeneously distributed with a higher level of methylation only in the telomeric regions in paternal chromosomes. The heterochromatic regions 1q12, 9q12, and 16q11.2 (marked with blue) contain little 5mC and no 5hmC.

of 5mC in the euchromatic arms, with a higher level of methylation only in the telomeric regions. In poorly hydroxymethylated (maternal) sets, the tendency to R-band-specific accumulation of 5mC was more pronounced (Fig. 5). All pericentric regions including 1q12, 9q12, and 16q11.2 were poorly methylated or

contained no 5mC at all in both parental sets (Fig. 5). Thus, the distribution of both 5hmC and 5mC is mostly confined to the euchromatic arms of zygotic chromosomes, and the chromosome pattern of 5hmC is distinguished from that of 5mC by its distinct specificity to R-bands.

Figure 6 QFH banding (QFH) (A, E, I, and M), immunostaining for 5-hydroxymethylcytosine (5hmC) (B, F, J, and N), 5-methylcytosine (5mC) (C, G, K, and O), and merge images (D, H, L, and P) of human metaphase chromosomes from the blastomeres of a 3-cell embryo (A, B, C, and D), a 5-cell embryo (E, F, G, and H), an 8-cell embryo (I, J, K, and L), and a blastocyst (M, N, O, and P). The chromosomes with asymmetrical hydroxymethylation of sister chromatids and totally unhydroxymethylated chromosomes are present in cleavage-stage embryos. The chromosome pattern of 5hmC coincides with that of 5mC: hydroxymethylated chromatids are also marked with 5mC, while non-hydroxymethylated chromatids are poorly methylated. At the blastocyst stage, rare chromosomes with asymmetrical hydroxymethylation are still present, but the 5mC asymmetry is less pronounced due to acquisition by the chromosomes of a new methylation pattern, in which hypomethylated chromatids are almost absent (for details, see *Pendina et al. (2011)*). QFH/AcD staining was performed before the 5hmC and 5mC immunodetection for chromosome banding and identification.

Table 1 The relative number of asymmetrically hydroxymethylated and asymmetrically methylated chromosomes in human oocytes, zygotes, and preimplantation embryos.

Developmental stage	Number of samples	Number of metaphases and metaphase fragments	Number of chromosomes	Relative number of asymmetrical chromosomes per developmental stage	
				Hydroxymethylated	Methylated
Oocyte	19	19	486	0	0
Zygote	20	20	669	0	0
3-cell	4	4	123	76.4 ± 3.8%	59.2 ± 4.5%
4–5-cell	5	10	275	40.7 ± 2.9%	33.4 ± 2.9%
7-cell-morula	15	16	368	14.2 ± 2.0%	17.3 ± 1.2%
Blastocyst	37	65	2271	6.5 ± 0.5%	6.6 ± 0.5%

To check whether 5hmC is maintained in human preimplantation development, we analyzed chromosome hydroxymethylation patterns at the subsequent cleavage divisions up to and including the blastocyst stage. The zygotic 5hmC pattern was not present any more. We observed chromosomes with asymmetrical 5hmC distribution in sister chromatids – hemihydroxymethylated chromosomes (Fig. 6). The hydroxymethylated chromatids maintained band-specific 5hmC distribution, as it appeared at the zygote stage. In some chromosomes, hydroxymethylated and non-hydroxymethylated regions alternated in the chromatids demarcating sister chromatid exchanges. Along with asymmetrically stained chromosomes, we detected the chromosomes with equally poor, if any, hydroxymethylation in both chromatids (Fig. 6). Notably, the chromosome pattern of 5hmC coincided with that of 5mC: hydroxymethylated chromatids retained the zygotic 5mC pattern, while non-hydroxymethylated chromatids were poorly methylated. At the blastocyst stage, asymmetrical hydroxymethylation was still present in rare chromosomes, but the methylation asymmetry was less pronounced due to acquisition of a new chromosome methylation pattern, in which hypomethylated chromatids were almost absent (described in detail in our previous study [Pendina et al. \(2011\)](#)) (Fig. 6). We counted the asymmetrically hydroxymethylated chromosomes in total metaphases and metaphase fragments of the analyzed embryos per developmental stage. At the 3-cell stage, $76.4 \pm 3.8\%$ of chromosomes featured asymmetrical 5hmC distribution in sister chromatids. At cleavages, from the 4-cell stage to the blastocyst stage, the number of asymmetrically hydroxymethylated chromosomes decreased. They amounted to $40.7 \pm 2.9\%$ at the 4–5-cell stage, $14.2 \pm 2.0\%$ at the 7-cell-morula stage, and $6.5 \pm 0.5\%$ at the blastocyst stage, while the remaining chromosomes were unhydroxymethylated (Table 1). The relative number of asymmetrically methylated chromosomes was more or less equal to that of asymmetrically hydroxymethylated chromosomes and amounted to $59.2 \pm 4.5\%$ at the 3-cell stage, $33.4 \pm 2.9\%$ at the 4–5-cell stage, $17.3 \pm 1.2\%$ at the 7-cell-morula stage, and $6.6 \pm 0.5\%$ at the blastocyst stage (Table 1). Hence, cleavage divisions in human

preimplantation development are accompanied with a decrease in both 5hmC and 5mC levels.

Discussion

We have analyzed 5hmC patterns during DNA methylation reprogramming in human preimplantation development. We based our experimental approach on the detection of 5hmC and 5mC in preparations of zygotic and embryonic metaphase chromosomes using highly specific antibodies. This method provides information on hydroxymethylation and methylation patterns on the largest scale of genomic organization with a resolution at the level of individual chromosomes and chromosome bands.

We show that chromosomes of human zygotes have unique 5hmC and 5mC patterns, which differ from those in parental gametes. The zygotic patterns are characterized by inverse 5hmC and 5mC levels: highly hydroxymethylated paternal chromosomes contain little 5mC, while maternal chromosomes have low levels of 5hmC and are heavily methylated (Figs 1 and 2). The inverse 5hmC and 5mC patterns can be explained by conversion of 5mC to 5hmC in the paternal genome after fertilization; in contrast, the maternal genome avoids massive conversion of methyl to hydroxymethyl groups and remains highly methylated. Our observations are in line with studies demonstrating inverse 5mC and 5hmC patterns in the parental pronuclei of non-human mammals ([Iqbal et al. 2011](#), [Wossidlo et al. 2011](#), [Salvaing et al. 2012](#), [Zhang et al. 2012](#)). An intensive decrease in DNA methylation in the paternal genome in a shared cytoplasm is suggested to be a part of chromatin remodeling with the purpose of providing unrestricted access to common cytoplasmic factors for both parental genomes in a zygote ([Dean 2014](#)).

It has long been believed that in mammalian preimplantation development, parental genomes are demethylated through different pathways: maternal genome is exclusively demethylated through a replication-dependent (passive) mechanism during cleavage divisions, while paternal genome undergoes global replication-independent (active) loss of methyl groups at the zygote stage and then is also demethylated passively ([Rougier et al. 1998](#), [Reik et al. 2001](#),

Inoue & Zhang 2011, Pendina *et al.* 2011). However, a number of studies have demonstrated that replication-dependent and -independent (through TET3-mediated oxidation of 5mC to 5hmC) demethylation mechanisms coexist in both male and female pronuclei of mouse zygotes (Salvaing *et al.* 2012, Guo *et al.* 2014a, Shen *et al.* 2014, Wang *et al.* 2014). In the human zygotes at the pronuclear stage, hydroxymethylation of both male and female pronuclei with lower immunofluorescent signal in female pronuclei has been reported by Guo *et al.* (2014b). Our results indicate that maternal chromosomes in human zygotes have low but readily detectable hydroxymethylation, which coexists with the high level of methylation. Hydroxymethylation pattern of maternal chromosomes can either, at least in part, come from oocyte or be a result of selective conversion of 5mC to 5hmC at the zygote stage. To shed light on the involvement of parent-specific 5mC-to-5hmC conversion in the genome reprogramming in human zygotes, experiments on expression, translation, and binding of TET enzymes in human gametes and embryos are needed.

The genome of human zygotes appears to be hydroxymethylated selectively. Genome regions with high accumulation of 5hmC coincide with cytogenetically detected R-bands (Figs 1 and 5). The R-band-specific hydroxymethylation is most pronounced in paternal chromosomes. The amount of 5hmC in the R-bands of maternal chromosomes is reduced but readily detectable. Irrespective of parental origin, hydroxymethylated regions have well-defined borders, which strictly correspond to the R/G-band boundaries (Fig. 5). The specificity of hydroxymethylation to R-bands is probably associated with their structural and functional properties. In contrast to G-bands, R-bands are enriched in CpG dinucleotides – DNA methylation, and thus, hydroxymethylation sites. GC enrichment positively correlates with gene density and ‘open’ chromatin structure (Holmquist & Ashley 2006). It has been previously suggested that there may be distinct demarcation between ‘open’ and ‘closed’ chromatin (Gilbert *et al.* 2004). R/G-band transition is also important for the early/late replication switch between transcribed and silenced regions (Watanabe & Maekawa 2013). Hence, the sharp transitions between hydroxymethylated and non-hydroxymethylated DNAs at the R/G-band boundaries may contribute to the marking of structural/functional genome ‘units’ corresponding to chromosome bands in the newly formed genome of a zygote. Our observations support the hypothesis of a dual function of 5hmC: as an intermediate product in the TET-mediated DNA demethylation pathway, and as an epigenetic modification with a role of its own (Branco *et al.* 2011, Ficiz *et al.* 2011, Salvaing *et al.* 2012, Li & O’Neill 2013).

In the view of epigenetic marking of genome ‘units’ within chromosomes, the establishment of heterochromatin signatures is of special interest. In human zygotes,

the largest heterochromatic regions 1q12, 9q12, 16q11.2, and Yq12 contain little or no 5mC, which is in line with our previous observations (Baranov *et al.* 2005, Pendina *et al.* 2011). In contrast, in the somatic cells of adults and fetuses, the constitutive heterochromatin is hallmarked by a high level of DNA methylation (Barbin *et al.* 1994, Montpelier *et al.* 1994, Kokalj-Vokac *et al.* 1998, Pendina *et al.* 2005, 2011, Pfarr *et al.* 2005). It can therefore be expected that heterochromatic regions in zygotes are enriched in 5hmC, an active demethylation intermediate. However, in the studied zygotes, heterochromatic regions 1q12, 9q12, 16q11.2, and Yq12 appear to be completely unhydroxymethylated in both parental genomes (Figs 1 and 5). Moreover, both 5mC and 5hmC are absent in the heterochromatic regions of the oocyte chromosomes. Our data suggest that DNA methylation reprogramming of euchromatic and heterochromatic regions in the genome of human zygote proceeds in a different manner, favoring a specific function of heterochromatin during preimplantation period.

At cleavages, chromosome 5hmC pattern changes dramatically with each division. Chromosomes with one non-hydroxymethylated and one hydroxymethylated chromatid appear. Such asymmetrical (or hemihydroxymethylated) chromosomes are present at all cleavage stages up to and including the blastocyst stage (Fig. 6). The zygotically derived level and pattern of 5hmC are intact only in one – hydroxymethylated – chromatid throughout cleavages and are not reproduced in its sister chromatid. At cleavages, chromatids with zygotic 5hmC signatures are distributed among blastomeres. With each division, the relative number of hemihydroxymethylated chromosomes decreases, whereas chromosomes with both non-hydroxymethylated chromatids become numerous. These result in a gradual decrease in the total genome hydroxymethylation level during cleavages. Our findings point toward passive (replication-dependent) loss of 5hmC, when 5hmC pattern is not reproduced in the newly synthesized DNA strands. The replication-dependent loss of 5hmC has been reported in mice (Inoue & Zhang 2011), suggesting that this mechanism is involved in the reprogramming in both human and mouse preimplantation embryos.

The passive loss of 5hmC in human cleavage-stage embryos coincides with that of 5mC. The chromosome patterns of 5mC passive loss have been previously reported in human (Pendina *et al.* 2011) and mouse (Rougier *et al.* 1998) preimplantation embryos and correlate with those observed in our study. The coincidence of 5hmC and 5mC in some chromatids or chromatid regions is explained by the permanent persistence of their patterns in these loci throughout all cleavages starting from the zygote stage. Notably, this unique fate of the chromatids with zygotic epigenetic signatures can be observed at cleavages only when studying metaphase chromosomes. The interphase

immunofluorescent studies reported only a gradual decrease in general 5mC level in human preimplantation development (Beaujean *et al.* 2004, Fulka *et al.* 2004, Santos *et al.* 2010), as the discreteness of chromosome epigenetic marking could not be detected due to chromatin decondensation.

In contrast to the R-band-specific hydroxymethylation at the zygote stage, which is most probably associated with conversion of 5mC to 5hmC, the passive loss of both 5hmC and 5mC is not selective and equally involves all bands of all chromosomes. The decrease in the number of asymmetrically hydroxymethylated chromosomes indicates that there are no new global hydroxymethylation events during cleavage divisions. The absence of global hydroxymethylation does not contradict the possibility of local hydroxymethylation at specific genome regions to provide tight epigenetic regulation of the embryonic genome. The rare hemi-hydroxymethylated chromosomes at the blastocyst stage are quite intriguing. It is obvious that the presence of such chromosomes is compatible with the DNA remethylation and the primary differentiation of blastomeres to the inner cell mass and the trophectoderm. The further fate and the biological role of the chromatids with zygotic 5hmC signatures is an open and very interesting question. Thus, the DNA methylation reprogramming in human preimplantation development is likely to include at least three mechanisms: at the zygote stage, R-band-specific conversion of 5mC to 5hmC, which is strongly pronounced in the paternal genome and, at subsequent cleavages, replication-dependent non-specific loss of 5hmC and 5mC from both maternal and paternal genomes.

In conclusion, the 5hmC pattern is dynamically changed during the reprogramming of DNA methylation in human zygotes and cleavage-stage embryos. At the zygote stage, the genome-wide hydroxymethylation is strongly pronounced in the paternal genome. The global 5hmC pattern in zygotes is composed of local hydroxymethylation events, which are confined to the cytogenetically defined chromosome regions – R-bands. The transitions of hydroxymethylated to non-hydroxymethylated DNA at the R/G-band boundaries may contribute to the marking of structural/functional genome ‘units’ corresponding to chromosome bands in the newly formed genome of a zygote. The selective hydroxymethylation at the zygote stage can be treated as a new characteristic of R-bands distinguishing them from G-bands, in addition to the known differences in replication timing, GC enrichment, and gene density. At cleavages, there are no new global hydroxymethylation events and the genome-wide loss of 5mC and 5hmC proceeds through a non-selective passive mechanism. We believe that our conclusions are essential to expand knowledge of the initial stages of human embryogenesis and will have value both for fundamental and practical aspects of human reproduction science.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

O A Efimova, A A Pendina, A V Tikhonov, and I D Fedorova contributed equally to this work.

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