Genome-wide methylation patterns in normal and uniparental early mouse embryos

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In the normal diploid mouse embryo, active demethylation of the paternal genome but not of the maternal genome occurs within only a few hours and in a highly coordinated fashion as the zygote proceeds through the first G1 phase. This zygotic demethylation may be necessary to reprogram the sperm genome for somatic development. Immunofluorescence staining with an antibody against 5-methylcytosine shows that the cellular machinery of the fertilized egg cannot demethylate the second maternal genome in parthenogenetic, gynogenetic and triploid digynic embryos or remethylate the additional (already demethylated) paternal genome in androgenetic and triploid diandric embryos. This suggests that differential zygotic demethylation results from differences in the remodeling of paternal and maternal chromatin structures after fertilization, i.e. sperm nuclear decondensation and protamine–histone exchange. A proportion of embryos derived from normal matings display abnormal methylation patterns some of which are indistinguishable from those in androgenetic or gynogenetic embryos. We conclude that methylation reprogramming defects in mammalian zygotes contribute to the high incidence of early pregnancy failure.

INTRODUCTION

In mammals, the one-cell embryo is formed from two very different sets of chromatin: the highly compacted nucleoprotamine of the sperm and the metaphase II-arrested chromosomes of the oocyte. After fertilization, the activated oocyte must remodel the two transcriptionally inactive gamete nuclei into a functional diploid somatic genome (1). Immunofluorescent data from mouse eggs fertilized with bromodeoxyuridine-labeled sperm and mouse interspecific hybrid embryos suggest that topological separation of chromatin according to parental origin is preserved up to the four-cell embryo stage and then gradually disappears (2). Genome separation in the early diploid embryo, when dramatic chromatin remodeling and epigenetic changes occur, may be functionally important for reprogramming the two parental genomes for somatic development, rather than being a passive relic of the different developmental histories of sperm and egg chromatin before fertilization.

Global demethylation during preimplantation development leads to indistinguishable alleles at most gene loci except for a small number of methylated CpG sites in imprinted genes (8,9). These differentially methylated regions could serve as primary imprinting signals (10,11).

RESULTS

Methylation patterns in uniparental embryos

Consistent with previous anti-MeC immunofluorescence studies (5), one-cell mouse embryos from normal matings at 10–16 h after fertilization exhibited a very low MeC density over the somewhat larger paternal pronucleus, compared with the highly methylated maternal pronucleus (Fig. 1A). In contrast, diploid parthenogenetic one-cell embryos, which were made by activating unfertilized eggs and suppressing second polar body extrusion, always contained two methylated
maternal genomes (Fig. 1B). Gynogenetic and androgenetic embryos were made concurrently from the same group of fertilized eggs between 10 and 13 h after fertilization. The paternal pronuclei were already demethylated at the time of operation (data not shown), since only eggs where the pronuclei were well formed and distinguishable by size could be used for transplantation. Gynogenetic and androgenetic embryos were processed for immunofluorescence staining ~3–4 h after nuclear transfer, ~16 h after fertilization. The maternal genomes in haploid (Fig. 1C) and diploid gynogenetic one-cell embryos (Fig. 1D) remained fully methylated. In contrast, the paternal genomes in haploid (Fig. 1E) and diploid androgenetic embryos (Fig. 1F) were always demethylated. The second polar body was highly methylated in both gynogenetic and androgenetic embryos and served as a staining control. Triploid one-cell embryos were made more or less synchronously from the same group of eggs and analyzed between 15 and 17 h after fertilization. Triploid diandric embryos (Fig. 1G) displayed one methylated maternal and two demethylated paternal pronuclei. In contrast, triploid digynic embryos (Fig. 1H) had one demethylated paternal and two methylated maternal pronuclei.

In normal two-cell embryos, both nuclei showed highly localized MeC staining, reflecting compartmentalization of the two differentially methylated parental genomes (Fig. 2A). In a control experiment, more than 50 normal embryos were stained with anti-DNA antibody (Fig. 2B). Since we never did observe differential DNA staining of the two nuclei or of the two genomes within the same nucleus, we exclude the formal possibility that the observed methylation patterns are caused by altered accessibility of the two nuclei or the two genomes to the anti-MeC antibody. Parthenogenetic (Fig. 2C) and diploid gynogenetic two-cell embryos (Fig. 2D) at ~34 h after activation/fertilization displayed a uniformly intense nuclear staining with anti-MeC antibody. Interphase nuclei of haploid (Fig. 2E) and diploid androgenetic two-cell embryos (Fig. 2F) showed very low methylation levels. In triploid diandric two-cell embryos (Fig. 2G), the larger part of the
nuclear volume corresponding to the two male genomes was demethylated, whereas the maternal third of the nucleus was highly methylated. In triploid digynic embryos (Fig. 2H), approximately two-thirds of the nucleus were methylated and one-third was demethylated.

Abnormal methylation patterns in embryos from normal matings

In this study, 100 two-cell embryos from 25 non-superovulated and mated females, as well as 100 embryos from eight superovulated females, were analyzed by anti-MeC immunofluorescence. The great majority (90 and 87%, respectively) showed differentially methylated and spatially separated parental genomes (Fig. 3A and Table 1). Two to 3% of embryos showed methylation patterns that were indistinguishable from those in androgenetic embryos (Fig. 3B). One to 2% had one completely demethylated nucleus and one differentially methylated nucleus (Fig. 3C). One and 4%, respectively, were endowed with one completely demethylated and one completely methylated nucleus (Fig. 3D). One to 3% displayed one completely methylated and one differentially methylated nucleus (Fig. 3E). Two to 4% exhibited gynogenetic methylation patterns with two methylated nuclei (Fig. 3F). In all two-cell embryos analyzed, the second polar body was highly methylated, demonstrating the sensitivity and specificity of MeC staining. Altogether, 10–13% of two-cell embryos from normal matings showed abnormal methylation patterns, which may be associated with disturbances in establishing parent-specific developmental programs during early embryogenesis.

DISCUSSION

Our nuclear transfer experiments suggest that the cellular machinery of the fertilized egg is not capable of changing the methylation patterns of transplanted maternal (methylated) or paternal (demethylated) pronuclei. The inability of the egg to demethylate the two maternal genomes in diploid parthenogenetic embryos demonstrates that the demethylation activity is restricted to paternal DNA, regardless of the number of genomes present. It is plausible that the active demethylation of the paternal zygotic genome has its basis in genomic remodeling of the sperm following fertilization. The highly condensed sperm chromatin must be decondensed in the egg and undergo protamine–histone exchange prior to pronucleus formation (1,12,13). During this period of protein exchange, the paternal DNA is unusually loosely packaged, perhaps even naked (14). This provides a unique opportunity for the binding of demethylating enzymes and/or other protein factors. Once the nucleosomal configuration is restored and nuclear envelope construction is complete, the paternal chromatin physically resembles the maternal chromatin and, thus, may no longer be susceptible to demethylase or methyltransferase activity in the egg. Although the existence of a demethylase(s) in the early embryo seems certain (5,6,15), its identity and specificity remain to be determined. Although several possible biochemical activities have been reported, i.e. a repair enzyme that excises MeC, a processive enzyme that cleaves the methyl group from MeC and a ribozyme-like demethylase, these studies could either not be reproduced or yielded conflicting results (16).

In contrast to the paternal pronucleus, which is actively and passively demethylated in the normal fertilized egg, the maternal pronucleus undergoes only gradual demethylation by a replication-dependent mechanism (5,7). It is currently thought that the maintenance methyltransferase Dnmt1 is retained in the cytoplasm from the oocyte to the blastocyst stage (with a transient entry at the eight-cell stage), which prevents binding to target sites in the nucleus (17). If no methyl groups are added to the newly synthesized DNA strands, half

Table 1. Frequency of two-cell embryos with normal and abnormal methylation patterns

<table>
<thead>
<tr>
<th>Embryos derived from matings with:</th>
<th>% Embryos with normal methylation pattern A % Embryos with abnormal methylation patterns Total</th>
</tr>
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<tbody>
<tr>
<td>Non-superovulated females</td>
<td>90</td>
</tr>
<tr>
<td>Superovulated females</td>
<td>87</td>
</tr>
</tbody>
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A–F represent the different methylation patterns which are shown in Fig. 3. One hundred two-cell embryos were analyzed for each experiment.
of the remaining methyl groups are lost with every round of DNA replication.

Although the biological significance of genome separation and differential methylation in the early diploid embryo remains unclear, it is likely to be associated with parent-specific genomic imprinting which is a hallmark of mammalian development (18,19). Active demethylation of the paternal zygotic genome may make it easier for the cellular machinery of the fertilized egg to remodel the paternal chromosomes, to erase male germ-line-derived methylation imprints, and to re-establish higher-order chromatin structures in order to reprogram appropriate patterns of gene expression for embryonic development (5,6).

The mechanism by which specific paternal methylation marks are protected at imprinted loci during this demethylation is unknown.

A proportion of one- and two-cell embryos from normal matings show abnormal methylation patterns, which may result from disturbances in topological separation and/or differential demethylation of the two parental genomes. Those embryos with androgenetic or gynogenetic methylation patterns may be expected to fail early in development. It is possible that epigenetic reprogramming defects in the early diploid embryo lead to spontaneous abortions during development. The higher incidence of methylation disturbances in embryos from superovulated females, compared to non-superovulated females, may reflect the overall reduction in embryo quality, and supports our hypothesis that abnormal MeC staining is an indicator of early developmental failure.

MATERIALS AND METHODS

Preparation of mouse embryos

Fertilized eggs for normal and for nuclear transfer embryos were collected, handled and cultured according to standard procedures (20) from superovulated B6CBAFI females mated with same genotype males, at 20 h after human chorionadotropin (hCG) injection. Normal fertilized embryos were flushed and collected from superovulated B6CBAFI (34–44 h post-hCG) and non-superovulated B6C3FI females (22–32 h after fertilization).

Nuclear transplantation for the construction of androgenetic and gynogenetic embryos, whose genome is, respectively, either wholly of paternal or maternal origin, and of triploid diandric and digynic embryos was performed as described previously (21). The time of pronuclear transfer between diandric and digynic embryos was performed as described either wholly of paternal or maternal origin, and of triploid diploid embryo lead to spontaneous abortions during development.

For diploid parthenogenetic embryos, unfertilized eggs were activated 18–19 h post-hCG injection by immersing in 7% ethanol in culture medium for 4.5 min, cultured in medium containing 5 µg/ml cytochalasin B for 4 h to prevent extrusion of the second polar body, then washed and cultured to the required stage.

Air-dried preparations were made according to the method of Tarkowski (22). The embryos were placed in hypotonic solution (1% sodium citrate) at room temperature for 10 min, then transferred in groups of two to four in a small drop of the same solution (~5 µl) to a well cleaned glass slide and fixed immediately with several drops of a freshly prepared mixture of methanol and acetic acid (3:1). After air-drying, the slides were stored in a covered box for up to several weeks.

MeC staining

Methylated DNA was visualized by a well-characterized monoclonal antibody against MeC (5,7). Some embryos were stained with a monoclonal anti-DNA antibody (23). For immunofluorescence staining, the embryo preparations were treated with 100 µg/ml RNase A in 2× SSC at 37°C for 60 min and with 0.01% pepsin in 10 mM HCl at 37°C for 10 min, and then dehydrated in an ethanol series (70, 85 and 100%). The embryo preparations were denatured in 70% formamid, 2× SSC for 1 min at 80°C and then dehydrated in an ice-cold ethanol series. After brief air-drying, the slides were first incubated with blocking solution (3% BSA, 0.1% Tween 20, 4× SSC) in a Coplin jar for 30 min and then with mouse anti-MeC antibody (hybridoma supernatant), diluted 1:50 with PBS, in a humidified incubator at 37°C for 30 min. The slides were then washed in PBS three times for 10 min each and incubated for 30 min with fluorescein-isothiocyanate (FITC)- or Cy3-conjugated anti-mouse IgG and IgM (Dianova), appropriately diluted with PBS. After three further washes with PBS, the preparations were counterstained with 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) in 2× SSC for 5 min. The slides were mounted in 90% glycerol, 0.1 M Tris–HCl pH 8.0 and 2.3% 1,4-diazobicyclo-2,2,2-octane.

Images were taken with a Zeiss epifluorescence microscope equipped with a thermoelectronically cooled charge-coupled device camera (Photometrics CH250), which was controlled by an Apple Macintosh computer. Gray scale source images were captured separately with filter sets for FITC, Cy3 and DAPI. Gray scale images were pseudocolored and merged using VYSIS and ADOBE Photoshop software. It is worth emphasizing that, although a digital imaging system was used, all signals were clearly visible by eye through the microscope.

REFERENCES
