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Methylation Dynamics in the Early Mammalian Embryo: Implications of Genome Reprogramming Defects for Development

T. Haaf (✉)

Johannes Gutenberg-Universität Mainz, Langenbeckstrasse 1, Bau 601, 55131 Mainz, Germany
haaf@humgen.klinik.uni-mainz.de

Abstract In mouse and most other mammalian species, the paternal and maternal genomes undergo parent-specific epigenetic reprogramming during preimplantation development. The paternal genome is actively demethylated within a few hours after fertilization in the mouse, rat, pig, bovine, and human zygote, whereas the maternal genome is passively demethylated by a replication-dependent mechanism after the two-cell embryo stage. These genome-wide demethylation waves may have a role in reprogramming of the genetically inactive sperm and egg chromatin for somatic development. Disturbances in this highly coordinated process may contribute to developmental failures and defects in mammals. The frequency and severity of abnormal phenotypes increase after interfering with or bypassing essential steps of gametogenesis, early embryogenesis, or both. Nevertheless, it is plausible that normal fertilization, assisted reproduction, and embryo cloning are all susceptible to similar dysregulation of epigenetic components. Although the mouse may be an excellent model for early human development, species and strain differences in the molecular and cellular events shortly after fertilization may have important implications for the efficiency of epigenetic reprogramming and the incidence of reprogramming defects. Some species, i.e., rabbit and sheep, do not require drastic genome-wide demethylation for early development, most likely because the transition from maternal to embryonic control occurs relatively late during preimplantation development. A better understanding of key reprogramming factors—in particular the demethylase activity in the fertilized egg—is crucial for improving human infertility treatment and the efficiency of mammalian embryo cloning.

In mammals, both the paternal and the maternal genome are required for normal development (McGrath and Solter 1984; Surani et al. 1986). Genomic imprinting is an epigenetic mechanism by which the expression of a subset of genes becomes dependent on their parental origin (Bartolomei and Tilghman 1997). Following the establishment of imprinting in the male and female germ lines, respectively, the two parental genomes exhibit functional differences at fertilization. Methylation of 5′-cytosine residues in CpG dinucleotides is critical for regulating the temporal, spatial, and parent-specific gene expression patterns. DNA methylation establishes and maintains an inactive chromatin structure by posttranslational histone modifications (Wolffe and Matzke 1999; Jaenisch and Bird 2003). The sperm and egg genomes that are combined at fertilization are both highly methylated; however, there are important germ line-specific differences in the methylation patterns of genomic sequences (Reik et al. 2001; Haaf et al. 2004). Whereas the genomic methylation patterns and levels in somatic cells are generally stable and heritable, dramatic genome-wide changes occur in early embryos, where the two complementary parental genomes must be reprogrammed for somatic development. Methylation reprogramming may help to "revive" the inactive sperm and egg genomes and to restore a broad developmental potential in embryonic cells. This entire process appears to be maternally driven. However, the cellular machinery and factors in the fertilized egg that can reprogram the two very different gamete nuclei as well as a somatic cell nucleus that has been introduced into an oocyte during cloning remain to be elucidated. This chapter reviews recent cytological and molecular experiments that have addressed fundamental questions related to the reprogramming mechanisms and capabilities of mammalian oocytes.

1
Methylation Reprogramming in Early Mouse Embryos

Immunofluorescent staining with an antibody against 5-methylcytosine (mC) provides a valuable tool to directly visualize the genome-wide demethylation and remethylation waves in preimplantation mouse embryos (Rougier et al. 1998; Mayer et al. 2000a; Santos et al. 2002). The oocyte genome completes its meiotic maturation after sperm entry by extrusion of the second polar body. Very shortly after fertilization, the mouse zygote shows equally high methylation levels of sperm nucleus, maternal meiotic metaphase II chromosomes, and second polar body (Haaf et al. 2004). The activated oocyte then remodels the gamete chromatin into functional male and female pronuclei that oppose each other. In the normal diploid mouse zygote, the paternal genome is rapidly and drastically demethylated before onset of the first DNA

replication. The maternal genome, although exposed to the same cytoplasm, is resistant to this active demethylation process. Bisulfite sequencing studies revealed that paternal zygotic demethylation affects widely different classes of repetitive and single-copy sequences (Oswald et al. 2000). Only the control regions of imprinted genes seem to be protected against the maternal demethylase activity.

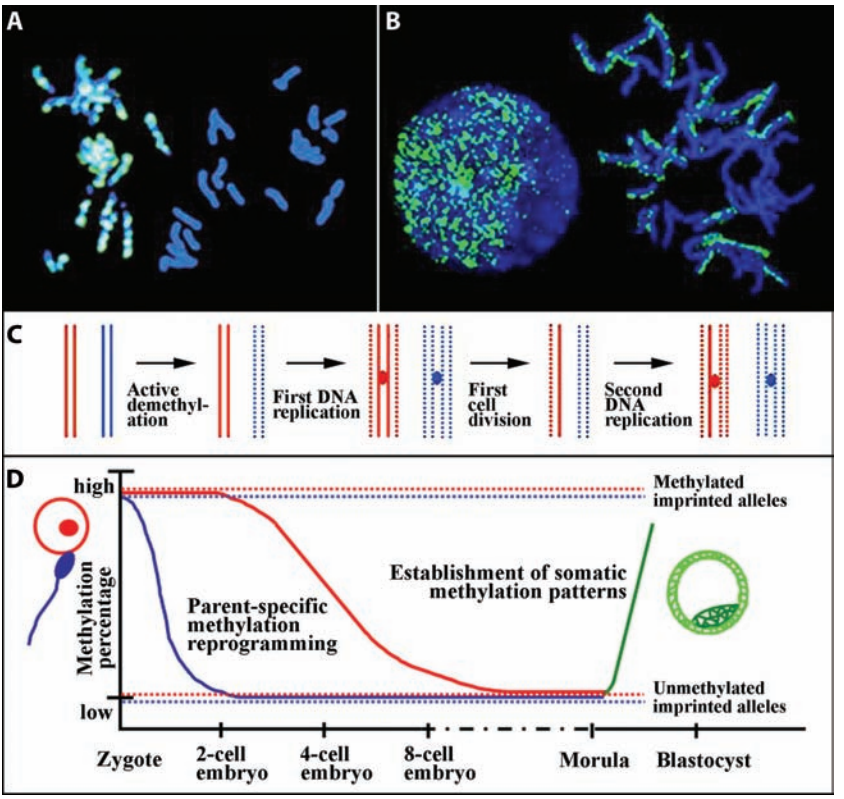
The mouse oocyte efficiently demethylates multiple male pronuclei in polyspermic embryos (Santos et al. 2002), whereas it cannot demethylate the additional female genome in parthenogenetic, gynogenetic, and triploid digynic embryos (Barton et al. 2001). This suggests that active demethylation depends on a sperm-derived factor. Before the male pronucleus can be formed, the highly compacted sperm chromatin must be decondensed and the protamines be exchanged by histones (Perreault 1992; Kanka 2003). During this period, the paternal DNA is unusually loosely packaged and provides a unique opportunity for binding of a demethylating enzyme whose molecular nature remains unknown. Paternal zygotic demethylation may be facilitated by strikingly different histone modifications in paternal and maternal pronuclei. The paternal zygotic genome becomes transiently associated with hyperacetylated histone H4 (Adenot et al. 1997; Santos et al. 2002), whereas the maternal genome is preferentially associated with methylated histone H3 (Cowell et al. 2002).

The global methylation level of the maternal genome is maintained up to the two-cell embryo stage. Interestingly, even breakdown of the pronuclear envelopes and first mitosis do not lead to an intermingling of the two parental chromosome sets (Fig. 1A). Topological genome separation is preserved at least up to the two-cell stage, each (the methylated maternal genome and the demethylated paternal genome) occupying approximately half of the nucleus (Fig. 1B, left nucleus). The existence of separate nuclear compartments may facilitate parent-specific methylation reprogramming in the early embryo (Mayer et al. 2000b; Haaf 2001). In contrast to first metaphase, where both sister chromatids of the maternal chromosomes are equally methylated, in the second metaphase only one of the two sister chromatids remains methylated (Fig. 1B, right metaphase). This sister chromatid differentiation is consistent with a replication-dependent demethylation mechanism of the maternal genome (Fig. 1C).

Because mC cannot be incorporated directly into replicating DNA, maintenance of DNA methylation patterns requires DNA methyltransferase 1 (DNMT1), which has a high affinity for hemimethylated sites that are generated transiently during DNA replication. DNMT1 detects methylated CpG sites in the parental DNA strand and adds methyl groups to the corresponding sites in the newly synthesized strand (Bestor 2000). Gradual demethylation of

Fig. 1 A–D **A, B** Parent-specific genome methylation patterns in early mouse embryos. Nuclei were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mC antibody (*green*) and counterstained with 4′-6′-diamidino-2-phenylindole (DAPI) (*blue*). **A** One-cell embryo during first mitosis. The maternal chromosomes are methylated in both sister chromatids and spatially separated from the fully demethylated paternal chromosomes. **B** Two-cell embryo in which one cell goes through second mitosis. The interphase nucleus exhibits a methylated maternal and a demethylated paternal compartment. During second mitosis, only one of the two sister chromatids of the maternal chromosomes remains methylated. **C** Differential demethylation of maternal (*red*) and paternal (*blue*) chromosomes during mouse preimplantation development. Both DNA strands of the paternal chromosome (*DNA double helix*) are already demethylated (*blue dotted lines*) in the zygote before onset of the first DNA replication. The maternal chromosome is protected from this active demethylation process. Following the first DNA replication cycle in the absence of maintenance DNA methyltransferase, the maternal chromosome consists of two hemimethylated sister chromatids (*DNA double helices*). After the first cell division and another round of DNA replication, the maternal chromosome consists of a hemimethylated and a fully demethylated sister chromatid, resulting in differential sister chromatid staining. **D** Methylation dynamics in early mouse embryos. The paternal (*blue*) genome undergoes active zygotic demethylation, whereas the maternal (*red*) genome is gradually demethylated after the two-cell stage. Both parental genomes are equally demethylated at the morula stage and then remethylated. The newly established somatic methylation patterns (*green line*) are identical on both parental alleles. Embryonic lineages derived from the inner cell mass (*dark green*) are more heavily methylated than the trophoblast (*light green*). Imprinted genes (*dotted lines*) escape this genome-wide methylation reprogramming after fertilization and maintain their germ-line methylation patterns. Methylated imprinted alleles do not become demethylated, and demethylated imprinted alleles are not remethylated

the maternal genome is achieved by sequestration of DNMT1 from the nucleus into the cytoplasm, which prevents binding to its target sites in hemimethylated DNA (Cardoso and Leonhardt 1999; Ratnam et al. 2002). When half of the methyl groups are lost with every round of replication, full double-stranded demethylation in one chromatid occurs after two cell cycles (Fig. 1C, red ideograms). Consequently, four-cell embryos have a much weaker mC density over the maternal half of the nucleus. After the eight-cell embryo stage, paternal and maternal chromosomes show equivalently low methylation levels (Mayer et al. 2000a). Later, in mouse blastocyst-stage embryos, genome-wide de novo methylation (Fig. 1D, green graph) occurs preferentially in the inner cell mass, establishing somatic methylation patterns in cells that give rise to the different embryonic lineages. Trophoblast cells that give rise to the extraembryonic lineages become less heavily methylated (Dean et al. 2001; Reik et al. 2001).



2
Species Differences in Methylation Reprogramming

If genome-wide methylation reprogramming in the early embryo is fundamental to the formation of totipotent embryonic cells, one would expect that the preimplantation methylation dynamics is conserved among mammalian species. Indeed, active demethylation of the paternal zygotic genome is observed in mouse, rat, pig, bovine, and human embryos (Mayer et al. 2000a; Dean et al. 2001; Beaujean et al. 2004a). However, the timing of remethylation already differs between species. In bovine embryos, considerable de novo methylation already occurs at the 8- to 16-cell stage, whereas in mouse, remethylation begins only in the blastocyst. In sheep and rabbit embryos anti-mC immunofluorescence revealed equally high methylation levels of the two parental genomes throughout preimplantation development (Beaujean et al. 2004a; Shi et al. 2004). The lack of detectable genome-wide methyl-

ation changes in these two species suggests that neither active nor passive demethylation is an obligatory requirement for epigenetic reprogramming after fertilization.

Interestingly, mouse sperm injected into sheep oocytes is significantly demethylated, although to a lesser extent than mouse sperm in murine oocytes. Ram sperm, which is not demethylated in sheep oocytes, can be partially demethylated in bovine oocytes (Beaujean et al. 2004b). Evidently, the demethylating activity of the ooplasm differs among species, being the highest in mouse, medium in bovine, and low in sheep (and rabbit) oocytes. However, the demethylation process must also involve a sperm-derived factor(s), i.e., differences in male pronuclear chromatin structure. The biological significance of the observed species differences in methylation reprogramming remains unclear. The timing and degree of demethylation are likely to play an important role for remodeling the two complementary germ line genomes into a diploid somatic genome (Haaf 2001; Haaf et al. 2004). The mouse embryonic genome, which is the most rapidly and drastically demethylated of all analyzed species, is already activated in the two-cell stage (Schulz 1993). Demethylation of the paternal genome in human, pig, and bovine zygotes is also associated with a relatively early transition from maternal to embryonic control of development (Memili and First 2000; Kanka 2003). By contrast, in rabbit and sheep embryos, which maintain high methylation levels after fertilization, maternal factors seem to control the preimplantation period, and transition to embryonic control of development occurs only at the 8- to16-cell stage (Manes 1973).

3 Methylation Reprogramming Defects

In mouse, cow, and most other mammalian species, the paternal and maternal genomes are demethylated by different mechanisms and at different times during preimplantation development (Fig. 1C, D). Disturbances in this spatially and temporally highly coordinated process provide one important explanation for the high rate of embryo loss after fertilization (Shi and Haaf 2002). Immunofluorescence staining demonstrated 20% abnormal methylation patterns in mouse two-cell embryos that were flushed from the oviducts of superovulated females, compared to 10% from non-superovulated females. Of the embryos, 14% from superovulated females, but only 5% from non-superovulated matings, failed to develop in culture to the blastocyst stage. This reflects an overall reduction in the reprogramming capability of the oocyte and embryo quality after hormone treatment. The dramatic differ-

ences in methylation reprogramming and development of in vitro fertilized mouse embryos that were cultured in different media may be due to a suboptimal environment at or shortly after fertilization. Since the preimplantation embryo is much less protected than the germ cells, this may be the time when environmental factors, i.e., nutrition and drugs, have the greatest impact on epigenetic reprogramming. Acetaldehyde, the toxic metabolic product of ethanol—and by extrapolation alcohol consumption—can cause methylation disturbances and developmental arrest in early mouse embryos. The efficiency of methylation reprogramming also depends on genetic factors (strain- and species-specific differences). Embryos from most inbred mouse strains or hybrids can efficiently develop in culture to the blastocyst stage, whereas embryos from Naval Medical Research Institute, USA (NMRI) and other so-called blocking strains show high incidences (20%–60%) of abnormal methylation patterns and arrest in in vitro development.

Disturbances in the establishment or maintenance of the appropriate parent-specific methylation patterns may also contribute to the medical problems of assisted reproductive technologies (ART). Genome-wide alterations cause early developmental failure and embryo loss (Barton et al. 2001; Shi and Haaf 2002), whereas methylation changes at specific gene loci have been associated with aberrant fetal growth and abnormal phenotypes. Initially it was shown in mouse and ruminants that isolation, manipulation, and culture of gametes and early embryos can affect the methylation and regulation of imprinted genes, leading to phenotypic defects (Koshla et al. 2001, Young et al. 2001). Recent studies in children conceived with ART also reported unexpectedly high incidences of certain rare human imprinting diseases, such as Beckwith-Wiedemann and Angelman syndromes, resulting from epigenetic DNA methylation defects, specifically an abnormal hypomethylation of the normally methylated maternal alleles (Maher et al. 2003; Ludwig et al. 2005). In light of growing concerns about epigenetic disturbances resulting from superovulation and embryo culture, there is clearly a need for both basic research on reproductive epigenetic events and long-term follow up studies of children born of ART.

Similar to ART, somatic cell nuclear transfer technologies interfere with essential reprogramming events in gametogenesis and early embryogenesis; however, the epigenetic insults in cloned embryos are much more frequent and pronounced. A somatic cell nucleus that has been introduced into an oocyte during cloning can be reprogrammed to some extent for somatic development, but the embryo cloning efficiency is generally low and somewhat variable between species (Solter 2000; Shi et al. 2003). Methylcytosine staining of cloned bovine embryos demonstrated incomplete or delayed demethylation of the donor genome (or both effects) (Bourc'his et al. 2001; Dean et al.

2001). The genome of cloned embryos is likely to be a mixture of normally and abnormally methylated sequences. Expression profiling of more than 10,000 genes showed frequent (4%) abnormal gene expression in placentas and livers of neonatal cloned mice (Humpherys et al. 2002). Reactivation of key embryonic genes that are necessary for the development of pluripotent cell lineages may be particularly inefficient in clones derived from somatic cell nuclei (Bortvin et al. 2003). Disruption of the allele-specific methylation and expression patterns of imprinted genes were observed in more than 95% of cloned mouse blastocysts (Mann et al. 2003). Collectively, these results suggest that the frequent developmental failures and defects resulting from cloning are largely due to epigenetic reprogramming defects.

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