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DNA methylation reprogramming and DNA repair in the mouse zygote

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ABSTRACT Here, we summarize current knowledge about epigenetic reprogramming during mammalian preimplantation development, as well as the potential mechanisms driving these processes. We will particularly focus on changes taking place in the zygote, where the paternally derived DNA and chromatin undergo the most striking alterations, such as replacement of protamines by histones, histone modifications and active DNA demethylation. The putative mechanisms of active paternal DNA demethylation have been studied for over a decade, accumulating a lot of circumstantial evidence for enzymatic activities provided by the oocyte, protection of the maternal genome against such activities and possible involvement of DNA repair. We will discuss the various facets of dynamic epigenetic changes related to DNA methylation with an emphasis on the putative involvement of DNA repair in DNA demethylation.

KEY WORDS: *DNA repair, zygote, DNA demethylation, epigenetic reprogramming*

Introduction

The cellular identity is defined by the composition of RNAs, proteins and other macromolecules. The blueprint of this information resides in the genome, which uses in various cell types and at various developmental stages different parts of its information content for gene expression. This differential gene expression is highly regulated and has its central basis in the differential organization of chromatin along the invariant genomes, established in so called epigenome. The epigenome, which is specific for different cell types, is controlled by a complex interplay between histone and DNA modifications (Jenuwein and Allis, 2001; Kouzarides, 2007). The unique distribution of differentially modified nucleosomes along genomic DNA, which itself is also specifically modified by methylation and hydroxymethylation at cytosine residues, allows the existence of virtually unlimited possible variations of a particular genome. Thank to such variability, cells containing the same DNA sequence can belong to thousands of different cell types comprising different tissues in complex multicellular organisms. The process, by which heritable phenotype is achieved through modification of a chromosome, that involves no alteration to the underlying DNA sequence, is defined as epigenetics (Berger *et al.*, 2009). The first level of epigenetic information is encoded in the distribution profile of 5-methylcytosine (5mC) residues which in animals are almost exclusively found within CpG dinucleotides and impose gene silencing (Bird and Wolffe, 1999). Another level includes various histone modifications, such as methylation, acetylation, phosphorylation, ubiquitination, sumolyation and ADP-ribosylation, as well as numerous variants of core and linker histones (Kouzarides, 2007). Since different cell types have different epigenomes, during development the epigenome of the progenitor cell changes or is programmed/reprogrammed upon differentiation into specialized tissue specific cell type.

The mammalian development begins with the fusion of two epigenetically distant cells: an egg and sperm. The resulting zygote within few hours reshapes both epigenomes and developes further to totipotent preimplantation embryo, which later gives rise to pluripotent cells, residing in the inner cell mass of a blastocyst (Evans and Kaufman, 1981; Sherman, 1975). The contact of a spermatozoa and an oocyte is the first trigger, which turns on the developmental program stored in the mature oocyte apart from the maternal DNA. Once being set off, the program can also act on genomes, which might have various origins and specifications, by converting their epigenetic features into embryo specific state, or in other words – reprogramming them. This ability allows different manipulations with the zygote such as the creation of parthenogenetic (monoparental) embryos, and nuclear/pronuclear

Abbreviations used in this paper: 5mC, 5-methylcytosine EGA, embryonic gene activation; PGC, primordial germ cell.

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transfer, including interspecies transfer (Chang *et al.*, 2003; Hammer *et al.*, 2001; Liu *et al.*, 2004b; McGrath and Solter, 1983; McGrath and Solter, 1984; McGrath and Solter, 1986). The mature oocyte, awaiting the fertilization at metaphase II, gets activated by the penetrating sperm, but it also can be artificially activated *in vitro* by chemicals, or even by temperature and pH shifts (Meo *et al.*, 2004; Nagai, 1987; Prather *et al.*, 1991). After activation or fertilization, the oocyte completes meiosis II and one set of maternal chromosomes is extruded as second polar body. The remaining haploid set decondenses and forms a separate maternal pronucleus. Also the sperm DNA penetrated into the oocyte decondenses, exchanging DNA bound protamines against histones derived from the oocyte and gets packaged into a separate pronucleus, that contains expanding interphase like chromatin. The structural reorganizations of the parental genome lead to functional changes from transcriptionally inactive state to transcriptionally active. Some transcriptional activity is detected already in the developing zygote and the total embryonic gene activation (EGA) occurs at 2-cell stage in mouse, or at 8-16 cell stage in bovine and rabbit embryos (Kanka, 2003).

DNA methylation reprogramming

The very first studies of genome wide DNA methylation by HpaII methylation sensitive digestion in mammalian gametes and preimplantation embryos revealed that sperm DNA is hypermethylated, while DNA from the oocyte is relatively undermethylated (Monk *et al.*, 1987). Further studies using bisulfite sequencing could verify this for selected single copy genes and repetitive elements (Oswald *et al.*, 2000). Nevertheless there were also repetitive elements found, which are highly methylated in both gametes, like IAP and Etn (Kim *et al.*, 2004; Lane *et al.*, 2003). The rapidly developing genome wide high-throughput DNA methylation analysis technologies provide insight into sperm DNA methylation profile. These data show, that, indeed, repetitive elements, intronic and intergenic sequences are highly methylated (Popp *et al.*, 2010), but promoter methylation patterns resemble these of ES cells, excluding promoters of pluripotency related genes (Farthing *et al.*, 2008). Further development of methods for comprehensive DNA methylation data analysis will soon enable the follow up research on oocytes and cleavage stage embryos.

After fertilization the overall methylation level goes down, reaching its minimum at early morula stage (Kafri *et al.*, 1993; Santos *et al.*, 2002). First *de novo* methylation event coincides with the differentiation of cells in the developing blastocysts into inner cell mass (ICN) and trophectoderm. DNA methylation is clearly detectable by 5mC specific antibodies in ICN, which gives rise to the embryo proper, but to lesser extent in the trophectoderm, which forms placenta after the implantation into uterus (Dean *et al.*, 2001). The most remarkable event is the loss of 5mC specific antibody signal, observed in the paternal pronucleus of zygote prior the first round of replication, which was interpreted as active paternal DNA demethylation (Mayer *et al.*, 2000; Oswald *et al.*, 2000; Rougier *et al.*, 1998; Santos *et al.*, 2002). The suggested reduction of DNA methylation in the paternal pronucleus was also confirmed by bisulfite sequencing (Oswald *et al.*, 2000; Wossidlo *et al.*, 2010). The thorough investigations revealed that paternal demethylation starts after the replacement of protamines by histones, proceeds up to the beginning of DNA replication (Lepikhov and Walter, 2004; Santos *et al.*, 2002) and probably continues further (Wossidlo *et al.*, 2010). According to 5mC antibody signal, the demethylation process affects most of 5mC content in the paternal pronucleus of the mouse zygote, while maternal DNA retains steady level of methylation. The loss of 5mC antibody signal is accomplished as early as 6-8 hours after fertilization (Santos *et al.*, 2002). At the same time bisulfite analyses of Line1 and Etn repetitive sequences reveal rather modest reduction in methylation (Wossidlo *et al.*, 2010).

Since both methods utilize different principles, each has its flaws and advantages. The genomic regions chosen for bisulfite analysis might not be representative for showing global changes in DNA methylation and the PCR followed after bisulfite treatment could be biased towards amplification of either methylated or non-methylated sequences. At the same time the antibody binding could be affected by the distribution pattern of the epitope, thus not reflecting the actual amount of the methylation (Weber *et al.*, 2005). The possibility of further modification of 5mC to 5-hydroxymethylcytosine (5hmC) should also be considered because, in this case, 5mC is no more detectable by specific antibody, but still indistinguishable from 5hmC by bisulfite analysis (Jin *et al.*, 2010). The potential sources of zygotic demethylation data variability and its dependence on the methods used are discussed elsewhere (Aranyi and Paldi, 2006).

Though sometimes being variable in the degree of progression, the zygotic paternal demethylation seems to be common among mammals and is so far reported for mouse (Oswald *et al.*, 2000; Rougier *et al.*, 1998), rat (Dean *et al.*, 2001; Zaitseva *et al.*, 2007), human (Fulka *et al.*, 2004), pig (Dean *et al.*, 2001; Fulka *et al.*, 2006), cattle (Dean *et al.*, 2001), sheep (Hou *et al.*, 2008) and rabbit (Lepikhov *et al.*, 2008). Mouse zygotes serve as typical and relatively well characterized model to study epigenetic reprogramming in mammalian preimplantation embryos, therefore most of research data described and discussed here relate to the experiments on mouse embryos and gametes.

It is important to mention, that differentially methylated regions (DMRs) of imprinted genes and certain classes of repeat sequences remain refractory to the general demethylation (Lane *et al.*, 2003; Oswald *et al.*, 2000). In fact both paternal and maternal imprints escape further demethylation during embryonic cleavage stages, where so-called “passive, i.e. replication dependent, DNA demethylation takes place. One of the intriguing questions concerning the demethylation in zygotes is the selectivity of the process. What protects the maternal DNA and paternal imprints against demethylation, or alternatively – what targets the demethylation machinery to the sequences being demethylated? Another alternative, which should also be considered is the selective re-methylation occurring immediately after non-selective global demethylation. The instructive role can be assigned to chromatin structure, to specific DNA binding factors or both.

Chromatin modifications in the zygote

Besides DNA methylation changes, the chromatin structure of gametes is also altered upon their fusion. The chromatin of sperm is dramatically different from that of oocyte. Unlike histone containing oocyte chromosomes, sperm DNA is packaged by protamines. Only a minor portion of sperm chromatin (1% in mouse and

up to 10% in human) has a histone containing nucleosome like structure. ChIP-on-chip analysis of histones in sperm chromatin revealed, that nucleosomal structures are maintained at promoter region of developmentally important genes (Brykczynska *et al.*, 2010; Hammoud *et al.*, 2009). After fertilization, the tight protamine packaging of sperm genome is quickly replaced by histone nucleosomes packaging. These maternally provided histones assemble into nucleosomes that lack histone methylation marks but are heavily pre-acetylated (Adenot *et al.*, 1997; Santos *et al.*, 2002). In contrast, the maternal chromatin in mature oocyte is hypoacetylated and contains various types of histone methylation marks (Arney *et al.*, 2002; Kim *et al.*, 2003; Santos *et al.*, 2005). In the first hours post fertilization the paternal pronucleus acquires mono-methylation marks, such as H3K4me1, H3K9me1 and H3K27me1 (Lepikhov and Walter, 2004; Santos *et al.*, 2005). During further development repressive and transcription-activating modifications behave differently – activating H3K4 methylation progresses up to tri-methylated form in late zygotes (Lepikhov and Walter, 2004; van der Heijden *et al.*, 2005), while the stable repressive methylation marks H3K9me2-3, H3K27me3 and H4K20me3 are mostly absent from paternal pronuclei through all zygotic stages. However, these histone methylation marks are detectable in the maternal chromatin through all the zygotic stages. Furthermore, the asymmetry between the two paternal pronuclei is also reflected by preferential accumulation of H3.3 histone variant in paternal pronuclei in PN2 – PN3 stage zygotes (Torres-Padilla *et al.*, 2006; van der Heijden *et al.*, 2005). The dynamics of histone modifications in relation to DNA methylation changes are schematically pictured on Fig. 1.

Links between DNA and histone modification reprogramming

The maternal nucleosomes are marked by repressive histone marks and retain the methylation status of DNA (Fuks *et al.*, 2003). Therefore it is tempting to assume, that the repressive chromatin structure prevents maternal DNA from being demethylated. Indeed, there are some indirect evidences supporting this assumption. Liu *et al.* have demonstrated the ability of germinal vesicle (GV) oocytes to re-methylate the implanted demethylated paternal pronuclei and this re-methylation was also accompanied by H3K9 methylation (Liu *et al.*, 2004a). However, at the same time the artificial induction of H3K9me2 in paternal pronuclei by treating the zygotes with protein synthesis inhibitor cycloheximide did not abolish DNA demethylation (Liu *et al.*, 2004a). These data provide evidences, that DNA demethylating activity is inactive in GV oocyte and is triggered by the fertilization and completion of the meiosis. Similarly, the role of differential histone acetylation was also suggested (Cervoni and Szyf, 2001). Spinaci *et al.* reported the induced demethylation of maternal DNA in mouse zygotes, which were derived from oocytes with artificially increased histone acetylation levels (Spinaci *et al.*, 2003). The induced maternal demethylation also required the presence of DNA methyltransferase inhibitor 5-Aza-cytidine. Unfortunately this work has not been followed up and we could not reliably reproduce these data. Overall the data do not show a clear pattern of interdependence between DNA methylation and the regulatory role of certain histone modification on DNA methylation reprogramming. The histone – DNA methylation hierarchy is still questionable because examples for both as the higher-ranked player exist.

The leading role of histone modifications was shown in the following examples: in *Neurospora crassa* the presence H3K9me3

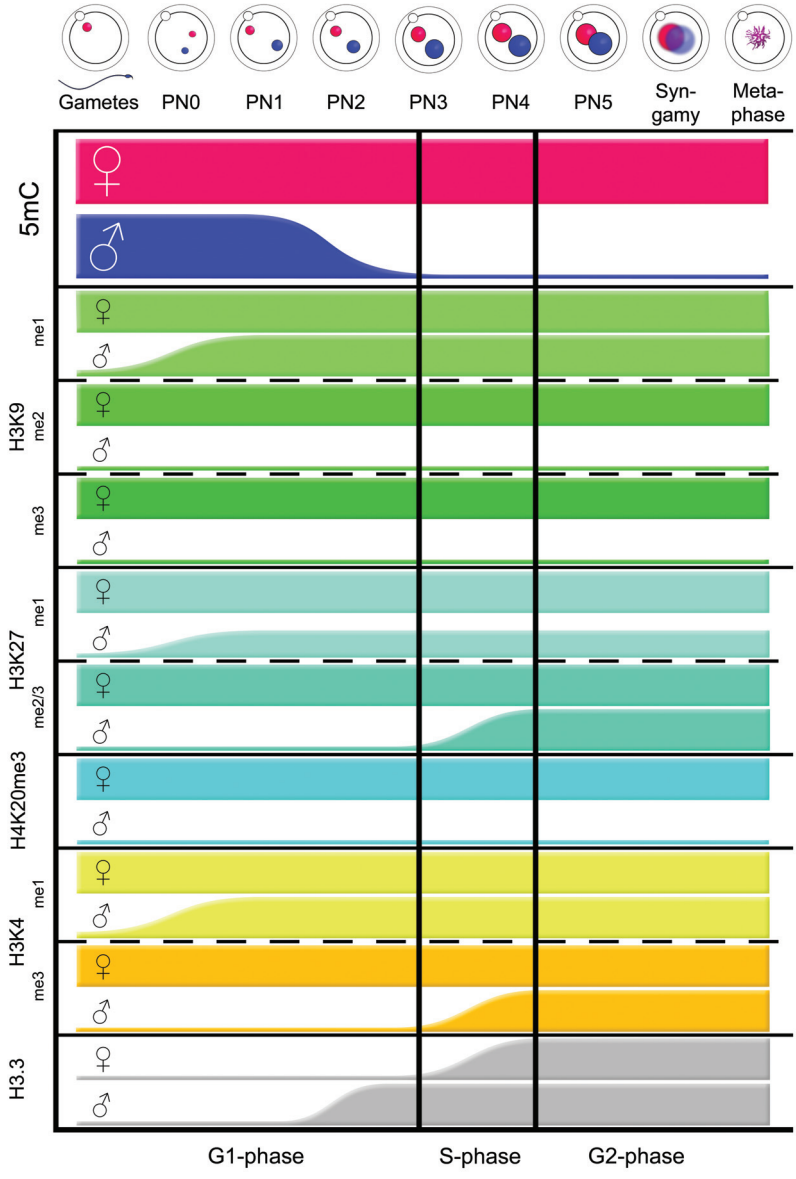

Fig.1. Dynamics of DNA methylation, histone exchange and histone modifications in the zygote. *The thickness of the bars reflects the relative amount of particular epigenetic mark in separate parental pronuclei. The data presented summarized from the following references: Arney* et al., *2002; Erhardt* et al., *2003; Lepikhov and Walter, 2004; Santos* et al., *2002; Santos* et al., *2005; Sarmento* et al., *2004; Torres-Padilla* et al., *2006; van der Heijden* et al., *2005.*

but not H3K9me2 is required for establishing DNA methylation (Tamaru *et al.*, 2003). In *in vitro* experiments the complex of Dnmt3a/Dnmt3L methyltransferases preferentially binds to histone H3 methylated at lysine 9 but not at lysine 4 thus indicating the guiding role of histone modifications in setting up DNA methylation by *de novo* Dnmt3a methyltransferases (Zhang *et al.*, 2010b). The physical and functional interaction between histone methyltransferases Suvar39h, which establishes repressive histone methylation marks, and DNA methyltransferases Dnmt3b further corroborates the superiority of chromatin modifications over DNA methylation (Lehnertz *et al.*, 2003). Similarly G9a and Ezh2 histone methyltransferases were also shown to direct DNA methylation in ES cells. (Epsztejn-Litman *et al.*, 2008; Vire *et al.*, 2006).

On the other hand, the regulation of histone modifications patterns by DNA methylation was also demonstrated. The depletion of DNA methylation by 5-Aza-cytidine treatment negatively influences H3K9me3 and H3K27me3 (Komashko and Farnham, 2010). The specially engineered transgene with regulated methylation status also associates with H3K9 methylation and looses histone acetylation if forced to be methylated (Hashimshony *et al.*, 2003).

In general these examples show that in many cases histone modifications and DNA methylation are closely related to each other. Therefore the proper or permissive chromatin environment might be one of the conditions required for selective DNA methylation reprogramming in mammalian zygote. Indeed, sperm DNA undergoes demethylation only after histone-protamine exchange and obviously not during the transition period, when DNA could be free from packaging proteins and hence accessible for demethylation enzymes (Lepikhov and Walter, 2004). In agreement with this suggestion is the fact, that in somatic cell nuclear transfer derived (SCNT) embryos the donor DNA is also subjected to demethylation though the protamine – histone exchange step is missing in this case (Lepikhov *et al.*, 2008; Wossidlo *et al.*, 2010).

Non-chromatin factors influencing DNA demethylation

The chromatin environment seems not to be the only essential prerequisite for zygotic demethylation. A couple of publications show that other DNA binding proteins are also involved in the regulation of the process. The depletion of maternally provided DNA/RNA binding factor PGC7/Stella from maternal pronucleus induces demethylation of maternal DNA alongside with paternal (Nakamura *et al.*, 2007). This factor, ubiquitously provided by oocyte, serves as protector against the demethylating activity in zygote though it is shown to be equally distributed among both parental pronuclei. Why the protection by PGC7/Stella is only effective in the maternal pronucleus – is still an open and very intriguing question. Methyl-CpG binding protein 3 (MBD3) has also been shown to provide the protection against demethylation. But its function is restricted to secure the paternal imprints maintenance such as methylation of differentially methylated domain (DMD) of H19 gene (Reese *et al.*, 2007). The role of MBD3 in the imprints protection is also associated with repressive chromatin remodeling complexes such as NuRD since MBD3 is part of the complex and its depletion negatively influences the complex formation (Ho and Crabtree, 2010; Reese *et al.*, 2007). Recent reports have also shown the implication of Elp3, a component of the elongator complex, into the paternal demethylation in zygotes. The knockdown of the protein resulted in impaired demethylation (Okada *et al.*, 2010). It is not yet clear if the protein is directly involved in demethylation, but it possesses so-called "radical SAM" domain which is known to be present in the enzymes, catalyzing radical reactions and using S-adenosylmethionine (SAM) as co-factor (Wang and Frey, 2007). The overexpression of Elp3, mutated within radical SAM domain, in mouse zygotes had similar effect as Elp3 knockdown – the paternal DNA demethylation was abolished (Okada *et al.*, 2010) suggesting the enzymatic involvement of Elp3 in the demethylation process.

DNA demethylation in primordial germ cells and the involvement of DNA methyltransferases in methylation reprogramming

Comparably to the preimplantation development also the primordial germ cell (PGC) development includes a demethylation process, which occurs in postimplantation mouse embryos between 10.5 dpc and 12.5 dpc. This demethylation is believed to be active because Dnmt1 was shown to be present in the nuclei and the process is very fast (Hajkova *et al.*, 2002; Seki *et al.*, 2005).

This demethylation process in PGCs has some similarities and differences to the process in the zygote and preimplantation embryos: in both cases the global reduction of methylation, i.e. detectable by immunofluorescence, is achieved. But in PGCs the imprints and IAP elements also undergo a rapid demethylation process (Hajkova *et al.*, 2002; Lee *et al.*, 2002). It is not clear if demethylation in PGCs and zygotes follow the same pathways and involve the same mechanisms. If the mechanisms are similar, then the differences in demethylated target sequences could be explained by a differential protection, targeting or re-methylation. The immunocytochemistry analyses show that the histone modification patterns share similar features, such as enrichment in H3K4 methylation, depletion of repressive H3K9me2, H3K9me3 and H3K27me3 (Hajkova *et al.*, 2008; Seki *et al.*, 2005). At the same time the composition and localization of DNA methyltransferases in PGCs differs from that in zygotes. Whereas in PGCs Dnmt3a is absent and Dnmt3b is located in the cytoplasm (Hajkova *et al.*, 2002), in zygotes Dnmt3a is present from one cell stage to 8 cell stage and Dnmt3b can be observed from two cell stage on in the nuclei (Hirasawa *et al.*, 2008). It could be assumed that Dnmt3a and Dnmt3b are responsible for maintaining methylation at specific sequences, but bisulfite sequencing of Dnmt3a/3b deficient blastocysts has shown that both enzymes are not essential for the imprint maintenance, at least for the H19 and Dlk/Gtl2 DMR, but Dnmt1s (somatic isoform) is required for the maintenance of DMRs methylation (Hirasawa *et al.*, 2008). That was quite surprising because Dnmt1o (oocyte-specific isoform) was reported to be absent from the nuclei in the preimplantation development except for 8 cell stage when it transiently enters the nuclei (Ratnam *et al.*, 2002) but later publications demonstrated the presence and nuclear localization of Dnmt1s of maternal origin and then after 2-cell stage of embryonic origin (Cirio *et al.*, 2008; Kurihara *et al.*, 2008). The continuous presence of Dnmt1s in the nuclei of preimplantation embryos argues against the simple mechanistic model of passive demethylation, which is

thought to be achieved by DNA replication without maintenance of methylation due to the retention of Dnmt1o in the cytoplasm (Grohmann *et al.*, 2005). Therefore the protection of specific sequences against active demethylation and targeting of the active and/or passive demethylation seems to be more likely.

The suggested mechanisms of DNA demethylation and potential demethylases

The mechanism behind the active paternal DNA demethylation is still unknown. Most likely the oocyte cytoplasm provides the demethylation factors, which are then directed or restricted to the paternal genome. In fact, DNA demethylation is not only limited to mammalian zygotes and PGCs; it is also observed in other cell types and under defined conditions, though it then affects only specific genomic regions and is not regarded as global (Kim *et al.*, 2009; Klug *et al.*, 2010; Metivier *et al.*, 2008). In all cases, active demethylation could be either accomplished by the removal of the methyl group in C5 position of the cytidine ring directly (*bona fide* demethylation) or by the removal of the complete cytosine base (indirect demethylation) (Morgan *et al.*, 2005).

So far, there are no clear evidences for the existence of a *bona fide* demethylase, which catalyzes the direct removal of the methyl group. Several candidates have been proposed, but none could be verified as a global demethylase. MBD2 was shown to demethylate DNA directly (Bhattacharya *et al.*, 1999), but this data could not been reproduced by the others (Ng *et al.*, 1999). In *Escherichia coli* the dioxygenases AlkA and AlkB are able to directly demethylate 3-methylcytosine (3mC) and their human homologs ABH2 and ABH3 were identified (Duncan *et al.*, 2002). Due to more stable C-C bond in 5mC compared to the less stable C-N bond in 3mC, it is biochemically unlikely that a dioxygenase directly demethylates 5mC. Nevertheless, the discovery of 5-hydroxymethyl-cytidine (5hmC) in mammalian DNA (Kriaucionis and Heintz, 2009; Tahiliani *et al.*, 2009) and corresponding dioxygenases TET1, TET2 and TET3 catalyzing the synthesis of 5hmC suggests a possible candidate mechanism for *bona fide* demethylation. Liutkeviciute *et al.* have shown that DNA methyltransferase DNMT1 is able to directly remove the hydroxymethyl group *in vitro* in absence of SAM (Liutkeviciute *et al.*, 2009), implying 5hmC as an intermediate compound in the direct DNA demethylation. 5hmC could also be the target for further enzymatic oxidation, which would lead to enzymatic decarboxylation and yielding the unmodified cytosine. The recent study by Ito *et al.* has demonstrated the presence of TET1 in mouse zygotes and thus providing the potential evidences of 5hmC presence in preimplantation embryos (Ito *et al.*, 2010).

The candidate pathways for indirect demethylation all include the removal of the whole nucleotide and subsequent repair of the resulting abasic site. There is growing evidence that DNA repair processes are involved in epigenetic reprogramming events in mammalian zygotes. The fertilized oocyte has the ability to perform different types of DNA repair, including non-homologous end joining (NHEJ) and homologous recombination (HR) (Derijck *et al.*, 2008). We and other research groups have shown that in late G1 mouse zygotes, during phase of active DNA demethylation, DNA strand breaks can be detected in the paternal pronucleus (Derijck *et al.*, 2006; Hajkova *et al.*, 2010; Wossidlo *et al.*, 2010). These strand breaks co-localize with PARP-1, a prominent factor of the base excision repair (BER) machinery. Also during S-phase we still find more pronounced DNA strand breaks in the paternal genome (Wossidlo *et al.*, 2010). Mouse zygotes will still initiate S-phase because of the missing G1/S-damage checkpoint (Shimura *et al.*, 2002) and therefore the repair of such demethylation events could be performed also during S-phase. The active DNA demethylation in PGCs is also shown to be mechanistically linked to the appearance of single-stranded DNA breaks and the activation of the base excision repair (BER) pathway (Hajkova *et al.*, 2010). Taken together these findings strongly suggest the involvement of DNA repair in the zygotic demethylation in mammals.

The indirect demethylation by direct removal of the 5mC nucleotide could be accomplished by DNA glycosylases. As it is shown for flowering plants, 5mC is actively demethylated by DNA glycosylases DEMETER (DME) and REPRESSOR OF SILENCING 1 (ROS1) (Agius *et al.*, 2006; Grossniklaus *et al.*, 1998; Kiyosue *et al.*, 1999). DME mediates the DNA demethylation, which is necessary for establishing the genomic imprints in the endosperm, while ROS1 is involved in trimming DNA methylation patterns in transposons and genic regions (reviewed in (Kinoshita *et al.*, 2008; Saze *et al.*, 2008; Zhang *et al.*, 2010a)). Both enzymes are bi-functional DNA glycosylases, which are able to excise 5mC and directly process the abasic site by intrinsic lyase activity. The resulting DNA gap is repaired by factors of BER machinery and an unmodified cytosine is then incorporated. In mammals so far no homologues for DME and ROS1 could be identified. The human and chicken DNA glycosylase MBD4 and also the thymine DNA glycosylase TDG were shown to demethylate 5mC *in vitro* (Zhu *et al.*, 2000a; Zhu *et al.*, 2000b). A very elegant publication by Kim *et al.* has shown that MBD4 plays an important role in hormone-induced transcriptionally regulated active demethylation of the cytochrome p450 27B1 gene (Kim *et al.*, 2009). However both MBD4 and TDG showed only weak catalytic activity on the demethylation of 5mC:G dinucleotides (Hardeland *et al.*, 2003) and oocytes derived from MBD4-knockout-mice are still able to demethylate the paternal genome (Santos and Dean, 2004).

Another mechanism for indirect demethylation involves the enzymatic deamination of 5mC to thymine, followed by T:G mismatch repair that replaces thymine with cytidine resulting in demethylation of 5mC. Two classes of enzymes have been proposed for this mechanism: DNA deaminases and DNA methyltransferases. The DNA deaminases AID (Activation-induced deaminase) or the Apobec´s have been shown to deaminate 5mC (Morgan *et al.*, 2004) and a very recent publication by Popp *et al.* verifies the involvement of AID in genome wide demethylation of PGCs (Popp *et al.*, 2010). In this study PGCs isolated from $AID^{-/-}$ embryos revealed higher DNA methylation levels compared to wild type embryos, but the demethylation activity was still prominent and offspring of $AID^{-/-}$ parents did not show phenotypic defects indicating that AID might be not the only player in demethylation. In concert with these findings the work on interspecies heterokaryons, derived by fusion of mouse embryonic stem cells with human fibroblasts, showed that AID is required for the epigenetic reprogramming to gain pluripotency (Bhutani *et al.*, 2010). Short interfering RNA mediated knockdown of AID in these heterokaryons abolished promoter demethylation and induction of OCT4 and NANOG gene expression. The promi-

nent role of AID for active demethylation is further affirmed by the findings in zebrafish (Rai *et al.*, 2008). Overexpression of AID and MBD4 in zebrafish embryos led to increased global demethylation of injected methylated DNA fragments and the zebrafish genome. If AID plays the same important role in mammalian zygotes remains to be clarified. The authors were also able to confirm the demethylating activity of another potential demethylating factor with unknown enzymatic activity, Gadd45a, which was controversially described as being involved in demethylation (Barreto *et al.*, 2007; Li *et al.*, 2010) but this statement was disapproved by other publications (Engel *et al.*, 2009; Jin *et al.*, 2008).

The DNA methyltransferases DNMT3A and DNMT3B are also proposed as indirect demethylation candidates. Metivier *et al.* have shown that in absence of SAM both DNMT3A and DNMT3B are able to deaminate 5mC to T (Metivier *et al.*, 2008) in similar fashion as it was reported for prokaryotic methyltransferase M.HpaII (Shen *et al.*, 1992). Furthermore DNMT3A associates with TDG and BER factors showing the functional link between 5mC deamination and coupled DNA repair. However, it is not clear whether SAM levels are reduced or restricted in the paternal pronucleus. In this aspect, the involvement of the demethylation factor Elp3 in influencing local SAM levels through its radical SAM domain activity could be of particular interest.

The indirect active demethylation could also involve the modification of 5mC coupled with repair of the modified 5mC. The presence of 5hmC in mammalian DNA fuels such an idea and a hydroxymethyl specific zygotic DNA glycosylase could initiate subsequent BER. It was shown that several DNA glycosylases like NEIL enzymes or SMUG1 possess the enzymatic activity to recognize and cleave 5hmC. 5hmC could also trigger passive DNA demethylation because the hydroxylation hinders DNMT1 to maintain DNA methylation in the newly replicated DNA strand (Tahiliani *et al.*, 2009; Valinluck and Sowers, 2007). The list of potential DNA demethylases is summarized in Table 1.

Influence of the environment on zygotic DNA demethylation

The extensive use of different artificial reproduction technologies (ART) in cattle breeding and human reproduction raised the question how ART affect reprogramming in mammalian zygotes and consequently during preimplantation development. Alterations in the DNA methylation states of preimplantation embryos have been reported in various mammalian species as a consequence of manipulations such as ICSI, SCNT, cryopreservation of embryos and gametes or even only due to *in vitro* culture conditions and superovulation (Santos *et al.*, 2003; Shi *et al.*, 2004; Shi and Haaf, 2002). Effects were documented on the whole genome scale using antibody stainings on developing embryos or by analysing DNA methylation at specific genomic regions such as imprinted loci and representative repetitive elements. The effects documented are sometimes variable, e.g. the responsiveness to environmental stress appears to vary between mammalian species. While *in vitro* culture conditions and ICSI manipulations lead to incomplete demethylation in rat embryos (Yoshizawa *et al.*, 2010; Zaitseva *et al.*, 2007) similar conditions and manipulations do not affect global demethylation in mouse embryos (Fulka and Fulka, 2006; Peters *et al.*, 2009). The maintenance of genomic imprints appeared to be more sensitive indicators in different mammals. Hypomethylation of paternal H19 allele was reported for individual mouse (Fauque *et al.*, 2007) and for human (Zechner *et al.*, 2009) embryos conceived by *in vitro* fertilization. In general, the fidelity of human ART is actively debated and the reports claiming ART being save for the imprints (Tierling *et al.*, 2009) are published along with those raising the concerns about the issue (Katari *et al.*, 2009). Therefore the impact of zygotic demethylation on imprinting requires additional investigations. With human artificial reproduction becoming now a common medical practice, further progress in cultivation and manipulation technologies is still needed.

TABLE 1

SUGGESTED DNA DEMETHYLASES

	Enzyme	Suggested demethylation mechanism	References
MBD2	methyl-binding domain protein 2	*bona fide* demethylation	(Bhattacharya *et al.*, 1999)
MBD4	methyl-binding domain protein 4	5mC specific DNA glycosylase	(Kim *et al.*, 2009, Zhu *et al.*, 2000a)
GADD45a	growth arrest and DNA-damage	nucleotide excision repair mediated process inducible protein 45 alpha	(Barreto *et al.*, 2007, Li *et al.*, 2010)
AID	activation-induced cytidine deaminase	5mC specific deaminase	(Morgan *et al.*, 2004, Popp *et al.*, 2010)
DNMT3a/b	DNA methyltransferases 3a/3b	oxidative deamination of 5mC	(Metivier *et al.*, 2008)
DEMETER ROS1 DML2, DML3	plant demethylases	5mC specific DNA glycosylases	(Gehring *et al.*, 2006, Gehring *et al.*, 2009)

Conclusions

The successful development of a newly formed mammalian embryo includes various epigenetic reprogramming events. These events equalize the extremely epigenetically different chromosomes of sperm and oocyte and make them competent for further development. Both DNA and histone modifications in parental pronuclei of the mammalian zygote should synergistically form a specific epigenetic landscape in order to achieve a certain degree of genomic plasticity. Such plasticity is the prerequisite for establishing the totipotency state first, which then transforms into pluripotency in ICM cells at blastocyst stage. It is the mammalian ooplasm which provides the machinery for this reprogramming, because not only incoming sperm genome, but also somatic cell donor nuclei in SCNT embryos are epigenetically altered. The DNA methylation reprogramming activity is part of this process. The mechanism of the DNA demethylation is still not solved, but more and more data show the involvement of DNA repair events. Beyond that, the biological reasons for the changes in zygotic DNA methylation and histone modifications are heavily debated. One obvious interpretation is the necessity of DNA methylation reprogramming for subsequent EGA. Indeed, in mouse and rat embryos EGA begins already at the end of the first cell cycle, but bovine and rabbit embryos activate their genomes few cycles later – at 8-16 cell stage (Kanka, 2003). However, data on sperm methylation profile show hypomethylation of promoter regions, which means the paternal genes are already in the stand by mode to initiate the transcription (Farthing *et al.*, 2008). On the other hand the genome wide epigenetic changes could probably serve as a "genomes compatibility proof" – a mechanism allowing the

transcription through the entire paternal genome (not only genic regions) in order to reveal its potential in supporting the development of the embryo. Furthermore it might also be compared with *tabula rasa* conception when the oocyte erases sperm inherited epigenetic information and then re-establishes it accordingly to the developmental program stored in the ooplasm. As recent data show, the latter could only be partially true, since some of sperm epigenetic heritage remains after zygotic reprogramming and plays not the least role in further development (Brykczynska *et al.*, 2010; Hammoud *et al.*, 2009). More detailed studies of different epigenetic reprogramming events in preimplantation mammalian embryos are hindered by the availability of the material. We hope that so far unresolved issues can be clarified with the development of new high throughput sequencing technologies requiring less material and providing not only conventional sequence information but also allowing discrimination of different base modifications, such as methylation and hydroxymethylation (Clarke *et al.*, 2009; Flusberg *et al.*, 2010).

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