

Epigenetic reprogramming in mammals

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Epigenetic marking systems confer stability of gene expression during mammalian development. Genome-wide epigenetic reprogramming occurs at stages when developmental potency of cells changes. At fertilization, the paternal genome exchanges protamines for histones, undergoes DNA demethylation, and acquires histone modifications, whereas the maternal genome appears epigenetically more static. During preimplantation development, there is passive DNA demethylation and further reorganization of histone modifications. In blastocysts, embryonic and extraembryonic lineages first show different epigenetic marks. This epigenetic reprogramming is likely to be needed for totipotency, correct initiation of embryonic gene expression, and early lineage development in the embryo. Comparative work demonstrates reprogramming in all mammalian species analysed, but the extent and timing varies, consistent with notable differences between species during preimplantation development. Parental imprinting marks originate in sperm and oocytes and are generally protected from this genome-wide reprogramming. Early primordial germ cells possess imprinting marks similar to those of somatic cells. However, rapid DNA demethylation after midgestation erases these parental imprints, in preparation for sex-specific *de novo* methylation during gametogenesis. Aberrant reprogramming of somatic epigenetic marks after somatic cell nuclear transfer leads to epigenetic defects in cloned embryos and stem cells. Links between epigenetic marking systems appear to be developmentally regulated contributing to plasticity. A number of activities that confer epigenetic marks are firmly established, while for those that remove marks, particularly methylation, some interesting candidates have emerged recently which need thorough testing *in vivo*. A mechanistic understanding of reprogramming will be crucial for medical applications of stem cell technology.

INTRODUCTION

During development of multicellular organisms, different cells and tissues acquire different programmes of gene expression. It is thought that this is substantially regulated by epigenetic modifications such as DNA methylation, histone tail modifications and non-histone proteins that bind to chromatin (1,2). Thus, each cell type in our body has its own epigenetic signature which reflects genotype, developmental history and environmental influences, and is ultimately reflected in the phenotype of the cell and organism. For most cell types in the body, these epigenetic marks become fixed once the cells differentiate or exit the cell cycle. However, in normal developmental or disease situations, some cells undergo major epigenetic 'reprogramming', involving the removal of epigenetic marks in the nucleus, followed by establishment of a different set of marks (3–5) (Fig. 1). In particular, this happens upon fertilization when many gametic marks are erased and replaced with embryonic marks important for

early embryonic development and toti- or pluripotency. Major reprogramming also takes place in primordial germ cells (PGCs) in which parental imprints are erased and totipotency is restored. Cells undergoing dedifferentiation, such as cancer cells, are also expected to undergo reprogramming (6), as do cells that can transdifferentiate (7–9). Finally, dramatic reprogramming is required following somatic cell nuclear transfer (SCNT) for the purposes of cloning and stem cell derivation for therapy (10–13).

Recently, substantial new insights have been obtained into epigenetic reprogramming in normal development and in SCNT. Information has accumulated on comparisons between different mammalian species and on links between different epigenetic marking systems. A number of candidate reprogramming factors have emerged, whose relevance to the process occurring *in vivo* needs to be tested. Although insights into the mechanisms of reprogramming and the factors involved are still rudimentary, the foundation knowledge is now reaching a stage at which more detailed concepts

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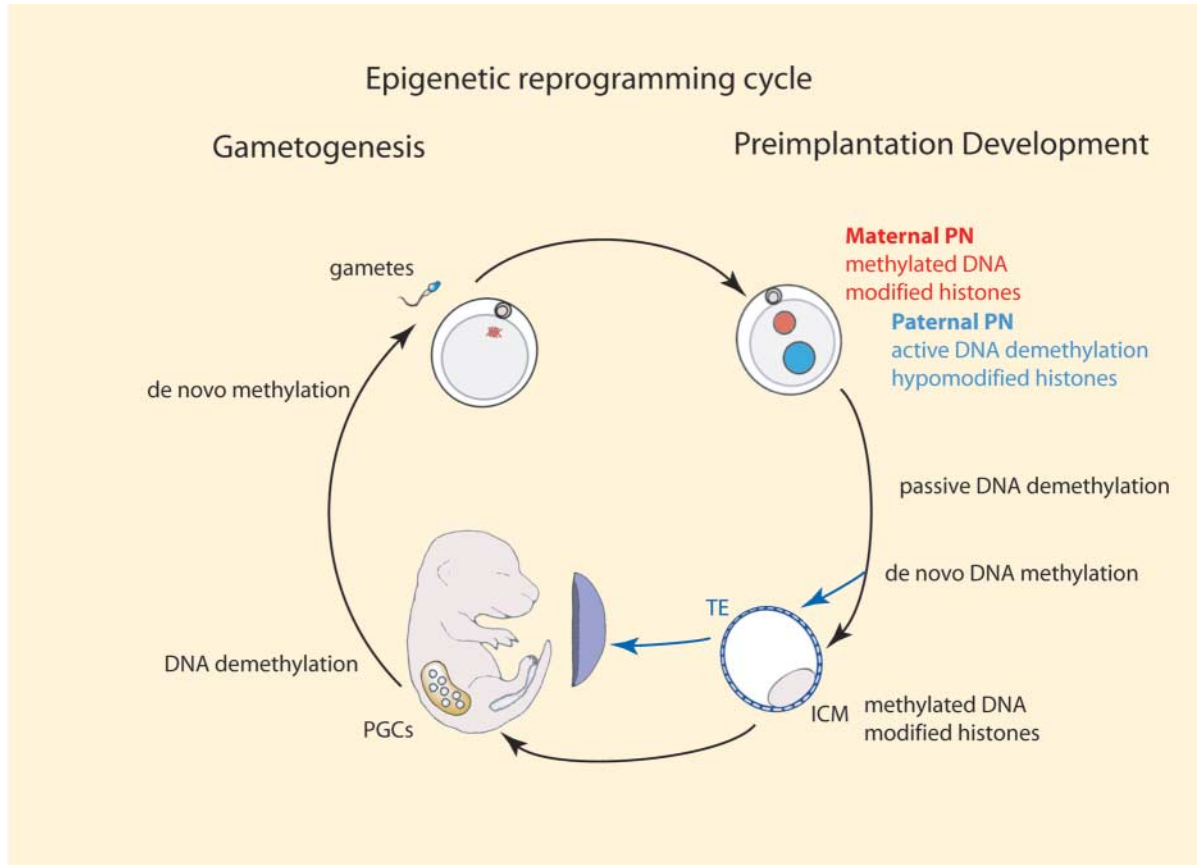


Figure 1. Epigenetic reprogramming cycle. Epigenetic modifications undergo reprogramming during the life cycle in two phases: during gametogenesis and preimplantation development. PGCs arise from somatic tissue and develop into mature gametes over an extended period of time. Their genome undergoes DNA demethylation in the embryo between E11.5 and E12.5, including at imprinted genes. Following demethylation, the genomes of the gametes are *de novo* methylated and acquire imprints; this process continues up to E18.5 in males and in maturing oocytes before ovulation in females. Fertilization signals the second round of reprogramming during preimplantation development. The paternal genome is actively demethylated and its histones initially lack some modifications present in the maternal pronucleus (PN). The embryo's genome is passively DNA demethylated during early cell cycles before blastulation. Despite this methylation loss, imprinted genes maintain their methylation through this preimplantation reprogramming. *De novo* methylation roughly coincides with the differentiation of the first two lineages of the blastocyst stage, and the inner cell mass (ICM) is hypermethylated in comparison to the trophoblast (TE). These early lineages set-up the DNA methylation status of their somatic and placental derivatives. Histone modifications may also reflect this DNA methylation asymmetry. Particular classes of sequences may not conform to the general genomic pattern of reprogramming shown.

can be developed and better experimental approaches can be devised to examine mechanistic aspects.

EPIGENETIC ASYMMETRY IN THE ZYGOTE

DNA methylation

At fertilization, the parental genomes are in different stages of the cell cycle with very different epigenetic marks and chromatin organization. The paternal genome has been delivered by the mature sperm, is single copy (1C), and is packaged densely for the most part with protamines rather than histones. In contrast, the maternal genome is arrested at metaphase II with its 2C genome packaged with histones. Upon fertilization, protamines in sperm chromatin are rapidly replaced with histones (Fig. 2A), whereas the maternal genome completes meiosis. The histones H3 and H4 that associate with the paternal chromatin are more acetylated than those already present in the maternal chromatin (14,15).

This may be a passive consequence of the pool of available histones in the cytoplasm being largely acetylated or due to active incorporation of a particular acetylated histone variant.

The precise timing and sequence of epigenetic changes in the mouse following histone deposition is becoming clearer (Fig. 2A). Closely following histone acquisition of the paternal genome is the initiation of genome wide loss of DNA methylation detectable both by indirect immunofluorescence (15–19) and by bisulphite sequencing of unique gene sequences and repeat families (20,21). This demethylation is completed before DNA replication begins in the paternal pronucleus. However, some regions of the genome do not become demethylated at this stage. These include heterochromatin in and around centromeres (15,22), IAP retrotransposons (21) and paternally methylated imprinted genes (23). These sequences may have to remain methylated for normal chromosome stability, the suppression of IAP transposition and the maintenance of parental imprinting, respectively. Other

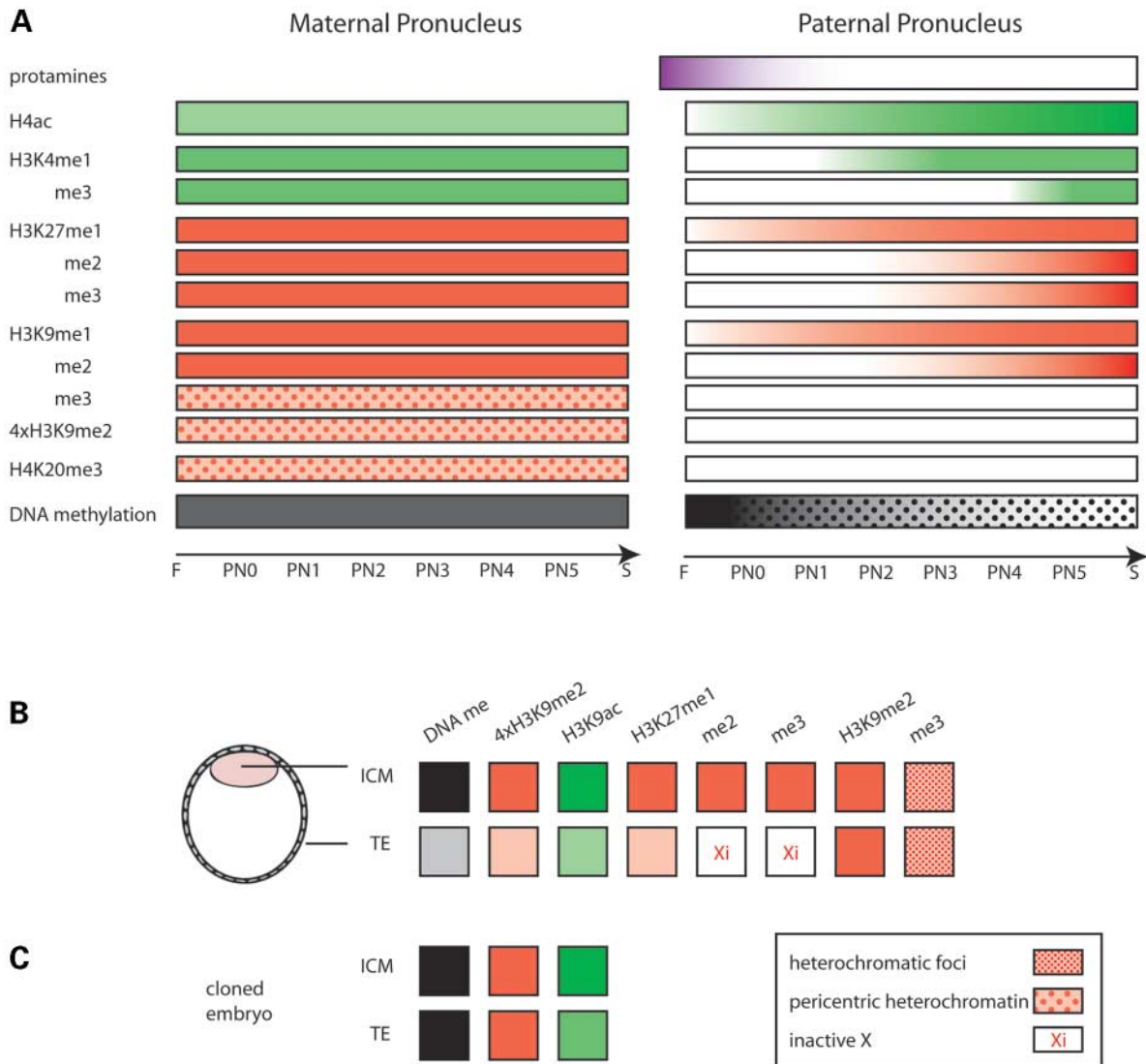


Figure 2. Preimplantation epigenetic reprogramming and cloning. (A) Remodelling in the mouse maternal and paternal pronuclei. Transcriptionally permissive modifications (green) and repressive modifications (red) are shown with shading intensity indicative of level of modification from fertilization (F) through pronuclear stages (PN0–PN5) (14) and syngamy (S). In the paternal pronucleus: protamines (purple) are exchanged for histones which are hyperacetylated (14,15), and carry H3K27me1 and H3K9me1 (42), but initially lack H3K4me1 and H3K9me3 (37), H3K27me2* and H3K9me2 (36) and H3K9me3* (38), heterochromatic histone methylation (4 × H3K9me2 antibody) (34,35) and H4K20me3 (39); DNA methylation (black) is lost actively before DNA replication at PN3–PN4, except at pericentric heterochromatin, some repeats (IAPs) and paternally methylated imprints (14–17,20,21); H3K4me1 and H3K9me3 (37), H3K27me2*, H3K9me2 (36) and H3K9me3* (38) accumulate during pronuclear development. H3K9me2 and H3K9me3*, heterochromatic histone methylation and H4K20me3 particularly mark pericentric heterochromatin in the maternal pronucleus (34–36,39). (B) Epigenetic differences between the ICM and trophectoderm. *De novo* methylation results in a methylated ICM, but less methylated TE in mouse (15,17); bovine (26,54); sheep (18). H3K9ac and heterochromatic histone methylation are re-established in the blastocyst preferentially in the ICM compared to the TE (54). H3K27me1, H3K9me2* and H3K9me3 are present predominantly in the ICM, and H3K27me2* and H3K9me3 only mark the inactive X chromosome in the TE (in the mouse) (38). H3K9me2 stains evenly between ICM and TE and across the genome (38). H3K9me3 marks heterochromatic foci in the ICM and TE (38). (C) In cloned embryos, DNA methylation (bovine and sheep), heterochromatic histone methylation (bovine) and H3K9ac (bovine) remain at abnormally high levels in the TE (17,18,26,54). Asterisk marks refer to data not shown (38).

heterochromatic and euchromatic sequence families need to be examined to see how general these patterns are.

Some interesting observations have emerged from comparisons of zygotic demethylation between mammalian species. Immunofluorescence studies have demonstrated loss of paternal methylation in mouse, rat, pig, bovine and human (17–19). The DNA methylation dynamics of sheep zygotes differ in some way (18,24–26). The sheep paternal pronucleus retains more DNA methylation than the mouse or human, but

is still hypomethylated relative to the maternal pronucleus (24,25). A comparison of total levels of methylation in sheep sperm and oocytes is required to know what methylation levels the parental genomes start with. It is currently difficult to exclude any reduction in methylation of the paternal genome in the first 6 h after fertilization (25,26). Indeed, sheep oocytes are capable of demethylating mouse sperm chromatin and conversely, sheep sperm becomes demethylated in mouse oocytes, attesting to the conservation of

paternal genome demethylation activity in sheep (27). In the rabbit, immunofluorescence studies failed to identify active or passive demethylation during preimplantation development (28), whereas bisulphite sequencing showed evidence for demethylation (29). These apparent discrepancies could arise from factors that can influence these analyses such as starting levels of methylation in both gametes, the relative amount of demethylation in different sequence classes, the distribution, density or organization of these sequences in the genome and the threshold of detection and signal saturation inherent to immunofluorescence. For example, as centromeric satellites seem to resist demethylation, their substantially different size in different species may result in different estimates by immunofluorescence of the extent of demethylation. More extensive analyses are clearly needed to understand better what aspects of demethylation are conserved, and what aspects differ, between mammalian species.

Both the mechanism and the function of paternal genome demethylation are unknown. It is likely that the oocyte cytoplasm contains demethylation factors (see Candidate mechanisms for demethylation) and that these are specifically targeted to or excluded from certain sequence classes in sperm chromatin. Paternal demethylation may have arisen in order to reprogramme paternal germline imprints by the maternally produced oocyte cytoplasm (30). This is consistent with the majority of germline methylation imprints being maternal and with zygotic demethylation of the paternal genome being uniquely observed in species which have imprinting. Paternal demethylation may also be needed for proper transcriptional activation of the paternally derived embryonic genome; the paternal genome is known in some species to be transcriptionally activated before the maternal genome (31). It is interesting to note that the species with an earlier stage of embryonic genome activation (mouse, pig and human) show a more extensive zygotic demethylation than species where genome activation occurs later (sheep, rabbit) in preimplantation development (Table 1) (32). Although it is intuitively clear that demethylation may be part of the process which returns the specialized gametic genomes to embryonic totipotency, it has yet to be shown that there are, for example, methylated genes in the sperm genome whose demethylation is needed for early transcriptional activity and hence for embryo development.

Histone modifications

Maternal chromatin is organized such that DNA methylation and chromatin modifications are already abundant at fertilization (Fig. 2A). These include both nucleohistone modifications and chromatin proteins associated with active and repressive configurations (14,15,34–40). In particular, different modifications (e.g. acetyl or methyl) of the same amino acid residues are detectable, but these occur in different parts of the genome. For example, histone modifications ordinarily associated with an active chromatin state such as acetylated lysine and H3K4me are found in the female pronucleus (14,15,37). Heterochromatic modifications such as H3K9me2/3, H3K27me1 and H4K20me3 largely associated with repressive chromatin organization are also in evidence (34–40). Higher resolution analyses are beginning to show more specific associations; centromeric

major satellites, for example, are marked by H3K9me3, whereas minor satellites are marked by H3K9me2 and DNA methylation (41,42), suggesting an association between H3K9me2 and the protection against DNA demethylation in the female pronucleus (42).

Histones incorporated into the male pronucleus are highly acetylated (14,15), however, immediately upon histone incorporation, H3K4me1, H3K9me1 and H3K27me1 are detectable (37,38,42) (Fig. 2A); this is at a time when DNA methylation is still present in the male pronucleus. Whether any of these early marks protect specific regions such as the centromeres from demethylation is not known. The early appearance of histone methylation marks implies that histone residues are rapidly deacetylated and then monomethylated by the appropriate histone methyltransferases (HMTases): SET 7/9 for H3K4me (43), G9a and ESET for H3K9me (44,45) and an unknown HMTase for H3K27me independent of Ezh2 (described subsequently). What is surprising is that me2 and me3 of these residues only occur in a protracted fashion, with for example H3K9me3 occurring as late as the four cell stage, despite the presence of Suvar39h and Eed/Ezh2 activities in the oocyte (38,42). It is intriguing that HP1 β is found apparently bound to H3K9me1, perhaps protecting it from further methylation at this stage (42). It is not clear why there might be a delay in further modification of H3K9me1 but this perhaps allows DNA demethylation to proceed unhindered. Nevertheless, the progressive histone modifications occurring to the paternal genome presumably lead to a chromatin state equivalent to that of the maternal genome. This does not exclude the paternal genome from acquiring unique epigenetic marks early on in development which are important for imprinting and X chromosome inactivation (46–48). Oocyte specific manipulations of the HMTases and modifying complexes by conditional gene targeting or RNAi are needed to address these interesting aspects of reprogramming.

PREIMPLANTATION DEVELOPMENT TO THE BLASTOCYST STAGE

Passive demethylation

From the one cell to blastocyst stage in the mouse (Fig. 1), there are further changes in global DNA methylation and histone modifications. By the blastocyst stage, the first two lineages have been set up: the inner cell mass (ICM, the embryonic lineage) and the trophectoderm (TE, the extraembryonic lineage) (Fig. 2B). DNA methylation is reduced progressively with cleavage divisions (49); this loss of DNA methylation depends on DNA replication (50) and results in unequally methylated sister chromatids (22). Indeed, Dnmt1 protein inherited from the oocyte (Dnmt1o) is seemingly excluded from the nucleus during the first three cleavage divisions (51,52) accounting for the loss of methylation by a passive mechanism. Many different types of sequences lose methylation at this stage but imprinted genes retain their germline imprints raising questions about the DNMT responsible and the recognition of regions where DNA methylation is to be maintained. Curiously, Dnmt1o only enters the nucleus at the eight cell stage, and it has been argued that this is

Table 1. Zygotic activation of transcription and 5mC in mammalian sperm (32,33)

	Minor transcription	Major transcription	α -amanitin arrest	Developmental block	5mC sperm/somatic
Mouse	late 1	2	2	2	0.87
Pig	n.d.	4	n.d.	4	0.64 ^a
Human	n.d.	4–8	4	4–8	0.95
Bovine	1–2	8–16	9–16	8	0.54
Ovine	n.d.	8–16	8–16	8–16	0.67
Rabbit	2	8–16	n.d.	n.d.	n.d.

Zygotic activation of transcription can be determined for a minor activation or major activation (32). If transcription is inhibited by α -amanitin, the point at which development stops is an indication of at which stage zygotic transcription is necessary (32). The developmental block is a characteristic stage at which some embryos of these species spontaneously arrest (32). The ratio of the proportion of 5mC in the genome was determined by HPLC in sperm/liver. Some aspects are not determined (n.d.) yet.

^aExcept pig which is sperm/kidney (33).

needed for the maintenance of imprinted methylation (53). It is not known whether Dnmt1s (the somatic form of Dnmt1) is required for specific maintenance of imprints during preimplantation. Unique histone modifications may guide DNA methylation maintenance of imprinted regions. There are quantitative differences in passive demethylation between mammalian species that have been analysed. Perhaps, this is related to the differences in timing of blastocyst cavitation and development of progenitors of the embryonic and extraembryonic lineages. To date, extensive changes of DNA methylation during this period have not been reported outside of mammalia, suggesting that this is a mammalian specialization.

To what precise extent histone modifications are reprogrammed during passive DNA demethylation is not yet clear. In the mouse, it seems that H3K4me, H3K9me and H3K27me are not globally altered (38), but in bovine embryos, both heterochromatic histone methylation (that detected by anti-4 \times H3K9me2 antibody) and H3K9ac decrease and then increase in advance of major genome activation (54). More transient histone marks, such as phosphorylation and arginine methylation, undergo changes during the cell cycle in mouse embryos (55,56) that are likely associated with DNA replication rather than reprogramming, but to what extent this differs from cell cycle regulation of these marks in more differentiated cells is not clear at present.

Epigenetic asymmetry and lineage commitment

The first lineage allocation event in mammalian embryogenesis occurs at the morula stage and results in the formation of the ICM and TE lineages in the blastocyst. It is not clear yet how cell fate, leading to two lineages, is determined. Two-cell blastomeres are still totipotent, whereas at the four-cell stage, some lineage bias is already present when the developmental potency of individual blastomeres is tested (57). It is also thought that cells in the eight and 16 cell morula stage that come to be located inside are more likely to become ICM cells, whereas those cells on the outside are more likely to become TE cells (58). The earliest markers detected so far which may delineate this restricted potential are Cdx2 for TE precursors and Nanog for ICM precursors (59).

What is interesting is that there are global differences in DNA methylation between the extraembryonic lineages

(placenta) and the embryonic ones (Fig. 2B), with the placenta relatively undermethylated for different sequence classes (60), and maintenance of imprinting and X chromosome inactivation relatively independent of DNA methylation in this tissue at later stages (46,61). These differences arise as early as the blastocyst stage, at which the combinations of active and passive demethylation have resulted in a low ground state of methylation in the TE (15,26,54,62). The ICM, in contrast, shows clear signs of extensive *de novo* methylation, which may begin as early as in the late morula stage (15); it is likely that this is caused by Dnmt3b as this *de novo* methylase is detectable in blastocysts in the ICM but not in the TE (63).

Histone H3K9me3 marks heterochromatic foci in the ICM, and H3K27me1, me2 and me3 are more abundant in the ICM than in the TE (38). The inactive X chromosome, and certain imprinted regions, are also marked in the TE by H3K27me and H3K9me. H3K27me is largely but not exclusively dependent on Ezh2, an HMTase (38,64). H3K9me2 arises in the blastocyst independently of Ezh2, whereas H3K9me3 is partly dependent on Ezh2, especially in the TE (38). The precise functions of Suv39h1/2 (a heterochromatic H3K9 HMTase), ESET (a H3K9 HMTase) and G9a (a euchromatic H3K9 and perhaps H3K27 HMTase) on various H3K9me marks at these stages are not yet completely characterised.

The global differences in DNA methylation between the two lineages are conserved among mouse, bovine, sheep and rabbit (15,18,54,62) and may, thus, be important for allocation or function of the lineages (35). As with DNA methylation, higher levels of specific repressive histone methylation marks are also found in the ICM when compared with the TE (Fig. 2B). The epigenetic asymmetry established in the blastocyst is indeed important for development. Embryos null for Dnmt1 (65), Dnmt3a and b (66), Ezh2 (38), ESET (45) and G9a (44) have more severely affected embryonic rather than extraembryonic lineages; in fact, Ezh2 and ESET are for the derivation of ES cells required (38,45). It is possible that much more sophisticated epigenetic control is needed in embryonic tissues, with the complexity of their differentiation and the longevity of their derivatives; in comparison, the differentiation potential and life expectancy of the extraembryonic tissues is limited. Reprogramming may extend beyond development up to the blastocyst stage. Although modifications are being re-established in much of

the genome in the ICM, reprogramming of marks already established may occur to restricted parts of the genome. The imprinted inactive X chromosome becomes reprogrammed and the X chromosomes are randomly inactivated (67). At some imprinted regions, though imprinted expression occurs in morulae and blastocysts, and parent of origin specific histone modifications are present in ES cells, differentiated ES cells and post-implantation embryos lack some of these histone modifications (46,47).

REPROGRAMMING IN THE GERM LINE

PGCs are derived from epiblast cells and first arise in the posterior primitive streak at E7.5 (embryonic day) from where they begin to migrate from E 8.5 to the genital ridge, arriving by E11.5 (68). Female PGCs enter meiotic arrest in prophase of meiosis I, and male PGCs enter mitotic arrest until about birth when mitosis of spermatogonial stem cells is resumed. Early PGCs are thought to have epigenetic marks similar to those of other epiblast cells, including random X chromosome inactivation, imprinted gene expression and DNA methylation. However, many of these epigenetic marks are erased about the time PGCs arrive at the genital ridges. Imprinted genes are biallelically expressed (69), and methylation in imprinted genes and single copy genes is erased with the bulk of the demethylation occurring between E11.5 and E12.5 (70–72). The extent of the loss of methylation over these few cell cycles in the presence of Dnmt1 in the nucleus suggests that this demethylation is likely to be active. It is unknown if there are any mechanistic similarities between active demethylation in PGCs and in zygotes. Although demethylation in PGCs appears genome wide, there are specificities. First, paternally methylated differentially methylated regions (DMRs), e.g. the DMR of H19, are demethylated in PGCs but not in zygotes (70). Secondly, some retrotransposon families seem relatively resistant to demethylation compared to the rest of the genome, in both PGCs and zygotes (21,70). Incomplete demethylation is seen both in Line 1 and in IAP families, and this may underlie the inheritance of epigenetic information that sometimes occurs at mutant gene loci with transposon insertions (73,74).

Not all epigenetic marks present in imprinted genes may be erased in PGCs. There is demethylation of paternal *H19* and maternal *Snrpn* alleles, however, later on in spermatogenesis or oogenesis when *H19* or *Snrpn*, respectively, becomes methylated again, it seems that the originally methylated alleles become *de novo* methylated at an earlier stage than the originally unmethylated ones (75,76). This raises the possibility that other types of epigenetic marks, perhaps those based on histone modifications, are only incompletely erased and that they provide signals for *de novo* methylation. To date, histone tail modifications have not been analysed in PGCs; it will be fascinating to see whether there are specific marks associated with genes that become demethylated, compared to those that do not. More sensitive ChIP protocols need to be developed urgently so that this type of investigation becomes feasible.

EXPERIMENTAL REPROGRAMMING

The most dramatic way of altering epigenetic marks experimentally is by SCNT or cloning. It is remarkable that cloning works at all, as it requires a differentiated somatic nucleus to become reprogrammed in an enucleated oocyte to a totipotent state without the reprogramming that normally happens during development. All epigenetic marks that have been examined in cloned embryos and adults so far (and in different mammalian species) show abnormalities, and most cloned embryos also differ from each other in the precise epigenetic profile they possess, indicating that epigenetic reprogramming during cloning is a haphazard and stochastic process whose outcome is impossible to predict (10–13,54). Cloned embryo development is as variable as their epigenetic makeup: cloned embryos die at all stages of development with a variety of abnormalities. Of those that develop to later gestational stages or to term, many have placental abnormalities, and a proportion die perinatally from maladaptation to extrauterine life. It is likely that most of the developmental problems of clones are caused by epigenetic defects, because offspring of cloned animals appear normal (77). Even genetic defects arising from chromosome abnormalities could have origins in epigenetic defects in centromeres. Both the developmental and the epigenetic abnormalities of SCNT embryos tend to be more severe the earlier they are examined, with less abnormal ones surviving to later stages (78,79). This also means that epigenetic studies carried out at different stages should be compared with caution. Because of the key theme of extreme variability, we will only review here the main classes of epigenetic abnormalities that have been described, without summarizing the detailed observations.

Epigenetic defects described in cloned offspring include errors in X inactivation (79,80), imprinting (78,81,82), DNA methylation in general and of specific gene and repeat sequences (17,78,83,84), histone acetylation and methylation (54) and widespread alterations in gene expression (85) including the failure to activate Oct4 and related key pluripotency genes (86–88).

Considering the different stages and mechanisms of reprogramming discussed earlier are there particular aspects that are defective in cloned embryos? Active demethylation of the somatic nucleus occurs to some extent (17), however, a detailed evaluation of quantitative and qualitative aspects of demethylation has not yet been done. In particular, aberrant demethylation of imprinted genes could lead to developmental defects (82).

Reduced passive demethylation has also been observed in a proportion of cloned embryos. This is perhaps because the somatic form of Dnmt1 continues to be expressed in the cloned embryos, and hence prevents proper passive decline of DNA methylation (89). Histone acetylation and methylation are also only very incompletely reprogrammed (54), and precocious *de novo* methylation of DNA may also occur. These events in the early cloned embryo lead particularly to aberrantly high levels of DNA methylation and histone modifications in the TE and later in the placenta (Fig. 2C); these extraembryonic tissues show the highest rate of abnormal development. Which gene targets are particularly

deregulated by abnormal epigenetic marks in cloned embryo placentae is not known. As pointed out before, there is great variability in epigenetic marks between individual cloned embryos, and a small proportion of cloned preimplantation embryos have modifications resembling those of natural embryos; these seem to have a better developmental potential than those with highly aberrant epigenetic marks (54).

In summary, both the erasure of the epigenetic memory of the differentiated donor cells (89,90), and the establishment of the epigenetic programme of the early embryo, appear highly defective in the majority of cloned embryos.

Besides reprogramming of somatic nuclei in oocytes, somatic cells have also been fused to EG and ES cells (91,92), injected into *Xenopus* oocytes (93) and treated with 'reprogramming' extracts, with limited success (94,95). EG and ES cell fusion appears to lead to appropriate reprogramming of the somatic nucleus of imprinted genes in EG cell fusion and Oct4 with ES cell fusion (91,92,96). Successful early development of cloned embryos may be particularly dependent on correct expression of pluripotency and related genes such as Oct4 (86,87,93). Injecting mammalian somatic cells into *Xenopus* leads to the demethylation of Oct4 (97). This is perhaps surprising because there is no global active demethylation in the *Xenopus* zygote (98); however, it cannot be excluded that there are more local gene specific demethylation events needed for early development. Reprogramming systems based on cell extracts may also be useful in the future for biochemical or genetic identification of reprogramming factors. Immunodepletion of *Xenopus* oocyte extracts of some candidate proteins identified the chromatin remodelling ATPase BRG1 as a factor capable of remodelling human somatic nuclei (94). The reprogramming of fibroblasts in a T cell extract produced activation of lymphoid cell-specific genes. This cell free form of transdifferentiation remains controversial but in theory offers much towards therapeutic applications of tissue replacement (95,99).

MECHANISTIC LINKS BETWEEN EPIGENETIC MARKING SYSTEMS

In what way are DNA methylation and histone modification reprogramming linked? There are some links that are established in other systems, but in mammals, the information is still scarce. In *Neurospora* and *Arabidopsis*, H3K9me can be a signal for DNA methylation, through links between HP1 homologues which bind to H3K9me, and in turn recruit DNA methyltransferases to the marked region (100, 101). In support of this model, ES cells mutant for Suv39h1/2 that lack H3K9me3 in pericentromeric heterochromatin have reduced DNA methylation in major, but not minor, centromeric satellites (41). Conversely, in *Arabidopsis*, DNA methylation may also signal histone methylation, though indirect control via transcription is difficult to exclude (102), and in cultured mammalian cells, the methyl-binding domain protein MeCP2 is associated with HMTase activity (103). These suggest that DNA methylation can also signal histone methylation. However, no large scale changes in histone methylation were observed in ES cells deficient for DNA methylation, suggesting that if there is such a link *in vivo*, it

may be limited to specific genomic regions or developmental stages (41).

The dynamics of DNA methylation and histone modifications during epigenetic reprogramming raise several questions about additional mechanistic links. First, histone methylation, particularly H3K9me2, could act to protect DNA in the pronuclei from active demethylation (42). More precise mapping of H3K9me and DNA methylation in the female pronucleus needs to be carried out, especially to see if there are regions that become actively demethylated. Conversely, sperm DNA exposed during protamine histone exchange, or DNA packaged in acetylated histones, may be targeted by or be accessible to an active demethylation complex present in the oocyte cytoplasm. Sequences that escape demethylation in the male pronucleus are of special interest; is there sequence preference for demethylation or are there regions in the sperm genome that contain histones rather than protamines, perhaps carrying particular modifications? Are the stepwise histone modifications of the paternal genome marks for later *de novo* methylation events?

During global passive demethylation, histone modifications do not appear to be modulated at the same rate as DNA methylation, if at all. Another mystery is the maintenance of germline methylation marks in imprinted genes during this period. Is there a low concentration of Dnmt1 actually in the nucleus which is targeted to imprinted genes by particular histone modifications? In PGCs, in contrast, imprinted genes are rapidly demethylated. Is a particular histone modification responsible for attracting demethylation to these regions?

Finally, the acquisition in the ICM of *de novo* methylation as well as several types of histone modifications again raises questions about mechanistic links and the order of acquisition. Perhaps some of these links can be further elucidated by genetic manipulation of ES cells.

Possibly, the most interesting insight into mechanistic links during reprogramming is that these appear to be developmentally regulated and may depend on the precise developmental stage, cell type and genomic region. This may account for developmental plasticity and regulation, which is typical of pluripotent cell types, in contrast to somatic differentiated cells. The fact that there are so many open questions means that this is an area with exciting discoveries ahead of us.

CANDIDATE MECHANISMS FOR DEMETHYLATION

Enzymatic removal of acetylation from histones is well understood mechanistically, whereas removal of methylation from DNA or histones is not. Last year a number of exciting candidate mechanisms for both have emerged (Fig. 3), whose significance to reprogramming can now be tested *in vivo*.

The loss of DNA methylation of the paternal genome in the zygote is likely an enzyme-catalysed, active demethylation. An oocyte can actively demethylate a transferred somatic nucleus, indicating that the activity responsible is likely to be found in the oocyte rather than the sperm. A number of candidate biochemical pathways have been suggested that either remove the methyl group in the C5 position of the cytidine ring directly (*bona fide* demethylation) or the entire

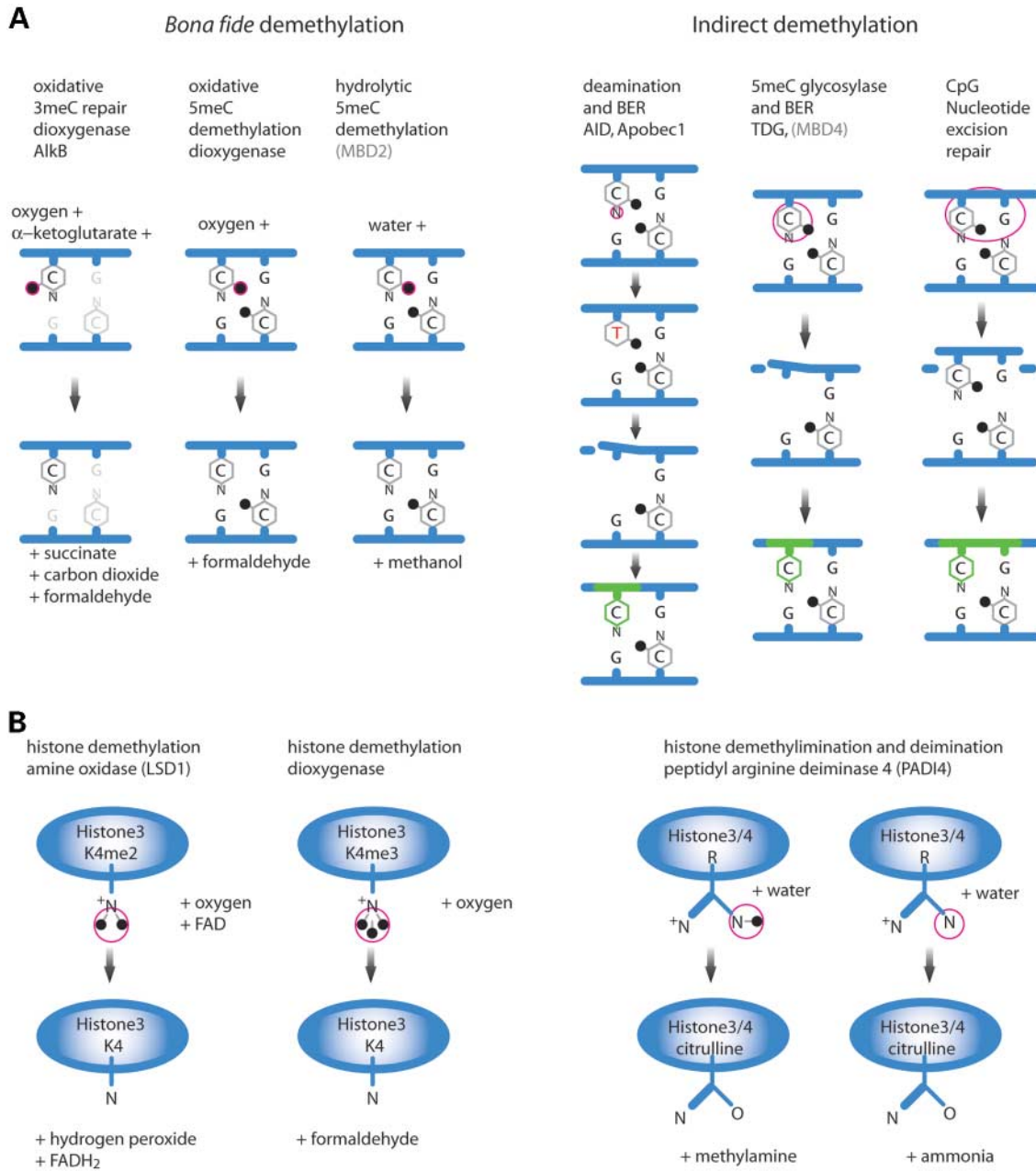


Figure 3. DNA and Histone bona fide and indirect demethylation. (A) Proposed pathways for DNA demethylation have different initial targets (purple ring). Demethylation does not occur in the oocyte by MBD2 or MBD4 (greyed) because oocytes deficient for these proteins still demethylate (15,26). Bona fide demethylation, i.e. direct removal of the methyl group, involves no DNA strand breaks. Oxidative demethylation of 5meC has been proposed by a mechanism akin to 3meC repair by AlkB (106) or hydrolytic reduction (akin to that proposed for MBD2) (104,105). Removal of a mismatched T following deamination of 5meC (111) or removal of the 5meC by a glycosylase (107,108), would require replacement of the cytosine (green), by a mechanism such as base excision repair (BER) (109). An activity that exchanges the meCpG and surrounding phosphates, through nucleotide excision repair (NER), by an unidentified enzyme has been described (110). (B) Histone H3K4me1 and me2, but not me3, can be demethylated by a bona fide demethylase, LSD1 (118). A possible mechanism for demethylating trimethylated histone may be through oxidation by a hypothetical dioxygenase (119). The deimination of H3 or H4 arginine or methylarginine to citrulline by PADI4 prevents further methylation and/or removes monomethylation, indirectly leading to histone demethylation (120,121).

cytidine base (or nucleoside or nucleotide; indirect demethylation) (Fig. 3A). The difficulty with direct demethylation is that the methyl group is attached by a carbon-carbon bond which is very stable. Although direct removal of the methyl group by MBD2 has been claimed (104), this has not been substantiated (105), and importantly oocytes that lack MBD2

remain competent to demethylate the paternal genome (15). The dioxygenases can remove methyl groups from the C3 position of cytidine (106), but this N-C bond is less stable than the C-C one in 5meC, and no enzymes have been found that catalyse the oxidative removal of the methyl group from 5meC.

The indirect pathways to demethylation all involve DNA repair. DNA glycosylases such as thymine DNA glycosylase (TDG) and methyl-binding domain protein 4 (MBD4) normally repair T:G mismatches thought to result from spontaneous deamination of 5meC. TDG and MBD4, however, have been shown to have weak activity on 5meC:G base pairs (107,108), leading to base excision repair (BER) (109) where C replaces 5meC. No information exists yet about the *in vivo* function of TDG, and oocytes lacking MBD4 are still able to demethylate (26). An activity that replaces the mCpG dinucleotide has been described (110), but the enzyme responsible or *in vivo* evidence of this activity has not been elucidated.

Recently, it has been shown that AID and Apobec1, cytidine deaminases, can deaminate 5meC to result in T and that these enzymes are expressed in oocytes and germ cells (111). This activity of cytidine deaminases coupled with BER could theoretically result in demethylation without DNA replication. This proposed mechanism circumvents the problem of the stable carbon-carbon bond but would require extensive BER in the zygote. Transcripts of DNA repair genes are indeed overrepresented in oocytes compared with later preimplantation stages (112), and HR6A (113) and poly(ADP-ribose) activity (114), which may be involved in DNA repair, have critical roles in the first cell cycle of the zygote. DNA methylation and deamination have other intriguing links in evolution. In plants, DNA glycosylases and presumably DNA repair are involved in overcoming silencing by methylation of imprinted genes and transgenes (115,116). In *Neurospora*, methylation and deamination regulate repeat induced gene silencing and mutation (117).

Recent evidence of reversal of histone methylation has revealed interesting mechanistic parallels with demethylation of DNA (Fig. 3B). The direct removal of methyl groups from H3K4me1 and me2 can occur in an FAD dependent oxidative process catalysed by lysine specific demethylase 1 (LSD1) (118), and this enzyme can repress the transcription of specific genes, at least in cell culture. Neither H3K4me3 nor H3K9me3 can be demethylated by this pathway, instead it has been proposed that dioxygenases might be involved (119).

In contrast to the direct demethylation of lysine by LSD1, histone arginine methylation can be reversed indirectly by deimination (120), the removal of nitrogen at arginine's site of methylation (leaving citrulline, not arginine) or demethyliminination (121), the removal of arginine's monomethylated site (also leaving citrulline), by peptidyl arginine deiminase 4 (PADI4). This would effectively result in histone demethylation as chromatin containing histones with citrulline cannot be methylated at these residues. Although the role of PADI4 *in vivo* is not clear yet, it is intriguing that there is apparent arginine demethylation in preimplantation embryos (56) and that the homologue ePAD is expressed in the oocyte (122,123).

CONCLUSIONS

Extensive epigenetic reprogramming of DNA and histone marks occur in mammalian development in totipotent early embryos and pluripotent germ cells. To what extent this reprogramming is a mammalian adaptation perhaps linked to

imprinting, viviparity and regulative embryonic development needs to be established. Handles on the molecular mechanisms are just beginning to emerge, and these highlight the complexity of the process and the challenges involved in reprogramming somatic cells. The challenge is highly worthwhile as it promises completely new avenues to human medicine.

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