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Epigenetic programming and reprogramming during development

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Cell identity is determined by specific gene expression patterns that are conveyed by interactions between transcription factors and DNA in the context of chromatin. In development, epigenetic modifiers are thought to stabilize gene expression and ensure that patterns of DNA methylation and histone modification are reinstated in cells as they divide. Global erasure of epigenetic marks occurs naturally at two stages in the mammalian life cycle, but it can also be artificially engineered using a variety of reprogramming strategies. Here we review some of the recent advances in understanding how epigenetic remodeling contributes to conversion of cell fate *in vivo* and *in vitro*. We summarize current models of epigenetic erasure and discuss the various enzymes and mechanisms that may operate in cellular reprogramming.

One of the most remarkable properties of complex genomes is their capacity to generate a range of different cell types in a highly ordered and reproducible manner. How this happens has intrigued geneticists and developmental biologists alike and has helped spur recent advances in epigenetics. Whereas 'epigenomes' of humans[1,2] and of other species have been sampled, we still know relatively little about how different gene expression patterns initially segregate in the developing embryo or how these are stably transmitted through cell division. In particular, the molecular details of the templating mechanisms that duplicate epigenetic marks through DNA replication remain uncertain[3–6]. Several different types of epigenetic modifications are thought to contribute to mitotic memory, including those that alter chromatin structure, modify DNA and histones, remodel nucleosomes and incorporate variant histones[7]. Collectively, these are thought to stabilize gene expression patterns in specialized cell types so that cellular identity and lineage fidelity is preserved. However, in at least two phases of the life cycle of mammals, epigenetic stability is globally perturbed: when gametes fuse to form the zygote and when gamete precursors (primordial germ cells; PGCs) develop and migrate in the embryo (**Fig. 1**). This *in vivo* 'reprogramming' of the epigenetic landscape signals the reacquisition of totipotency in the zygote and the formation of the next generation through PGCs.

Epigenetic reprogramming can also be achieved *in vitro* using several different approaches in which somatic cells are induced to regain pluripotency. Studies of the factors and dynamics of *in vivo* and *in vitro* reprogramming have begun to uncover how epigenetic information can be erased or maintained. Here we summarize some recent reports describing chromatin-based and DNA-based changes that are associated with reprogramming and the reacquisition of pluripotency in the mouse.

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Epigenetic reprogramming in the preimplantation embryo

Chromatin dynamics. At fertilization, two specialized cell types (gametes) fuse to generate the first cell of the developing embryo, the zygote. Initially, the gamete genomes remain physically separate in the zygote, where they undergo different chromatin changes while under the influence of a common set of maternally inherited factors (**Fig. 2a**).

Soon after fertilization, the paternal genome exchanges protamines (arginine-rich nuclear proteins that replace histones during spermatogenesis[8]) with maternally inherited histones[9]. Upon histone incorporation acetylation is detected[9,10], most likely because newly synthesized histones carry the evolutionary conserved acetylation of Lys5 and Lys12 on histone H4 (ref. 11). Methylation of histones is detected later, and the onset of mono-, di- and trimethylation exhibits a timing that is specific for each progressive modification[10]. In the early zygote, the acquisition of a hyperacetylated and hypomethylated chromatin state may increase the accessibility of the paternal genome and allow additional remodeling to occur. Conversely, the maternal genome maintains the histone modifications that were acquired during oocyte growth (such as methylation on Lys9 and Lys27 of histone H3; refs. 12,13) in both the zygote[10,14] and during subsequent cell divisions[13,15]. This creates an asymmetry between the male and female genomes that is detected, for example, on the basis of H3K9me3 abundance, up to the 4-cell stage[13,14], whereas other chromatin marks such as H4K20me3 (ref. 16), H3K64me3 (ref. 17) and H3K4me3 (refs. 18,19) are equalized by the two-cell stage. Asymmetry is also apparent at the level of DNA methylation, which is globally lost in the paternal pronucleus of the zygote but is retained in the maternal genome (**Fig. 2a**), as shown by microscopy[20–22] and molecular analyses[23].

This asymmetric program in the zygote is probably a consequence of inheriting gametes from the previous generation that had widely different epigenetic profiles. However, its functional importance remains unclear. It has been hypothesized that chromatin modifications established in the gametes may be part of a transgenerational program that is required for proper embryonic development[24]. Notably, the time of activation of embryonic gene expression parallels epigenetic

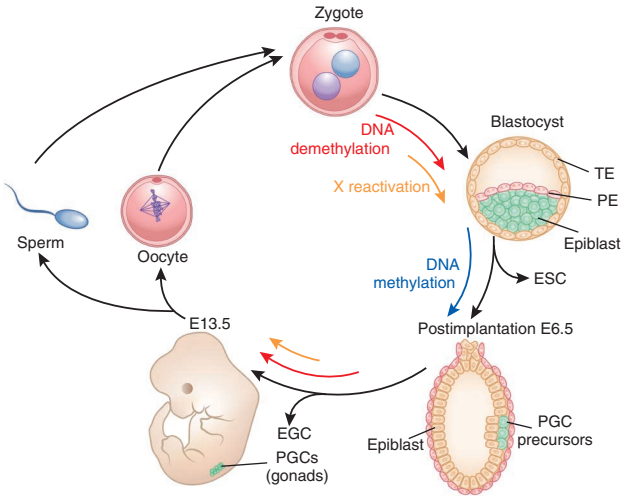


Figure 1 Epigenetic programming and reprogramming during the mouse life cycle. Epigenetic modifications are important for 'programming' lineage determination and cellular identity during development. Global 'reprogramming' of the epigenetic landscape instead marks the conversion of differentiated cells to totipotent or pluripotent states, upon fusion of the gametes (sperm and oocyte) in the zygote and in the PGCs after their specification from the somatic epiblast of the postimplantation embryo. It is notable that two populations of pluripotent cells can be established *ex vivo* within the time window in which extensive epigenetic reprogramming takes place. These cells are ESCs and embryonic germ cells (EGCs) that are derived from the inner cell mass of the blastocyst and from the PGCs at E8.5–E13.5, respectively. Major remodeling events (for example, DNA demethylation and X-chromosome reactivation) are highlighted in the figure by colored arrows. TE, trophoectoderm; PE primitive endoderm.

reprogramming, with the male genome being more permissive to transcription from the late zygote stage[25–27], whereas transcription increases in both genomes thereafter at the 2-cell and 4-cell stages (reviewed in ref. 28). Recent evidence has shown that loss of early zygotic demethylation in the male genome perturbs the activation kinetics of paternal alleles of several pluripotency-associated genes and impairs development[29]. This suggests that distinct epigenetic programs may be required to tune expression of key genes during the sequential activation of the two parental genomes. Understanding which genes are sensitive to this remodeling and documenting the differential activation kinetics of male and female alleles may give us some insight into the role of epigenetic asymmetries in development of the early embryo.

Global DNA demethylation. The 5-methylcytosine (5mC) modification at CpG dinucleotides is a widespread DNA modification that is associated with gene silencing. Inheritance of this modification through cell division is achieved via the maintenance DNA methyltransferase Dnmt1, which copies this modification onto the newly synthesized DNA strand. In the absence of Dnmt1 activity, replication of DNA can lead to reduced overall 5mC levels (so-called 'passive' demethylation of the genome), and this has been hypothesized to occur in the maternal genome during cleavage divisions in the preimplantation embryo[20–22]. Conversely, the paternal genome is thought to be demethylated in the zygote in a manner that is independent of DNA replication (so-called 'active' DNA demethylation)[20,21,23,30,31].

How active DNA demethylation is accomplished is the subject of intense study. Recent studies have shown that 5mC can be sequentially oxidized to 5-hydroxymethylcytosine (5hmC)[32], 5-formylcytosine (5fC) and 5-carboxymethylcytosine (5caC)[33,34] by the Tet dioxygenases (Tet1–3). A member of this family of proteins, Tet3, is highly expressed in oocytes and zygotes but is downregulated in 2-cell-stage embryos. Microscopy studies have shown that 5hmC progressively accumulates at the paternal genome while 5mC levels decrease[35,36] (**Fig. 2a**). This presumed conversion of 5mC to 5hmC is impaired upon silencing[36] or knockout[29] of maternal Tet3, leading to a delayed activation of key pluripotency factors in the paternal genome and partial embryonic lethality. These data suggest that loss of 5mC in the paternal genome is at least in part due to Tet3-mediated oxidation. The molecular mechanisms by which 5hmC is resolved to yield unmodified cytosine are, however, still hotly debated.

High-resolution analyses of metaphase cells in the preimplantation embryo have shown that 5hmC—and its oxidative products 5fC and 5caC—marks both paternal sister chromatids at the first zygotic metaphase but only one set of chromatids at the second, with a progressive reduction at subsequent cell divisions[22,37]. These studies have suggested that 5hmC may be lost through a 'passive' or 'replication-dependent' mechanism. However, as these results have relied on antibody staining to assess the genome-wide distribution of different modifications, they should be viewed cautiously. A recent genome-wide study that characterized the global methylation profile of gametes and embryos at sequential stages of preimplantation development using reduced-resolution bisulfite sequencing showed a drop in 5mC modification from the sperm to the zygote but no additional changes subsequently[23]. Although bisulphite treatment cannot be used to distinguish between 5mC and 5hmC or 5caC and cytosine, this analysis challenges previous data and their interpretation.

On the basis of studies in plants and zebrafish, enzymatic pathways that can trigger base (or nucleotide) excision and thereby replace 5mC and/or 5hmC for unmodified cytosine (reviewed in ref. 38), have been implicated in embryogenesis. In the zygote there is some evidence that the base excision repair (BER) pathway may participate in DNA demethylation. This is based on chromatin binding of BER components (that is, Parp1 and Xrcc1) at single-stranded DNA breaks in the paternal pronucleus[36,39] and the observation that inhibition of BER results in increased DNA methylation[39]. However, an enzymatic activity that can efficiently target 5mC and recruit BER machinery has yet to be identified, and mammalian thymidine glycosylases that could act downstream of 5mC and/or 5hmC deamination have not been detected in the zygote[39].

Although there is still much to learn about these enzymatic pathways, current evidence suggests that both 'passive' and 'active' mechanisms may contribute to the DNA demethylation of the paternal genome. Loci that are demethylated in the zygote[23] and in cloned embryos[40] resemble genomic features and promoter classes enriched for 5hmC in embryonic stem cells (ESCs), namely repetitive elements of some LINE (long interspersed nucleotide elements) *L1* family and germline-associated gene promoters[41,42]. This supports the idea that at least some genomic regions may be targeted for demethylation by a 5hmC-dependent mechanism. For the maternal genome, in which conversion of 5mC to 5hmC is less evident, demethylation has been assumed to occur via replication-dependent dilution of the 5mC marks. However, as Dnmt1 is present throughout and is sufficient to maintain methylation at imprinted regions[43,44], the basis of the loss of 5mC as cells divide still awaits a mechanistic explanation.

Chromatin-based protection of DNA methylation. In the zygote, methylation of the maternal genome is protected from Tet3 oxidation via Dppa3 (also called PGC7 or Stella), a protein that is essential for development during preimplantation[45]. Zygotes lacking Dppa3 lose

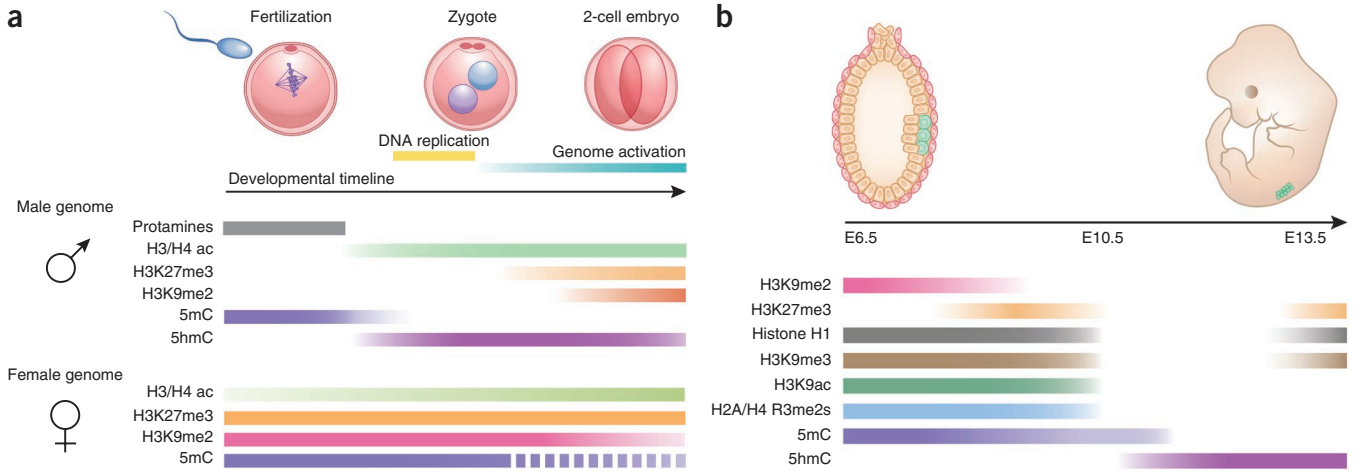


Figure 2 Epigenetic changes during *in vivo* reprogramming. (**a**) Schematic of global DNA and histone modifications that lead to transcriptional activation of the embryonic genome between the late zygote (paternal genome only) and the 2-cell stage. Gamete genomes undergo different epigenetic programs after fertilization with the paternal genome being mostly subject to epigenetic remodeling at the zygote stage and the maternal genome gradually losing repressive modifications during the subsequent cleavage divisions. (**b**) Global epigenetic changes during germline development from PGC specification (E6.5) to the mitotic/meiotic arrest at E13.5. Two major reprogramming phases can be distinguished during PGC migration toward the genital ridges (E7.5–E10.5) and upon their arrival into the gonads (E10.5–E12.5).

asymmetric DNA methylation and exhibit a global loss of 5mC and acquisition of 5hmC in both paternal and maternal pronuclei[36,46]. Recent evidence has shown that Dppa3 binds H3K9me2 in the maternal genome (**Fig. 2a**) and prevents Tet3 access[47]. Loss of maternal Dppa3 or Tet3 alters the kinetics of DNA demethylation in female and male genomes, respectively. In both cases, embryonic lethality occurs, emphasizing the potential importance of epigenetic asymmetry during early preimplantation development.

Although loss of 5mC appears to be a global phenomenon as observed by immunostaining, some genomic sequences (for example, *Iap* retrotransposon, centromeric heterochromatin and imprinted loci) escape demethylation during preimplantation development[48–50]. Maintenance of DNA methylation at these regions is thought to be essential for proper development by preventing the activation of retrotransposons and maintaining imprints and chromosomal stability. Recent evidence suggests that histone modifications, DNA methylation and maternally inherited *trans* factors cross-talk to protect these sequences from the global demethylation events that take place during preimplantation development. The protective effect of Dppa3 is in fact not restricted to the maternal genome but also involves imprinted regions of both maternal and paternal origin[46]. It has been shown that in mature sperm, some imprinted regions are enriched for H3K9me2 (for example, *H19* and *Rasgrf1*), relative to other paternal imprints that are maintained independently of Dppa3 (for example, *Dlk1-Gtl2*)[47], thus opening the interesting perspective that the protection of DNA methylation at some imprints may rely on the inheritance of histone modifications from the gametes to the next generation. This scenario is supported by the findings that histones are retained in the sperm at nonrandom genomic locations[51,52] and can be inherited in the zygote[53].

A recent study has shown that sequence-specific DNA-binding factors can cooperate with the histone modification system to protect against the indiscriminate genome-wide DNA demethylation. The recruitment of Zfp57 and Trim28, two proteins that are essential for the maintenance of DNA methylation at different subsets of imprinted regions[54,55], is preceded in ESCs by the trimethylation of H3K9 and in turn induces DNA methylation[56]. Zfp57 has been shown to bind to a hexanucleotide motif, which is present at all its protected imprinted genes, in a methylation-dependent manner[57] and through Trim28 can recruit both Dnmt proteins[58] and histone modifiers, including the H3K9me3 methyltransferase Setdb1 (ref. 59). Zfp57 and Trim28 therefore mediate a reinforcing feedback loop between DNA methylation and histone modifications, thus enhancing the maintenance of DNA methylation at specific genomic sequences. Taken together, these studies suggest that an elaborate temporal and spatial program of chromatin modifications is necessary to establish the developmental program in the zygote. This is probably needed to choreograph the interplay between inheritance and epigenetic reprogramming of gametes that will give rise to the totipotent zygote.

Epigenetic landscape during development and differentiation

The global epigenetic remodeling that takes place in the early preimplantation embryo is thought to be required for unveiling the entire potential of the genome. However, totipotency is lost as cleavage divisions progress and positional clues start to have a role. The question of when in mammalian preimplantation development the embryonic lineages are first specified is much debated[60,61]. The first well-documented differentiation event, which discriminates between cells that mainly contribute to the extraembryonic lineage and others that will give rise to the embryo proper, probably occurs at the 8–16-cell morula stage[62,63] and has been associated with the level of H3R26me2 in individual blastomeres of 4-cell embryos. The finding that perturbing the levels of H3R26me2 in blastomeres influences their fate suggests that histone modifications may have an instructive role in determining lineage fate[64]. However, as most of the reported studies are dependent on immunofluorescence assays in which antibodies are used to reveal modified histones, the results probably reflect a global change or redistribution of chromatin features. In the future, genome-wide profiling of individual blastomeres by chromatin immunoprecipitation will be necessary to reveal and substantiate more subtle changes, so that the relationship between gene expression, chromatin and cell fate decisions in the early embryo can be clarified.

Currently, much of our understanding of the roles of histone modifications and DNA methylation during development relies on studies in ESCs and *in vitro* differentiation. These models allow access to some of the critical events in the differentiation of embryonic tissues derived from the epiblast cells of the inner cell mass. Histone modifications and DNA methylation are thought to be important for regulating lineage induction, as ESCs lacking specific histone methyltransferase and DNA methyltransferase activity show impaired differentiation[65]. Polycomb repressor complexes, for example, appear to be required to maintain key developmental regulator genes in a silent yet transcriptionally poised state[66,67]. Consistent with this idea, the regulatory regions of many Polycomb target genes in ESCs are marked with histone modifications associated with gene activation and repression on the same nucleosome[68] and this functional 'bivalency' is important for preserving the lineage flexibility of the undifferentiated state.

ESCs that lack Polycomb repressive complex 1 (PRC1) or PRC2 components are reported to be susceptible to unscheduled or biased differentiation[65,69]. PRCs are also known to control gene expression during *in vitro* differentiation and, in this context, recent studies have emphasized the importance of the cross-talk between different histone modifications for 'programming' the genome upon lineage transitions. For example, PRC2 has been shown to be recruited to promoters of active genes through the interaction between one of its subunits, Phf19, and H3K36me2/me3, two marks associated with actively transcribed genes[70,71]. This interaction enhances PRC2 catalytic activity and H3K27me3 deposition while recruiting two different H3K36me2/me3 histone demethylases (Kdm2b and NO66) that facilitate the silencing of transcribed genes[70,71]. Another H3K36me3 demethylase Kdm2a is recruited to unmethylated CpG islands[72], which are enriched for Polycomb target genes[73]. This suggests that a generalized but intricate connection between H3K36me2/me3, Polycomb silencing and DNA methylation could allow the continued repression of lineage-inappropriate genes during cell-fate transition.

Epigenetic remodeling and germ cell precursors

A second wave of global remodeling occurs during the development of germ cells in the embryo (**Fig. 1**). PGCs are specified from the epiblast cells of the postimplantation embryo—cells that have already been primed to a somatic fate[74]. Reprogramming must ensure that germ line–specific genes are primed and that an epigenetic landscape that is compatible with restoring totipotency to the next generation is established. This remodeling appears to be a multistep and coordinated process that requires the timely expression of key transcriptional factors as well as appropriate epigenetic modifiers. The earliest global chromatin change occurs as PGCs migrate and undergo a reciprocal loss of H3K9me2 and increase in H3K27me3 (embryonic day (E)7.5–E10.5)[75,76] (**Fig. 2b**).

The switch between these two repressive chromatin marks has led to the suggestion that germ cells may need to use a more flexible silencing mechanism at this stage. This idea stems from the description that Polycomb-mediated H3K27me3 in ESCs allows the promoters of many developmental regulator genes to be transcriptionally silent, yet poised to be rapidly activated upon differentiation[69]. Transient increases in H3K27me3 may represent the acquisition of a silent but poised state that on the one hand compensates for the loss of H3K9me2 and on the other hand allows fast derepression. Consistent with this, a recent report has shown that the timely downregulation of H3K27me3 during PGC development is required both for maintaining the expression of pluripotency genes (that is, *Oct4*, *Nanog*, *Sall4* and *SSEA1*) *in vivo* and for the derivation of embryonic germ cells *in vitro*[77].

In addition, loss of H3K9me2 may be required to allow DNA demethylation to occur and is probably triggered by the downregulation of Ehmt1 (Glp), a methyltransferase that is required for the deposition of this mark in complex with Ehmt2 (G9a)[78]. In ESCs, lack of the Ehmt1–Ehmt2 complex results in reduced H3K9me2. This is associated with reduced DNA methylation of single-copy genes and retrotransposable elements, even in the presence of all the three Dnmt proteins[79,80]. It is therefore possible that in PGCs the repression of Ehmt1 along with the downregulation of both *de novo* methyltransferases (Dnmt3a and Dnmt3b) and Uhrf1 (a protein that is essential for recruiting Dnmt1), may trigger the initiation of DNA demethylation at this early stage[81,82].

Reduced 5mC levels are reported to occur co-incident with the loss of H3K9me2 from approximately E8.0 (ref. 76; **Fig. 2b**). Several studies have shown that at least some imprinted loci[83–85], transposons (such as LINE *L1* and *Iap*)[48] and a subset of germline-specific genes that are involved in genome defense against active transposons (that is, *Tex19.1* and *Piwil2*)[86] are fully or partially demethylated at E10.5. A recent time-course analysis of both germline and somatic genes has shown a gradual loss of DNA methylation from E8.5 followed by a rapid erasure between E11.5 and E12.5 (ref. 87). On the basis of these kinetics data, it has been proposed that replication-dependent demethylation occurs during early PGC development, whereas an active mechanism operates at a later stage.

Extensive and rapid DNA demethylation has been detected when PGCs enter the gonads (E11.5–E12.5) at which time most of the cells are in G2 phase[75]. It is thought that this second phase of reprogramming depends on an active process independent of DNA replication. Complete demethylation is achieved by E13.5 in gene bodies or intergenic regions, including imprinted domains and repeat elements that were previously protected from erasure in the zygote[87,88]. The only exceptions known are transposable elements belonging to intracisternal A-particle and LTR-ERV1 families and some loci that are located close to these elements or within subtelomeric regions[87,88]. This widespread reprogramming has been thought to be important for preventing the transmission of inappropriate or information to the next generation. Therefore, whereas sex-specific imprinted DNA methylation needs to be reestablished, epimutations that may be accumulated during the organism's life (and may be detrimental) require erasure. This global demethylation is associated with a cascade of chromatin-remodeling events, including the transient loss of linker histone H1, H3K27me3 and H3K9me3, and stable loss of H3K9ac and H2A/H4 R3me2 (ref. 75; **Fig. 2b**) and subsequently, reactivation of the X chromosome in females[89].

Although the mechanisms of DNA demethylation in PGCs remain hotly debated, both replication-dependent and replication-independent mechanisms probably cooperate to achieve appropriate removal of DNA methylation. Parallel strategies may help to confer robustness to the process, an aspect that is particularly relevant in the germline so that genetic and epigenetic information can be faithfully conveyed to the next generation. Different strategies may also be required to orchestrate the timing of demethylation and the subsequent activation of genes with different biological functions. For example, a specific demethylation pathway that operates early during PGC development may target pluripotency genes to allow their activation in a specific time widow. Similarly, it has been postulated that demethylation of germline-specific genes that belong to the genome defense pathway is required to activate gene expression in a phase that precedes the demethylation and potential activation of retrotransposons to safeguard the genome[86]. Different mechanisms may therefore have evolved for specifically targeting different classes of genes. Supporting this hypothesis, it has been

recently shown that loss of Tet1 activity in PGCs leads to the retention of DNA methylation and the downregulation of a subset of genes that is crucial for meiosis and the formation of oocytes[90]. A comprehensive genome-wide analysis of 5mC and its derivatives during PGC development, however, has not been performed and such an analysis will help us in the near future to understand the functional importance of different demethylation pathways.

It is interesting to speculate that reduced DNA methyltransferase activity could favor a replication-dependent loss of DNA methylation in the early PGC migratory phase, whereas later erasure (after E11.5) could be the result of active enzymatic pathways and BER repair. A plausible mechanism for the deamination of 5mC (or 5hmC) into uridine creates mismatches that could be recognized by the mammalian glycosylases (such as Tdg, Mbd4 or Smug1). This has been supported by a genome-wide study showing reduced demethylation in E13.5 PGCs lacking the putative deaminase Aicda[88]. The contribution of Aicda to this process, however, remains controversial as the ablation has a mild effect, and knockout mice are both viable and fertile. Although the mild phenotype could be due to compensation of other deaminases, it is worth noting that neither Aicda nor Apobec1 are expressed at E11.5 when substantial demethylation occurs[39]. Furthermore, neither Tdg, a mammalian glycosylase that has been implicated in the demethylation of some imprinted loci in PGCs[91], or Mbd4 have been detected in PGCs between E10.5 and E13.5, as judged by immunofluorescence analysis[39]. However, Tet1 and Tet2 enzymes are expressed at E11.5 but not at earlier stages of PGC development[39], and Tet1 has been recently shown to be required for the demethylation (at E13.5) and the subsequent expression of meiotic genes during later stages of gametogenesis[90]. Clearly, the role of these enzymes in the development of PGCs awaits clarification, and studies of 5mC and 5hmC distribution and dynamics in normal and mutant embryos will be needed to resolve these mechanisms.

Lessons from *in vitro* models of cell fate reprogramming

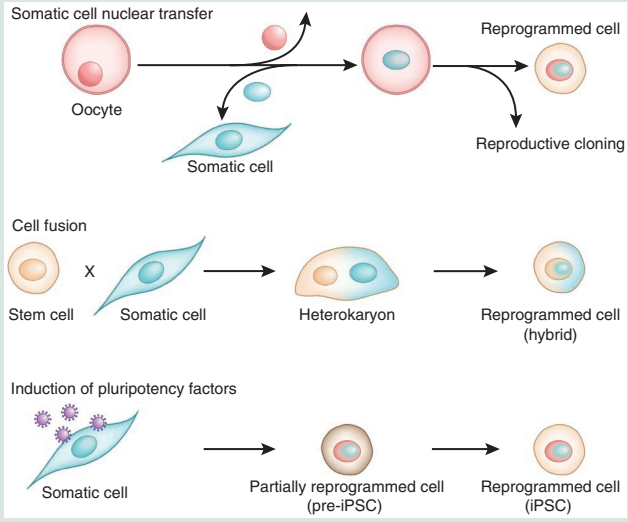
Several different experimental approaches have been used to reprogram somatic cells toward pluripotency (**Box 1**). In these systems, the somatic cell is induced to change its gene expression program through the action of key transcription factors. Exactly how these transcription factors access the somatic genome to reactivate the pluripotency network is still largely unknown. It has been argued that the forced expression of a set of key transcription factors enables each individual cell in a target population to be reprogrammed, provided sufficient time is allowed[92]. However, as only a small proportion of the progeny of a single cell is successfully reprogrammed, stochastic epigenetic events are probably important for reprogramming, and these precede activation of the pluripotency network[92,93].

Several studies have shown that by perturbing the epigenetic landscape of the somatic target, cellular plasticity and reprogramming success can be increased. For example, enhancing the levels of histone modification associated with gene activation increases reprogramming efficiency, most likely by favoring chromatin accessibility. Inhibition of histone deacetylases by various drug treatments (such as valproic acid or sodium butyrate) also enhances reprogramming[94–99]. Similarly, the deposition of H3K4me3 (another histone modification associated with gene activation) facilitates the activation of pluripotency genes[100,101], whereas downregulation of Wdr5, an effector of H3K4me3 and core member of mammalian Trithorax complex, impairs induced pluripotent stem cell (iPSC) reprogramming[102]. In contrast, preventing the deposition of repressive histone marks (or facilitating their removal) enhances reprogramming. For example, inhibition of Dnmt1 (ref. 100) or G9a[103,104] (an H3K9me2-specific methyltransferase) improves the generation of iPSCs. Similarly, loss of H3K9me3 by G9a depletion, or overexpression of the H3K9 demethylase Kdm3a (Jhdm2a), has been reported to facilitate the reactivation of pluripotency genes upon cell fusion–mediated reprogramming[105,106]. Somatic cells that lack the H3K27me3 histone demethylase Kdm6a (Utx) cannot stably reactivate the pluripotency network and cannot generate stably reprogrammed Nanog-positive iPSCs[77].

In addition to modifications at histone tails, nucleosome spacing also influences chromatin accessibility and is regulated by ATP-dependent chromatin remodeling complexes[107]. Components of two of these families of complexes (that is, SWI/SNF and CHD) are required to maintain pluripotency in ESC[108–110] and enhance iPSC reprogramming[108,111]. Depletion of the histone variant MacroH2A,

BOX 1 Experimental approaches for *in vitro* reprogramming

Fifty years ago, Sir John Gurdon showed for the first time that cell differentiation can be reversed. In a pioneering experiment, normal adult frogs were regenerated from terminally differentiated cells of the intestinal epithelium by transplanting their nuclei into enucleated eggs[125]. Since this finding, several techniques have been developed for reprogramming different somatic cell types toward a pluripotent state. In the somatic nuclear transfer approach (top schematic), the nucleus of a somatic cell is reprogrammed upon transfer into an enucleated egg or oocyte by factors present in the ooplasm. Although inefficient, this technique can lead to reproductive cloning of the entire organism, and after the first experiments with frogs, several species have been successfully cloned, including mouse[126]. Fusion of somatic cells with stem cells of different origin gives rise to tetraploid hybrids that acquire a pluripotent phenotype, as shown by the reactivation of pluripotency genes and the capacity to form chimeric embryos[127] (middle schematic). Reactivation of the pluripotency network has been detected a few days after fusion, when the nuclei of the fused cells are still separated (heterokaryon), thus showing that reprogramming can be achieved through the action of *trans*-acting factors[115]. A more recent technique is the induction of pluripotent stem cells (iPSCs) by the overexpression of a defined set of transcription factors[128] (bottom schematic). This has been used to reprogram a wide range of fully differentiated cell types of both mouse and human origin and has therefore a huge potential for clinical application in personalized cell-replacement therapy.



which reduces the binding of SWI/SNF complexes and is involved in heterochromatin formation and gene silencing[112], can enhance the reprogramming of fibroblasts injected into *Xenopus laevis* oocytes[113]. Collectively, these studies suggest that the removal of repressive chromatin modifications and establishment of more 'accessible chromatin' domains generally facilitates the interconversion of cell fates by allowing specific transcription factors to access the genome.

Kinetic studies of reprogramming have shown that somatic cells regain pluripotency via a multistep process in which structural changes to chromatin and loss of DNA methylation precede activation of pluripotency-associated genes[36,62,100,101,114–118]. Silencing of the somatic program has been reported to occur faster than reactivation of the pluripotency network in iPSCs and cell fusion–based systems[114,115,119,120]. It has been proposed that genes that are actively transcribed in the somatic nucleus are accessible for silencing, whereas pluripotency-associated genes are relatively inaccessible or 'occluded' by the stable binding of repressive complexes[119]. Although histone modifications and structural changes that are associated with a more open chromatin conformation can be observed very early during reprogramming, it is possible that stable expression of pluripotency genes requires more extensive chromatin remodeling.

Several studies in different systems have suggested that DNA methylation is a crucial barrier for reprogramming that must be removed for the effective reactivation of pluripotency genes[100,116,117,119]. How this occurs is still much debated. In somatic cell nuclear transfer experiments, demethylation of the *Oct4* (*Pou5f1*) promoter occurs independently of DNA replication[31,117], and the BER and Tet3-mediated 5hmC pathways have been implicated[36,62]. A similar replication-independent mechanism has been suggested to account for demethylation of the human *OCT4* (*POU5F1*) promoter in fibroblasts fused with mouse ESCs[116]. Silencing of Aicda was reported to prevent DNA demethylation and activation of *OCT4* in this system. The role of Aicda in cell fusion–mediated reprogramming has, however, been called in question by a similar study in which no evidence of Aicda expression was found before or after hybrids between rat fibroblasts and mouse ESCs were generated[119]. This study also claimed that inhibition of DNA replication abolished demethylation of *OCT4*.

A more recent study has shown that Tet2 and Parp1 (a multifunctional protein that is involved in both DNA repair and chromatin remodeling) were rapidly induced in fibroblasts after iPSC induction and were essential for reprogramming[121]. However, although both proteins were required for establishing accessible chromatin at the promoters of key pluripotency target genes, they did not have an obvious role in demethylation of these targets at least at early stages, as silencing of Tet2 and loss of 5hmC did not lead to increased methylation at the promoters of pluripotency genes. Loss of Parp1 resulted instead in increased DNA methylation at pluripotency gene promoters, although demethylation of the same promoters was not normally evident at early stages. The authors concluded that Parp1 might act by counteracting the activity of *de novo* DNA methyltransferass (and other epigenetic modifiers) rather than by actively promoting DNA demethylation. Others have suggested that DNA demethylation is a critical step for the stable conversion of partially reprogrammed cells (pre-iPSCs) into fully pluripotent iPSCs[100,122] and that this step requires, and is accelerated by, cell proliferation. The observation that inhibition of Dnmt1 accelerates the conversion adds weight to the assumption that a replication-dependent mechanism underlies this process.

Conclusions

In vivo and *in vitro* studies have demonstrated the intrinsic reversibility and plasticity of the differentiated state. Here we have discussed the roles of different epigenetic modifiers that can confer or remove histone and DNA modifications during *in vivo* and *in vitro* programming and reprogramming. The emerging data suggest that 'active' enzymatic activities can be complemented by the 'passive' loss of DNA and chromatin modifications during DNA replication, and that the relative contribution of each is probably context dependent. In this regard a very recent paper exemplifies how the combination of Tet-mediated 5mC to 5hmC enzymatic conversion with cell divisions has a central role in the DNA demethylation during PGC development[123]. DNA synthesis and mitosis constitute two critical phases in the cell cycle when histone and DNA modifications need to be faithfully conserved. Modulation of epigenetic 'readers' or 'modifiers/writers' during these intervals could, in principle, precipitate the loss or dilution of chromatin-based marks. Thus, a replication-dependent mechanism for remodeling chromatin could be particularly important for reactivating genomic regions or loci during specific stages of development (as discussed for the zygote and PGC) or for allowing cell fate conversions *in vitro* by promoting early stochastic changes in rapidly dividing cells[92,93]. This may also be relevant in refining the epigenetic state during the transition from partially to fully reprogrammed stable states[100,124]. In this context, it will become increasingly important to describe the chromatin profiles of partially reprogrammed cells at successive cell divisions and to genetically deplete 'candidate erasers', to dissect the relevant players and mechanisms in reprogramming.

To date, most of the histone modification analyses in the preimplantation embryo and PGCs have been done by immunofluorescence, and detection of gross changes might obscure more distinct and perhaps important locus-specific events. In the next years, it will therefore be key to adapt genome-wide mapping techniques to the nanoscale to characterize the different combinations of histone and DNA modifications in small reprogramming populations or even in single cells.

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