Epigenetic Reprogramming in Mammalian Development
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led to the assembly of large imprinted clusters. Although the epigenetic asymmetry between parental genomes remains enigmatic, it appears to have been a vital accompaniment to mammalian evolution, viviparity and placentation, and possibly for the necessary emergence of the trophectoderm lineage for the first time, since it is essential for blastocyst implantation. Consequently, its emergence has had a profound and wide-ranging impact on development in mammals.

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REVIEW

Epigenetic Reprogramming in Mammalian Development

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DNA methylation is a major epigenetic modification of the genome that regulates crucial aspects of its function. Genomic methylation patterns in somatic differentiated cells are generally stable and heritable. However, in mammals there are at least two developmental periods—in germ cells and in preimplantation embryos—in which methylation patterns are reprogrammed genome wide, generating cells with a broad developmental potential. Epigenetic reprogramming in germ cells is critical for imprinting; reprogramming in early embryos also affects imprinting. Reprogramming is likely to have a crucial role in establishing nuclear totipotency in normal development and in cloned animals, and in the erasure of acquired epigenetic information. A role of reprogramming in stem cell differentiation is also envisaged.

DNA methylation is one of the best-studied epigenetic modifications of DNA in all unicellular and multicellular organisms. In mammals and other vertebrates, methylation occurs predominantly at the symmetrical dinucleotide CpG (*1*–*4*). Symmetrical methylation and the discovery of a DNA methyltransferase that prefers a hemimethylated substrate, Dnmt1 (*4*), suggested a mechanism by which specific patterns of methylation in the genome could be maintained. Patterns imposed on the genome at defined developmental time points in precursor cells could be maintained by Dnmt1, and would lead to predetermined programs of gene expression during development in descendants of the precursor cells (*5*, *6*). This provided a means to explain how patterns of differentiation could be maintained by populations of cells. In addition, specific demethylation events in differentiated tissues could then lead to further changes in gene expression as needed.

Neat and convincing as this model is, it is still largely unsubstantiated. While effects of methylation on expression of specific genes, particularly imprinted ones (*7*) and some retrotransposons (*8*), have been demonstrated in vivo, it is still unclear whether or not methylation is involved in the control of gene expression during normal development (*9*–*13*). Although enzymes have been identified that can methylate DNA de novo (Dnmt3a and Dnmt3b) (*14*), it is unknown how specific patterns of methylation are established in the genome. Mechanisms for active demethylation have been suggested, but no enzymes have been identified that carry out this function in vivo (*15*–*17*). Genomewide alterations in methylation—brought about, for example, by knockouts of the methylase genes—result in embryo lethality or developmental defects, but the basis for abnormal development still remains to be discovered (*7*, *14*). What is clear, however, is that in mammals there are developmental periods of genomewide reprogramming

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of methylation patterns in vivo. Typically, a substantial part of the genome is demethylated, and after some time remethylated, in a cell- or tissue-specific pattern. The developmental dynamics of these reprogramming events, as well as some of the enzymatic mechanisms involved and the biological purposes, are beginning to be understood. Here we look at what is known about reprogramming in mammals and discuss how it might relate to developmental potency and imprinting.

Reprogramming in Germ Cells

The genomes of mature sperm and egg in mammals are highly methylated—comparable to methylation of somatic cells—although there may be differences in specific patterns (*4*). However, genomewide demethylation occurs early in the development of primordial germ cells in the mouse (Fig. 1A). This demethylation is completed by embryonic day (E) 13 to 14 in both male and female germ cells; before that time the primordial germ cells are also highly methylated and appear to have normal patterns of imprinting (*18*–*22*). At this developmental stage the primordial germ cells have entered the gonads, and most, if not all, differentially methylated regions (DMRs) in imprinted genes become demethylated over a period of a couple of days; the same is true of single-copy gene sequences. To what extent this demethylation occurs in other regions of the genome is not yet clear.

Whether this reprogramming occurs by passive or active demethylation is not known. Fusion of embryonic germ cell lines with somatic cells results in dominant demethylation of the same sequences in the somatic nucleus, suggesting that a demethylating activity acts in trans, and/or that the maintenance function of Dnmt1 has been inactivated in trans (*23*). Once the genomes of the male and female primordial germ cells have been demethylated, the cells enter mitotic (male) and meiotic (female) arrest, respectively. There may be no particular link between these two developmental events. Alternatively, there may be advantages in not replicating relatively demethylated genomes. For example, demethylated centromeres are decondensed and may be functionally altered (*24*). Demethylation also leads to a higher frequency of structural abnormalities in chromosomes (*25*).

Remethylation takes place several days later. It appears to occur earlier in the male germ line, at the prospermatogonia stage (E15 to E16 and onwards) (*20*, *21*, *26*). Remethylation thus precedes reentry of the cells into mitosis, and then meiosis. From the limited analysis carried out so far, it appears that remethylation of single-copy gene sequences and imprinted genes occurs at a similar time (*27*, *28*). Curiously, however, there seem to be differences in the timing of remethylating the maternal and the paternal *H19* gene; for example, the paternal allele is remethylated earlier than the maternal one, suggesting that epigenetic marks other than methylation are still present, particularly on the maternal allele, which therefore initially resists de novo methylation (*27*).

Remethylation in the female germ line takes place after birth during the growth of oocytes. Because oocyte growth is a protracted developmental process, it is conceivable that different sequences might become methylated at different time points. Although an oocyte-specific isoform of Dnmt1 has been shown to enter the nucleus at the beginning of oocyte growth (*29*) when de novo methylation begins, a role for this isoform in de novo methylation has now been disproved (*30*). Thus, the enzymes that lead to de novo methylation in germ cells are still unknown, although Dnmt3a and Dnmt3b are probably good candidates.

Reprogramming in germ cells is needed for the resetting of imprints. Whether it only occurs in species with imprinting is not known, because no comparative data are available for other vertebrates (such as amphibians or birds) in which imprinting is absent. Another likely purpose is the removal of acquired epigenetic modifications, which can be influenced by individual genetic and environmental factors (*31*–

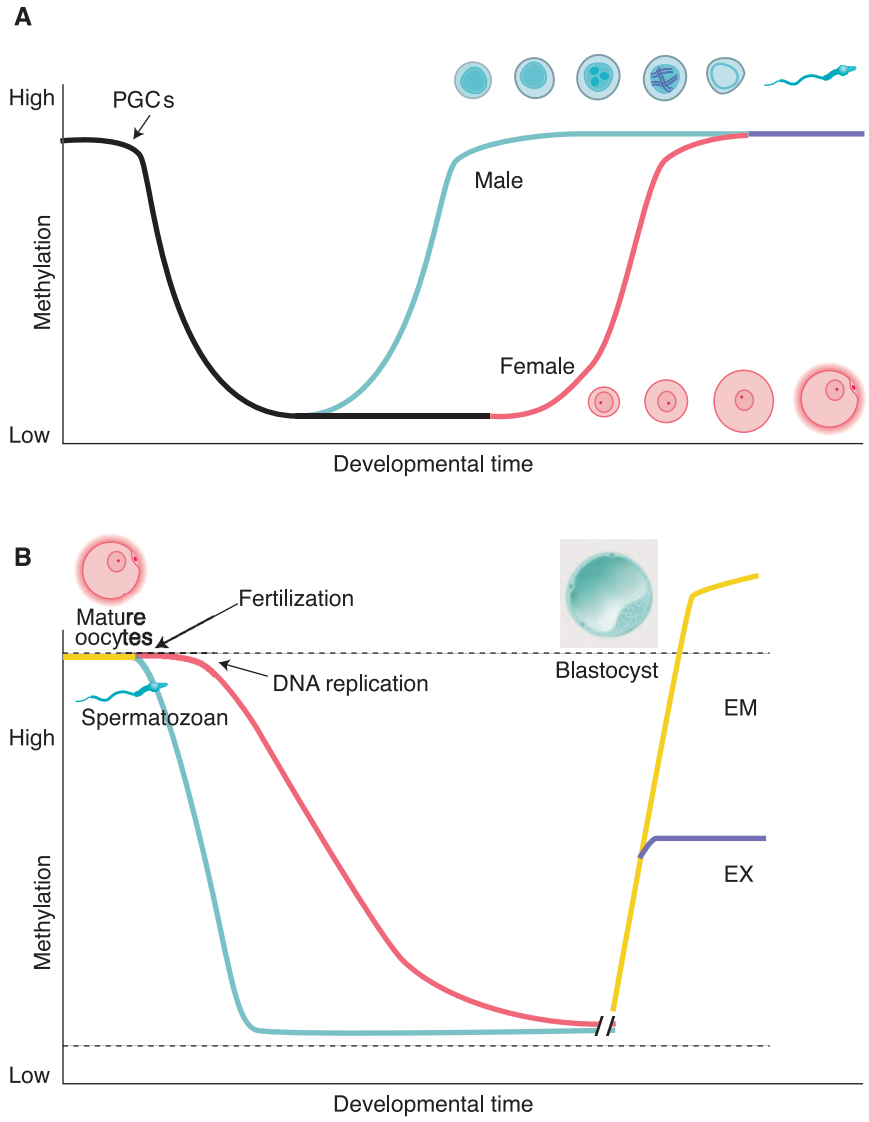

Fig. 1. (**A**) Methylation reprogramming in the germ line. Primordial germ cells (PGCs) in the mouse become demethylated early in development. Remethylation begins in prospermatogonia on E16 in male germ cells, and after birth in growing oocytes. Some stages of germ cell development are shown [modified from (*29*)]. (**B**) Methylation reprogramming in preimplantation embryos. The paternal genome (blue) is demethylated by an active mechanism immediately after fertilization. The maternal genome (red) is demethylated by a passive mechanism that depends on DNA replication. Both are remethylated around the time of implantation to different extents in embryonic (EM) and extraembryonic (EX) lineages. Methylated imprinted genes and some repeat sequences (dashed line) do not become demethylated. Unmethylated imprinted genes (dashed line) do not become methylated.

35). Occasionally, though, epigenetic information appears to be inherited through the germ line (*32*, *36*–*38*), and this is likely to be a result of incomplete erasure in the germ line (or after fertilization). Whether most epigenetic information is erased in germ cells is not clear; there might be a need to keep transposable elements silent by methylation, particularly in the germ line (*8*). Thus, it will be important to investigate if all transposable elements become demethylated. Finally, demethylation in the germ line may have the additional benefit of reducing the mutation rate caused by deamination of 5-methylcytosine (*39*).

Reprogramming in Early Embryos

Reprogramming in early embryos occurs both by active and passive mechanisms (Fig. 1B). The paternal genome undergoes a remarkable transformation in the egg cytoplasm, where remodeling of sperm chromatin through removal of protamines and replacement by (acetylated) histones is closely followed by genomewide demethylation, which is complete before DNA replication commences (*40*–*42*). Although this is the best evidence so far for active demethylation in vivo, the mechanisms by which it occurs are not known. Because acetylated histones are present in both parental genomes while the demethylation reaction takes place, it is unlikely that their presence confers either susceptibility or protection from demethylation (*42*). It is possible that demethylation is intricately linked to chromatin remodeling in the egg. Again, this by itself is not sufficient because the sperm genome in nonmammalian species (for example, *Xenopus*) undergoes chromatin remodeling when introduced into oocytes, but not active demethylation (*43*).

From in vitro experiments, demethylation reactions have been proposed to proceed by direct removal of the methyl group from the cytosine base (*16*), or by replacement of 5-methylcytosine by cytosine through a base-excision mechanism (*15*, *17*). Demethylation could also occur by initial deamination of 5-methylcytosine followed by mismatch repair [MBD4 has T/G mismatch glycosylase activity (*44*)]. The methylcytosine binding protein MBD2 has been proposed as a candidate enzyme for the direct removal of the methyl group (*16*), but this work has not been confirmed by others (*45*, *46*). In oocytes homozygous for a MBD2 knockout, the demethylation reaction occurs normally, demonstrating that MBD2 is not required for demethylation in vivo (*42*).

Some sequences in the paternal chromosomes are protected from demethylation at fertilization. These include the imprinted genes *H19* (*47*–*49*) and *Ras Grf1* (*50*) [but not *Igf2* (*40*)] and some repeat sequences (*51*). During the subsequent cleavage divisions, passive demethylation takes place because Dnmt1 is excluded from the nucleus (*29*, *30*, *52*, *53*). In mouse eight-cell embryos, however, Dnmt1 is relocated to the nucleus for just one replication cycle. In a knockout of this oocyte and early embryo form of Dnmt1 (Dnmt1o), the methylated allele of imprinted genes lost precisely 50% methylation, demonstrating that maintenance methylation by Dnmt1o during the fourth cell cycle is crucial for the maintenance of imprinted methylation (*30*). Before and after this stage when Dnmt1o is in the cytoplasm, it is possible that other enzymes maintain imprinted methylation. Alternatively, Dnmt3a and Dnmt3b could maintain the methylated allele, provided the unmethylated allele is protected from the action of these enzymes. Indeed, maintenance of methylation in DMR2 of *Igf2* requires both Dnmt1 and Dnmt3a,b (*14*). In some cases, continued methylation after fertilization may be required to establish fully maternal methylation imprints (*54*). Protection of the unmethylated allele of imprinted genes continues to be needed around the time of implantation when genomewide de novo methylation is carried out by Dnmt3a and Dnmt3b, and may be conferred by specialized chromatin structures on the unmethylated allele (*55*). In general, genomic sequences are likely to require other epigenetic marks to be protected from, or to attract, de novo methylation at this stage (*35*).

The basic reprogramming events of paternal demethylation in the zygote—passive demethylation in early cleavage stages and de novo methylation thereafter—appear to be conserved in eutherian mammals (*56*), although their relative timing with respect to developmental stage can differ. In mouse embryos, for example, de novo methylation occurs in the inner cell mass (ICM) cells of the expanded blastocyst, whereas in bovine embryos de novo methylation occurs from the 8- to 16-cell stage (*56*).

Postzygotic demethylation and remethylation are likely to play a role in the removal of acquired epigenetic modifications (as does germ-line reprogramming), particularly of those that might have been acquired during gametogenesis. Another intriguing possibility is that de novo methylation and nuclear reorganization are linked to the first-lineage decisions during mammalian development (*56*). This could lead to a reexamination of the original proposal linking methylation and differentiation (*57*–*59*). The question therefore arises whether genomewide reprogramming is limited to germ cells and preimplantation embryos, or conversely, whether it may occur much more generally during stem cell differentiation. Indeed, recent evidence supports transient global demethylation during muscle development in vitro (*60*).

Reprogramming and Cloning

The apparent conservation of early embryonic reprogramming in mammals raises important questions about animal cloning with somatic donor nuclei. Somatic donor nuclei that have been used include fetal and adult fibroblasts, embryonic stem (ES) cells, and other somatic cells that were presumably differentiated (*61*–*64*). Where tested these cells have a highly methylated genome, which is characteristic of somatic cells (*56*, *65*). Two studies have now addressed the question of whether reprogramming occurs to somatic nuclei in clones. In cloned bovine morulae and blastocysts, methylation levels of several repeat and unique sequences were found by bisulfite analysis to be much higher than in normal embryos, and thus resembled methylation levels in the donor-cell genome (*65*). Higher methylation levels in cloned bovine morulae were also found by immunofluorescence detection of 5-methylcytosine in interphase nuclei (*56*). Although the fibroblast nuclei appeared to lose some methylation on introduction into enucleated oocytes (consistent with active demethylation), further demethylation was not observed, and instead, precocious de novo methylation occurred in a substantial proportion of cloned embryos. In addition, reorganization of the nuclear pattern of methylation to resemble that of differentiated nuclei occurred prematurely in cloned embryos (*56*). These observations suggest that reprogramming is deficient in most cloned preimplantation embryos; in particular, demethylation seems to be inefficient, perhaps because the somatic nuclei contain the somatic form of Dnmt1, which, unlike the oocyte form, is capable of maintaining methylation levels. It is conceivable that the aberrant sequence of reprogramming events leads to developmental problems in clones, especially if de novo methylation events are linked to nuclear reorganization and cellular differentiation. Most cloned embryos die at preimplantation or various postimplantation stages, and even those that develop to term often have specific abnormalities, particularly of the placenta (*64*).

These observations on bovine embryos should be compared with those on cloned mice, in which the inactive X chromosome in extraembryonic tissues was the same as that in the somatic donor nuclei, but X inactivation was random in somatic tissues of clones (*66*). Thus, *Xist* methylation was presumably not affected initially by active demethylation, but was then reset (perhaps by passive demethylation) in descendants of ICM cells in the blastocyst. This particular aspect of reprogramming seemed thus to occur normally in cloned mice; however, an important difference between the studies is that the few surviving fetuses or offspring were studied in mice, but preimplantation embryos were examined in the bovine studies.

Of particular interest is the fate of imprints in cloned animals. Aberrant active demethylation could lead to loss of imprinting. While

germ line imprints are generally retained in differentiated tissues, it is not known whether all cells in a tissue retain imprints. In mouse ES cells, considerable heterogeneity in imprinting patterns can lead to developmental abnormalities when these cells are used to derive chimeric or cloned offspring (*67*, *68*). Some of the abnormalities seen in cloned animals such as fetal and placental overgrowth are consistent with aberrant expression of imprinted genes (*64*). A better understanding of reprogramming, in particular, those aspects that are necessary for the attainment of developmental totipotency—and perhaps also the criteria that could be applied to select or to create better donor nuclei—should lead to improved cloning efficiency.

Reprogramming and Imprinting Mechanisms

It is likely that embryonic reprogramming had a significant effect on shaping the evolution of imprinting mechanisms (Fig. 2) (*69*, *70*). First, paternal-genome reprogramming appears to be conserved in mammals with imprinting, but does not occur in the Zebrafish (*71*) or *Xenopus* (*43*), which do not have imprinting. Curiously, imprinting has been shown to occur in flowering plants (*72*), and in tobacco a sudden demethylation step consistent with reprogramming has been observed in pollen (*73*). The occurrence of active paternal demethylation in mammals has been interpreted in the context of the genetic conflict theory of imprinting as an "anti-imprinting weapon" (*74*). In this scenario, the maternal genome uses demethylation of the sperm genome to remove paternal methylation imprints (which normally enhance fetal growth, for example). Those genes with paternal germ line methylation (*H19*, *Rasgrf1*) must have evolved a special protection mechanism, perhaps one based on a protective chromatin structure, to escape demethylation (*74*). In addition, all DMRs with a germ line imprint must be resistant to passive demethylation. How this resistance is brought about is not yet clear; however, it is instructive to compare the structural features of DMRs with those of retrotransposons, which are also highly resistant to demethylation. It has thus been suggested that direct tandem repeat sequences found in retroviral long-terminal repeats and often in or near DMRs are involved in imprinting (*75*, *76*). If so, it is likely that they help to protect methylated alleles from (passive) demethylation. Protection from de novo methylation in the embryo of DMRs could occur by mechanisms similar to those underlying the general protection of CpG islands (*77*, *78*), or may require specialized chromatin structures.

Most maternally silenced imprinted genes are repressed by promoter methylation originating from DMRs that are methylated in the oocyte (*74*). By contrast, no protein-coding imprinted gene has been found that is repressed by paternal methylation derived from the sperm, presumably because of active demethylation of the paternal genome. One mechanism by which paternal repression is achieved is by overlapping antisense transcripts, which may act in cis. Promoter methylation of the antisense transcript (usually resulting from oocyte-derived methylation) represses its transcription and thus activates the protein-coding gene "epigenetically" (*74*, *79*). Epigenetic activation is not limited to maternally expressed genes (for unknown reasons) and can occur by mechanisms other than antisense overlap [discussed in more detail by Ferguson-Smith and Surani in this issue (*70*)].

Most of the imprinted genes occur in clusters in the genome (*69*, *70*). Two well-characterized clusters (on distal and middle chromosome 7 in the mouse, respectively) have at least one (middle 7) or two (distal 7) imprinting control regions (ICRs). These contain DMRs with germ line–derived methylation differences and are needed in cis for the coordinate control of several imprinted genes in the clusters. Nevertheless, several genes in the clusters, in addition to the ICR, have DMRs that are required for appropriate imprinting as well (*69*, *70*). Recently, it has been shown that sperm-derived methylation in DMRs of the *Igf2* gene is lost in the zygote, but differential methylation is reestablished at early postimplantation stages (*40*, *80*). Correct reestablishment requires the ICR in cis (*81*), thus suggesting that coordinate control of epigenetic modifications in clusters can overcome the loss of germ line imprints due to reprogramming. A similar observation has been made in the cluster on middle chromosome 7, in the *Nectin* gene (*82*). Hence, embryonic reprogramming may be a factor that contributes to clustering of imprinted genes.

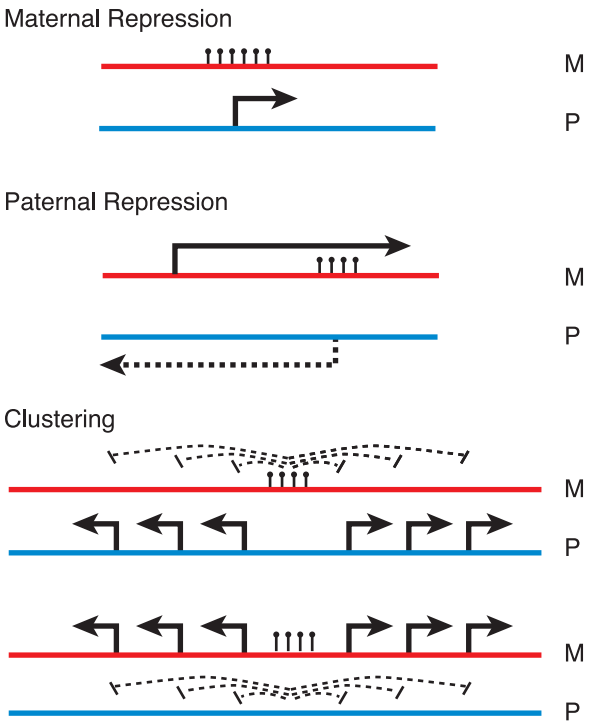

Fig. 2. Impact of reprogramming on imprinting mechanisms. Major imprinting mechanisms are shown schematically. Maternal repression can be achieved by maternal methylation (lollipops). Paternal repression can be achieved in cis by overlapping antisense transcripts (dashed line); maternal methylation leads to inactivation of the antisense transcript and hence maternal expression of the sense gene. Imprinting control in clusters can be achieved by a maternally methylated ICR (lollipops) leading to repression in cis (dashed lines), or a paternally unmethylated IC leading to repression in cis (dashed lines). Imprinting mechanisms are discussed in more detail by Ferguson-Smith and Surani (*70*).

Conclusions

In mammalian embryos there are two major cycles of epigenetic reprogramming of the genome: during preimplantation development and during germ cell development. Reprogramming in germ cells is necessary for imprinting; reprogramming in preimplantation embryos paradoxically can interfere with imprinting and has shaped imprinting mechanisms. It is possible that both demethylation cycles involve active demethylation, and it is crucial to identify the mechanisms. Reprogramming mechanisms in preimplantation embryos affect epigenetic modifications and genome function of cloned embryos. Sequences that escape reprogramming may be involved in epigenetic inheritance. It is important to examine whether in addition to germ cells and early embryos, reprogramming is also involved in stem cell differentiation.

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REVIEW

Nuclear Cloning and Epigenetic Reprogramming of the Genome

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Cloning of mammals by nuclear transfer (NT) results in gestational or neonatal failure with at most a few percent of manipulated embryos resulting in live births. Many of those that survive to term succumb to a variety of abnormalities that are likely due to inappropriate epigenetic reprogramming. Cloned embryos derived from donors, such as embryonic stem cells, that may require little or no reprogramming of early developmental genes develop substantially better beyond implantation than NT clones derived from somatic cells. Although recent experiments have demonstrated normal reprogramming of telomere length and X chromosome inactivation, epigenetic information established during gametogenesis, such as gametic imprints, cannot be restored after nuclear transfer. Survival of cloned animals to birth and beyond, despite substantial transcriptional dysregulation, is consistent with mammalian development being rather tolerant to epigenetic abnormalities, with lethality resulting only beyond a threshold of faulty gene reprogramming encompassing multiple loci.

Epigenetic modification of the genome ensures proper gene activation during development and involves (i) genomic methylation changes, (ii) the assembly of histones and histone variants into nucleosomes, and (iii) remodeling of other chromatin-associated proteins such as linker histones, polycomb group, nuclear scaffold proteins, and transcription factors (*1*). The two parental genomes are formatted during gametogenesis to respond to the oocyte environment and proceed through development (Fig. 1A). The zygote biochemically remodels the paternal genome shortly after fertilization and before embryonic genome activation (EGA) occurs. To successfully recapitulate these processes, the somatic nuclei transferred into an oocyte must be quickly reprogrammed to express genes required for early development.

Epigenetic reprogramming after fertilization and nuclear transfer has been studied in *Xenopus* and mammals (*1*). Here we will concentrate on aspects of epigenetic gene regulation that are pertinent to our understanding of the reprogramming process after mammalian somatic cell nuclear transfer including chromatin structure, DNA methylation, imprinting, telomere length adjustment, and X chromosome inactivation with a focus on experimental data from the mouse. Also, we will compare and contrast the outcome of cloning experiments when either somatic or embryonic stem (ES) cells are used for nuclear transfer.

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