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Review

Reprogramming mammalian somatic cells

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Abstract

Somatic cell nuclear transfer (SCNT), the technique commonly known as cloning, permits transformation of a somatic cell into an undifferentiated zygote with the potential to develop into a newborn animal (i.e., a clone). In somatic cells, chromatin is programmed to repress most genes and express some, depending on the tissue. It is evident that the enucleated oocyte provides the environment in which embryonic genes in a somatic cell can be expressed. This process is controlled by a series of epigenetic modifications, generally referred to as "nuclear reprogramming," which are thought to involve the removal of reversible epigenetic changes acquired during cell differentiation. A similar process is thought to occur by overexpression of key transcription factors to generate induced pluripotent stem cells (iPSCs), bypassing the need for SCNT. Despite its obvious scientific and medical importance, and the great number of studies addressing the subject, the molecular basis of reprogramming in both reprogramming strategies is largely unknown. The present review focuses on the cellular and molecular events that occur during nuclear reprogramming in the context of SCNT and the various approaches currently being used to improve nuclear reprogramming. A better understanding of the reprogramming mechanism will have a direct impact on the efficiency of current SCNT procedures, as well as iPSC derivation.

Keywords: Epigenetics; Somatic cell nuclear transfer; IPSCs; Embryo

Contents

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1. Introduction

The majority of cells in an organism differ both morphologically and functionally from one another (i.e., epithelial, muscle, connective, neural cells). However, they all originate from a single cell, the zygote, which through several cell divisions gives rise to all cell types. With very few exceptions, most cells in an organism contain exactly the same DNA sequence. Differences in gene expression among cell types are therefore not genetic, but rather epigenetic. The term "epigenetics" was introduced during the 1940s by Conrad H. Waddington to describe "the events which lead to the unfolding of the genetic program" [1]. Epigenetics was applied 40 yrs later to describe "the interactions between genes and the cellular environment that produce a change in the cell phenotype" [2].

As cells differentiate and specialize to become a particular cell type, "cellular memory" is established, ensuring that only a specific set of genes will be transcribed and others will be silent [3]. Once differentiated, each cell passes its specialized character on to the daughter cells, thereby ensuring the preservation of the appropriate tissue type. Transcriptionally active genes are roughly the same for a particular type of cell and the pattern of gene expression is "remembered" through subsequent cell divisions. Methylation of DNA, chromatin packaging, and remodeling of chromatin-associated proteins, such as linker histones, polycomb group, and nuclear scaffold proteins [4,5], are some of the epigenetic mechanisms stably passed from cell to cell during cell division, ensuring the maintenance of distinctive cell types.

Although epigenetic marks in somatic cells are stable, they can be altered to a certain degree and, as a general rule, most somatic cells can be reprogrammed into becoming a different cell type [6,7]. Furthermore, the nucleus of a somatic cell can be reprogrammed to develop into an embryo and become a new organism. One of the ways in which reprogramming of a differentiated cell can be achieved is Somatic Cell Nuclear Transfer (SCNT), commonly referred to as cloning. The somatic nucleus or even the whole somatic cell is transferred into what is commonly referred to as an enucleated oocyte from which its own genomic DNA has been removed [8]. Following nuclear transfer, the oocyte is activated to start embryogenesis and finally generate a new organism [9]. Table 1 summarizes the reports of live offspring from 20 mammalian species obtained from a wide range of somatic cells as nuclear donors for SCNT.

Table 1
First reported offspring in various mammalian species obtained by somatic cell nuclear transfer from differentiated cells.

Year	Species	Donor cell type	Reference
1997	Sheep	Mammary epithelium	Wilmut, et al. [211]
1998	Cow	Fetal fibroblasts	Cibelli, et al. [212]
1998	Mouse	Cumulus cells	Wakayama, et al. [56]
1999	Goat	Fetal fibroblasts	Baguisi, et al. [213]
2000	Pig	Granulosa cells	Polejaeva, et al. [214]
2000	Gaur	Skin fibroblasts	Lanza, et al. [215]
2001	Muflon	Granulosa cells	Loi, et al. [216]
2002	Rabbit	Cumulus cells	Chesne, et al. [217]
2002	Cat	Cumulus cells	Shin, et al. [218]
2003	Horse	Skin fibroblasts	Galli, et al. [219]
2003	Rat	Fetal fibroblasts	Zhou, et al. [220]
2003	African wild cat	Skin fibroblasts	Gómez, et al. [221]
2003	Mule	Fetal fibroblasts	Woods, et al. [222]
2003	Banteng	Skin fibroblasts	Janssen, et al. [223]
2003	Deer	Skin fibroblasts	Westhusin [224]
2005	Dog	Skin fibroblasts	Lee, et al. [225]
2006	Ferret	Cumulus cells	Li, et al. [226]
2007	Wolf	Skin fibroblasts	Kim, et al. [227]
2007	Buffalo	Skin fibroblasts	Shi, et al. [228]
2009	Camel	Skin fibroblasts	Wani, et al. [229]

Despite the technological advances in SCNT during the last decade, and its scientific and medical importance, the molecular processes involved in nuclear reprogramming remain largely unknown and the overall efficiency of SCNT in mammals remains very low. The efficiency of cloning, defined as the proportion of transferred embryos that result in viable offspring, is approximately 2 to 3% for all species. However, in cattle, average cloning efficiency is higher than in other species, ranging from 5 to 20% [10–15]. Among the factors thought to contribute to the greater success in cloning cattle are the relatively late embryonic genome activation specific for this species [16–18] and the optimization of reproductive technologies, such as *in vitro* embryo production and embryo transfer, brought about by the cattle industry [19]. Additionally, the efficiency of nuclear transfer technology may be enhanced by better understanding the nature of reprogramming using the cow model, since approximately half of all SCNT's worldwide are performed in this species [20].

Failure to reprogram the donor genome is thought to be a main reason for the low efficiency of cloning [5,21–23]. Various strategies have been employed to improve the success rate of SCNT. Most of these focus on the donor cell, including: a) cell type, or tissue of origin [24–27]; b) passage number [28–30]; c) cell cycle stage [31–35]; and d) use of chemical agents and cellular extracts to modify the donor

cell's epigenetic state [36–38]. The influence of various oocyte enucleation, fusion, and activation methods on cloning efficiency has also been analyzed to a lesser extent [39–41].

Although the cellular and molecular events that occur during nuclear reprogramming are integrated, in this review they will be analyzed separately for the sake of simplicity. We will first describe the reprogramming machinery of the oocyte and the changes in chromatin structure that occur after fertilization and nuclear transfer. We will then cover epigenetic modifications, including DNA methylation, gene imprinting, and X-chromosome inactivation, and their modifications after nuclear transfer. The expression patterns of genes that are crucial for embryonic development are discussed, focusing on differences among embryos produced by fertilization and those produced by nuclear transfer. Finally, we examine current strategies for improving nuclear reprogramming and the future application of these to enhance cloning efficiency.

2. Mechanisms of reprogramming

During fertilization, components in the oocyte cytosol reprogram the paternal genome. Although the entire process is not completely understood, it is known that sperm reprogramming involves remodeling of chromatin through removal of protamines and replacement by maternal histones. This event is closely followed by genome-wide demethylation, thereby creating the basis for appropriate gene regulation during embryogenesis [42–45].

Erasing the epigenetic marks of a somatic nucleus is a complex process that requires global changes in DNA methylation, chromatin structure, gene imprinting, X chromosome inactivation, and restoration of telomere length [46]. It is likely that the oocyte's machinery that reprograms the sperm and oocyte genomes is also responsible for erasing the "cellular memory" and reprogramming a somatic nucleus after SCNT. Since spermatozoa and somatic cells have such different chromatin structure and DNA methylation patterns, it is conceivable that the oocyte may not reprogram a somatic nucleus with the same efficiency it reprograms sperm DNA. Somatic nuclear reprogramming is delayed and incomplete when compared to sperm nuclear reprogramming [47]. It can be argued that the reprogramming of a somatic genome resembles the reprogramming of the maternal pronucleus undergoing a gradual replication-dependent demethylation. The nuclear reprogramming event caused by SCNT could be considered a transdifferentiation process that implies the molecular dominance of one distinct cell type (the oocyte cytoplasm) over another (the somatic nucleus), resulting in transformation of the somatic nucleus into a totipotent nucleus [48].

Epigenetic reprogramming by the oocyte is not an all-or-nothing event. There is ample evidence that demonstrates the presence of multiple degrees of reprogramming; some states are compatible with life, whereas others are not. The epigenetic marks in cloned embryos, fetuses, and adults from several species do not always correlate to those of their counterparts produced by fertilization. High levels of embryonic death suggest that some errors in epigenetic reprogramming are lethal [49,50]. The outcomes from an SCNT procedure varies from embryos that fail to develop, to cloned animals that reach adulthood with no evident pathology. Between these two extremes, there is a range of cloned animals that reach various stages. Some cloned embryos die during the earliest or later stages of pregnancy, whereas others make it all the way to term, but die during the perinatal period. Strong evidence from multiple independent laboratories, using various species, agrees that complete thorough reprogramming occurs only in a small proportion of nuclear transfers [51].

3. Extreme chromatin make over

3.1. Role of histones

The basic unit of chromatin is the nucleosome, which is comprised of 147 bp of DNA wrapped around an octamer of histones, formed by pairs of each of the four core histones (H2A, H2B, H3, and H4). Each nucleosome is linked to the next by small segments of linker DNA. Chromatin is further condensed by winding in a polynucleosome fiber, which may be stabilized through binding of histone H1 to each nucleosome and to the linker DNA [52]. Enzymatic modifications of histones include phosphorylation, methylation, acetylation and ubiquitination, or removal of these modifications [53]. These modifications are recognized by other structural proteins and enzymes, which together stabilize the pattern of gene expression.

Little is known about the initial molecular events that ensure nuclear reprogramming in the mammalian oocyte. In efforts to fill this gap of knowledge, new insight was brought by studies in which mammalian somatic cells were transferred into frog oocytes [54] which, due to their size and availability, represent an appropriate system for the study of nuclear reprogramming. Within 1 h after nuclear transfer, the mammalian

somatic nuclear membrane breaks down, mimicking the breakdown of the sperm nuclear envelope after fertilization [55]. The second event after SCNT appears to be condensation of somatic cell chromosomes upon exposure to the M-phase ooplasm, which directs the formation of a new spindle [56]. In *Xenopus laevis*, somatic nuclei lose more than 85% of their own protein when transferred to an enucleated oocyte, while simultaneously incorporating a substantial amount of protein from the cytoplasm [57]. Oocyte activation leads to the formation of "pseudopronuclei" that resemble the pronuclei formed after fertilization, but contain a random assortment of maternal and paternal chromosomes. Often two "pseudopronuclei" are formed, but, the formation of only one or more than two has been observed in the mouse [58]. The successful union of the pseudopronuclei occurs at the first mitotic division, as it does in normal fertilized embryos [47].

Significant histone reallocation takes place during the first few hours after SCNT. The linker histone H1 may be involved in the regulation of gene expression in early embryos [59]. Somatic H1 is lost from most mouse nuclei soon after transfer. The rate of loss depends on the cell cycle stage of donor and recipient cells [60]. Bovine linker histone H1 becomes undetectable in somatic nuclei within 60 min after injection into bovine oocytes, and is completely replaced with the highly mobile oocyte-specific H1FOO linker histone variant [55,61]. More recent findings suggest that Histone B4, an oocyte-type linker histone, also replaces H1 during reprogramming mediated by SCNT [62]. Together, these findings suggest an important role for linker exchange in nuclear chromatin remodeling. Histone 1 remains absent or in very low concentration in early cloned embryos, but becomes detectable at the 8- to 16-cell stages, when major transcriptional activation of the embryonic genome occurs. At these stages, oocyte molecules are replaced by the embryo derived H1 in a fashion similar to what happens in normally fertilized embryos [63]. It seems that nucleoplasmin, along with other proteins in the oocyte, are involved in the H1 removal [52]. In contrast, core histones of somatic nuclei, especially H3 and H4, are not removed, but remain stably associated with somatic DNA [64,65].

Histone tails are subjected to a wide range of postranslational modifications, including acetylation, phosphorylation, and methylation, which are implicated in transcriptional activation. Acetylated lysines on core histones (H3K9, H3K14, H4K16) of somatic cells are quickly deacetylated following SCNT. Their reacetylation was observed following the oocyte activation treatment in cloned mouse embryos. However, acetylation of other lysine residues on core histones (H4K8, H4K12) persisted in the genome of cloned embryos [66]. In somatic cells, transcriptionally active 5S rRNA genes are packaged with hyperacetylated histone H4; in the transcriptionally silent oocyte, these residues are hypoacetylated [67,68]. It could be argued that after SCNT, the cloned embryo establishes a histone acetylation pattern that partially resembles that of embryos produced by fertilization. The same has been reported for histone phosphorylation, whereby histone H3-S10 and H3-S28 were phosphorylated and dephosphorylated in the somatic chromatin in a manner paralleling changes in oocyte chromosomes [69].

3.2. Non-histone changes

Along with histones, several non-histone nuclear proteins are also actively released from or incorporated into the somatic chromatin after nuclear transfer [70]. One such example is the basal transcription factor TATA binding protein (TBP) that is released from somatic chromatin by a chromatin remodeling protein complex (ISWI, a member of the SWI2/SNF2 super family) in the oocyte cytoplasm [52,70]. The helicase activity of these multisubunit ATP-dependent enzymes exposes DNA and redistributes nucleosomes in a tissue-specific manner [53]. The loss of a principal component of the basal transcriptional complex from somatic nuclei that have been incubated in frog oocyte extract provided the first indication that members of the SWI/SNF family of enzymes may have roles in the development of cloned embryos [70,71]. Members of the high mobility group proteins (HMG), particularly those corresponding to the Nucleosomal subfamily (HMGN), are also actively removed from chromatin and later incorporated into it [72,73]. A schematic representation of the "nuclear reprogramming" and "chromatin remodeling" molecules that meet a somatic nucleus upon its entry into the enucleated oocyte are shown (Fig. 1).

4. DNA methylation has a say

In mammalian cells, stable silencing of genes is frequently correlated with DNA methylation of promoter regions, along with specific modifications in the N-terminal tails of histones. Methylation of DNA is restricted to cytosine (C) residues in CG dinucleotides. DNA methylation is the most studied epigenetic mechanism used by the cell for the establishment and main-

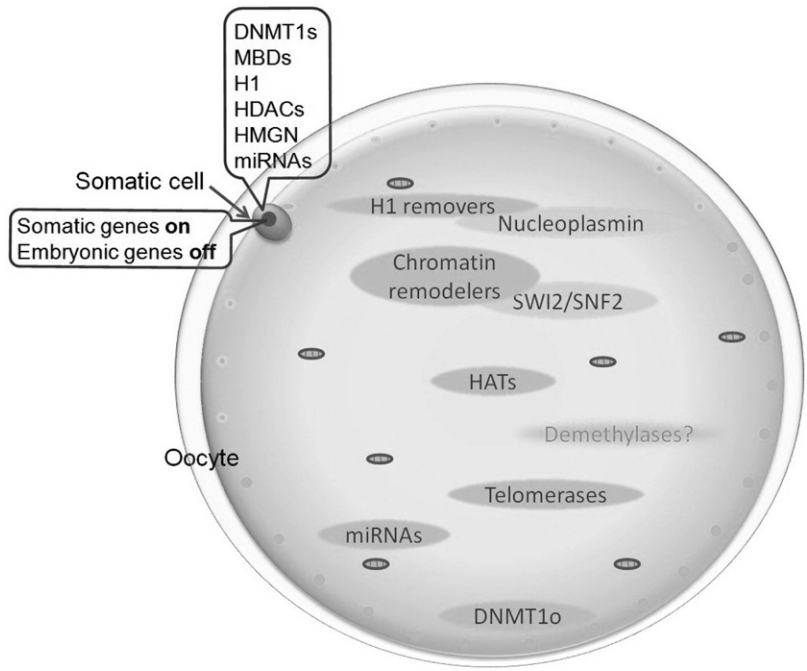

Fig. 1. Schematic representation of oocyte factors that participate in chromatin remodeling and reprogramming of the somatic nucleus after somatic cell nuclear transfer (SCNT). The chromatin structure of a somatic cell ensures expression of somatic and silencing of embryonic genes. The somatic cell contains the somatic isoform of the maintenance DNA methyltransferase DNMT1s, whereas the oocyte specific isoform DNMT1o is present in the egg cytoplasm and is translocated to the nucleus during the 8-cell stage. Somatic linker histone H1, present in the somatic nucleus, is removed by nucleoplasmin and replaced by the oocyte-specific variant H1FOO. The methyl CpG-binding domain (MBD) family of proteins and histone deacetylases HDACs contribute to silencing of embryonic genes in the somatic cell. Members of the high mobility group nucleosomal proteins HMGN are removed from somatic chromatin by chromatin remodeling factors, such as the ATP dependent family SWI2/SNF2. Histones of embryonic genes are acetylated by HATs. Demethylation of the somatic genome may be accomplished passively or actively. The cytidine deaminase AID and elongator-complex proteins contribute to the extensive removal of DNA methylation in mammalian cells. Telomere length is restored by telomerases in the oocyte. Oocyte microRNAs (miRNAs) play important roles in early embryonic development. The role of somatic miRNAs in early embryonic development remains to be established.

tenance of a controlled pattern of gene expression [74]. DNA methylation provides a genome-wide means of regulation, usually associated with the inheritance of lineage-specific gene silencing between cell generations [75]. Patterns of DNA methylation are distinct for each cell type and confer cell type identity [76]. With few exceptions, unmethylated DNA is associated with an active chromatin configuration, whereas methylated DNA is associated with inactive chromatin [77].

Methylation of DNA is accomplished by four DNA methyltransferases and an additional protein that collaborates with the enzymes. The first DNA methyltransferase to be discovered, DNMT1, maintains the methylation pattern following DNA replication, using the parental DNA strand as a template to methylate the daughter DNA strand. Therefore, an unmethylated CG sequence paired with a methylated CG sequence is methylated by DNMT1 [78,79]. The smallest mammalian DNA methyltransferase, DNMT2, contains only the five conserved motifs of the C-terminal domain. Its function in DNA methylation has been enigmatic [80,81]. Whereas some studies report that DNMT2 has a role in DNA methylation [82–84], others have detected little DNA methylation activity for this enzyme [85,86]. Recent research has demonstrated that DNMT2 methylates $tRNA^{Asp}$ in the cytoplasm [87,88]. The remaining enzymes, DNMT3a and DNMT3b have been identified as *de novo* methyltransferases, as they establish new DNA methylation patterns by adding methyl groups onto unmethylated DNA, particularly during early embryonic development and gametogenesis [89,90].

Analysis of methylation reprogramming in uniparental (parthenogenetic, gynogenetic, and androgenetic) embryos indicates that the reprogramming machinery in the egg cytoplasm treats the paternal and the maternal genomes in markedly different ways [91]. Prior to fertilization, the genomes of both sperm and oocytes are transcriptionally inactive and highly meth-

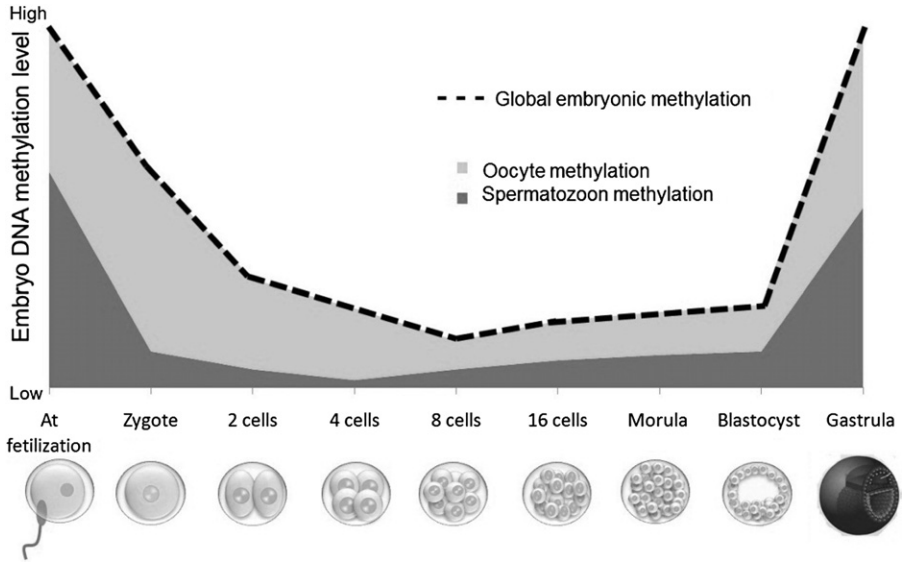

Fig. 2. Schematic representation of the changes in DNA methylation in the bovine embryo throughout preimplantation development. The DNA methylation is shown as arbitrary units in the Y axis. The DNA methylation level of the preimplantation embryo is the sum of the spermatozoon (blue) and oocyte (pink) methylation. Before the first mitotic division, the sperm genome undergoes active demethylation, whereas the oocyte genome undergoes passive demethylation throughout several cell divisions. Paternal and maternal genomes remain separated until after the 4-cell stage. After the 8-cell stage, a small wave of *de novo* methylation is observed. By the blastocyst stage, the DNA methylation level in the trophectoderm cells is markedly lower compared to cells of the inner cell mass ICM. At the peri-gastrulation stage *de novo* DNA methylation is completed throughout the entire embryo [210].

ylated [42,92]. Within hours after fertilization, a dramatic genome-wide loss of DNA methylation occurs in the male pronucleus [93,94].

Several mechanisms have been suggested for active demethylation of the paternal genome. Firstly, the removal of the methyl group from the cytosine; secondly, removal of the methyl-cytosine base by glycosylation; and thirdly, removal of a number of nucleotides (excision repair) [49]. The nature of the mechanisms involved in the active demethylation of the paternal genome remains unknown. However, recent studies reported a component of the elongator complex, elongator Protein 3 (ELP3), to be required for the removal of DNA methylation in the paternal pronucleus of the zygote [95]. The elongator complex was first described as a component of RNA polymerase II holoenzyme in transcriptional elongation, and has histone acetyltransferase activity [96]. Conversely, cytidine deaminase AID deficiency interferes with genome-wide erasure of DNA methylation patterns, suggesting that AID has a critical function in epigenetic reprogramming and potentially in restricting the inheritance of epimutations in mammals [97].

After several cleavage divisions, the female pronucleus is also demethylated. This process seems to be passively caused by a loss of methyl groups during each round of DNA replication [93,94]. The only methylation marks preserved in the embryonic genome are the ones in imprinted genes [42,94,98]. A schematic representation of the demethylation of paternal and maternal genomes after fertilization is shown (Fig. 2).

By the blastocyst stage, the embryonic genome is hypomethylated [99]. New methylation patterns are established, around the blastocyst stage, by DNMT3A and DNMT3B. The protein DNMT3L interacts with DNMT3A forming a dimer. The *de novo* methylation activity of DNMT3A, depends upon its dimerization with DNMT3L [100]. The exact biological function of this dynamic reprogramming of DNA methylation in early development is unknown. Several studies support the hypothesis that DNA methylation is crucial for establishment of gene expression during embryonic development [101,102]. However, recent data suggest that DNA methylation may only affect genes that are already silenced by other mechanisms, indicating that DNA methylation could be a consequence rather than a cause of gene silencing during development [103–105]. The establishment and maintenance of appropriate methylation patterns are crucial for normal development. Mutations in either the maintenance or the *de novo* methyltransferases result in early embryonic death in mice [98,106].

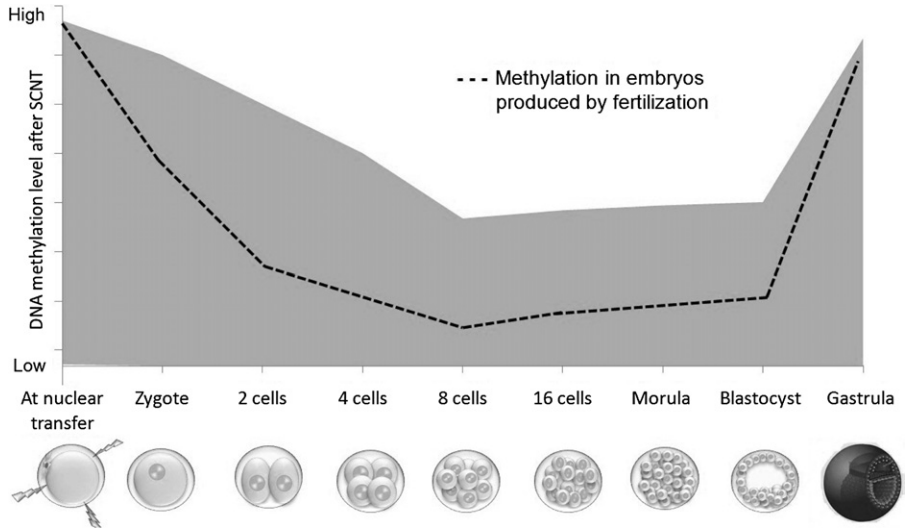

Fig. 3. Schematic representation of the changes in DNA methylation in a somatic nucleus after nuclear transfer. DNA methylation is shown as arbitrary units in the Y axis. The extent of DNA demethylation of a somatic nucleus after SCNT is incomplete, compared to that of embryos produced by fertilization (dashed line). Although by the 8- to 16-cell stage the DNA methylation level of the cloned embryo has decreased considerably, the pattern of methylation is heterogeneous in the blastomeres. The trophectoderm and ICM cells of cloned blastocysts have similar methylation levels, unlike the differential methylation observed in embryos produced by fertilization [210].

It was believed that the established methylation pattern was reliably and irreversibly maintained for the life of the organism [77]. However, recent data suggest that DNA methylation is reversible and can change in response to intrinsic and environmental signals [107]. The study of DNA methylation after SCNT has shown that somatic cell chromatin undergoes only limited demethylation after SCNT [108]. Some embryos derived from nuclear transfer have an abnormal pattern of DNA methylation, which in some cases resembles that of donor cells and is retained through several cell divisions in cloned embryos [109]. The somatic-like methylation pattern maintained in cloned embryos up to the four-cell stage indicates that active demethylation is absent in nuclear transfer [21]. Other studies suggest that cloned embryos undergo active demethylation, but lack passive demethylation [22]. It has also been reported that *de novo* DNA methylation starts precociously at the 4- to 8-cell stage in cloned embryos. By the 8- to 16-cell stage, cloned embryos had a heterogeneous methylation pattern, with some nuclei appearing hypomethylated and others hypermethylated. By the blastocyst stage, most nuclear transfer derived embryos seem to have a global DNA methylation level comparable to that of embryos produced by fertilization. However, abnormally high methylation patterns are detected in some regions of the genome [46,110,111]. A schematic representation of the level of DNA demethylation after nuclear transfer comparing it to the one occurring in embryos produced by fertilization is shown (Fig. 3).

It is not clear to what extent the DNA methylation pattern observed during normal development needs to be mimicked for cloning to succeed. Individual blastocysts display significant alterations in the methylation pattern. However, such aberrant reprogramming of DNA methylation does not seem to be lethal, since several of the cloned embryos with hypermethylated DNA developed beyond the blastocyst stage [21,22,111]. Variation in imprinted gene expression has been observed in cloned mice. Interestingly, many of these animals survive to adulthood despite widespread gene misregulation, indicating that mammalian development may be rather tolerant to epigenetic aberrations of this kind [112]. These data imply that even apparently normal cloned animals may have subtle abnormalities in their DNA methylation pattern. Other studies, however, have inversely correlated aberrant DNA methylation with developmental potential of cloned embryos [49,113].

In female mouse embryos at approximately the morula stage, nearly all genes in one of the two X chromosomes are inactivated by a dosage compensation mechanism known as X-chromosome inactivation (XCI) [114]. In fetal tissues this inactivation is random; in some cells the inactivated X chromosome is paternal, whereas in others it is maternal. However, in the trophectodermal cells, the paternal X-chromosome seems to be the only inactivated one [115,116].

The timing of XCI and the regulatory network(s) involved in the establishment of the inactive X chromosome state in other species has not been well characterized. Female embryos, obtained by nuclear transfer, receive a somatic nucleus, which already has one inactivated X chromosome. The recipient enucleated oocyte has to transiently activate the inactive X chromosome so that the embryo can later accomplish the random X chromosome inactivation that occurs in normal embryos. The inactivation of the X chromosome has been monitored in cloned mouse embryos to study reprogramming of a somatic female nucleus. Normal XCI patterns have been reported in cloned female tissues. Cloned female mice obtained from somatic cells with a transcriptionally "inactive" paternal X-chromosome had a random X-chromosome inactivation with an active paternal X-chromosome in some cells and an inactive one in other cells [117]. However, the trophectoderm cells maintained the inactivation of the X chromosome that was silent in the somatic cell, even when it was the maternal one [118]. Similar results have been reported for bovine cloned calves. Additionally, aberrant XCI patterns were detected in fetal and placental tissues from deceased cloned bovine and mouse fetuses [119,120]. Other studies reported significant failures in XCI in cloned mice and pigs [121,122]. Thus, to date, it is not clear how abnormal XCI affects cloning efficiency.

A novel cytosine modification, 5-hydroxymethylcytosine (5-hmC), has recently been reported in murine embryonic stem cells, gametes, and preimplantation embryos [123,124]. Methyl cytosine is converted to 5-hmC by the action of the Tet (Ten-eleven translocation) oncogene family member proteins [125]. Methyl-CpG binding proteins do not interact with 5-hmC-containing DNA [126] and since the biological functions of genomic 5-hmC have not been determined, hmC could play a different role in development than that of 5-mC. Interestingly, it seems that bisulfite sequencing cannot discriminate between 5 mC and 5hmC [127,128], rendering these two distinct cytosine modifications to seem indistinguishable. It could be necessary to take into consideration the lack of specificity of the current DNA methylation identification methods when interpreting DNA methylation data, since 5-hmC could have a different functional role.

5. The right set of genes

Differentiated cells have cell-specific gene expression. Genes transcriptionally active in one type of cell may be silenced in another cell type. There are genes, not all of them identified yet, whose activation means the difference between development and failure in a cloned embryo. After SCNT, global transcriptional silencing was detected in mouse, cattle and rabbit clones [129–131], followed by reappearance of the first signs of transcriptional activity at the two-cell stage, resembling embryonic genome activation after fertilization [5,129]. The expression of these genes might ensure blastocyst formation, implantation, and development to term, and their expression is the result of chromatin remodeling and DNA methylation modifications. These modifications not only ensure the activation of embryonic genes associated with a state of totipotency, but also the downregulation of somatic genes that are not necessary and could even be detrimental for the embryo.

The global transcriptome profile of cloned embryos, relative to that of donor cells and embryos produced by fertilization, has been studied using microarray technology. Global alteration of gene expression has been reported in cloned embryos, which may represent persistent expression of donor cell-specific genes [132]. Abnormal expression of genes with important roles in early embryonic development, implantation and fetal development is of particular interest. The expression of imprinted genes was abnormal in cloned blastocyst at three levels: total transcript abundance, allele specificity of expression, and allelic DNA methylation. Mann, and coworkers reported methylation and gene expression abnormalities for nearly all embryos, despite their morphologic quality, with considerable heterogeneity among individual embryos [133]. These observations indicate that epigenetic marks associated with imprinted genes are not faithfully retained in the majority of cloned embryos. The low proportion of embryos exhibiting a comparatively normal pattern of imprinted gene expression at the blastocyst stage is consistent with the proportion of live-born clones.

Conversely, other studies have reported a significant reprogramming of SCNT embryos by the blastocyst stage and transcriptome profiles comparable to those of embryos produced *in vitro* or *in vivo*, suggesting that defects in gene expression for SCNT embryos may occur later during redifferentiation and/or organogenesis [134–136]. Identifying key genes responsible for the general developmental failure in cloned embryos is not an easy task, since the alterations may be caused by a variety of factors, including donor cell type, cell cycle stage, nuclear transfer protocol, source of the oocytes,

embryo culture system, embryo transfer procedure, recipient management, and operators' skills [20].

Several studies have described misregulation of specific genes. For example, *POU5F1*, the gene encoding the transcriptional regulator Oct4, which is induced in somatic nuclei after nuclear transfer, has been one of the more studied markers of pluripotency [54,137,138]. Demethylation of the Oct4 promoter is a prerequisite for its activation [139]. Some studies have reported *POU5F1* misregulation in SCNT embryos [140,141], whereas others report it at the expected concentration [142,143]. The amount of POU5F1 transcripts were comparable in bovine cloned embryos and embryos produced by *in vitro* fertilization [144] No significant difference in *POU5F1* mRNA levels among cloned blastocysts and blastocysts produced by *in vitro* fertilization and artificial insemination were detected by microarray analysis and real-time PCR [145].

The imprinted gene Insulin-like Growth Factor 2 Receptor (*IGF2R*) [146] has been extensively studied, due to its implication in the large offspring syndrome (LOS) [147]. This gene has had altered expression values in embryos produced *in vitro* and a marked misregulation in cloned embryos [148,149].

Genes reported to be abnormally expressed in bovine cloned embryos include *IL-6, FGF4, FGFr2, FGF4, DNMT1, Mash2, HSP70*, interferon *tau, histone deacetylases*, and *DNMT3A* [141,142,150]. Oligonucleotide microarray analysis and Real Time PCR, showed that developmentally crucial genes, such as Desmocollin 3 (DSC3), a transmembrane glycoprotein involved in cell adhesion, and the high mobility group nucleosomal binding Domain 3 (*HMGN3*) were significantly down regulated in cloned bovine embryos compared to *in vitro* produced embryos [151]. The same study reported a significant down regulation in the Signal Transducer and Activator of Transcription 3 (*STAT3*) in cloned bovine blastocysts, contrary to a report of upregulation of this gene in cloned blastocysts [145]. Further study of these genes and functions of their products could provide insights into the poor developmental rates of cloned embryos.

A recent study reported abnormal gene expression of DNMTs, interferon tau (*INFT*) and major histocompatibility 1 complex Class 1 (MHC1 1) transcripts in the majority of cloned bovine embryos. This study reports down regulation of *DNMT3B* in the majority of cloned embryos on Day 7 [152]. Conversely, another study reported a significant upregulation in *DNMT3A* and *DNMT3B* transcripts in cloned bovine embryos compared to their *in vitro* produced counterparts [153]. The roles of DNMT3A and DNMT3B in *de novo* methylation could link these enzymes with high methylation levels in cloned embryos, as previously discussed.

The lack of consistency in the pattern of gene misregulation in cloned embryos in various studies has lead several authors to suggest that nuclear reprogramming after somatic cell nuclear transfer is stochastic in nature. According to this hypothesis, the number and the role of misregulated genes determine the fate of each cloned embryo. A complementary explanation to the possible stochastic nature of gene deregulation is the possibility that reprogramming is not a sudden event that happens in the nucleus of the donor somatic cell as soon as it is fused with the oocytes, but it is instead a rather dynamic process that occurs progressively.

6. The best is yet to come

Improving the efficiency of SCNT is directly related to knowledge regarding molecular reprogramming which is important for embryo formation and development after nuclear transfer. Factors contributing to nuclear reprogramming are being sought in hopes of regulating chromatin remodeling, histone modifications, and transcriptional activity [154–156], providing a better understanding of mammalian embryogenesis and improving the outcome of SCNT [157].

Assisted relaxation of chromatin structure (which corresponds to a transcriptionally permissive state) by histone-deacetylase inhibitors (HDACi) might increase H3K9ac levels and improve the reprogramming capacity of somatic cells, thereby increasing their cloning efficiency. Trichostatin A (TSA), a natural product isolated from *Strpetomyces hygroscopicus*, is a frequently used HDACi, which enhances the pool of acetylated histones and induces overexpression of imprinted genes in embryonic stem cells [158,159]. Trichostatin A seems to improve the genomic reprogramming of SCNT-generated embryos in mice [155], pigs [160], and cattle [161]. Scriptaid (SCR), a relatively new synthetic compound, which shares a common structure with TSA, seems to have low toxicity and has also been used to improve cloning efficiency in porcine [160] and bovine embryos [161]. Other HDAC inhibitors that have been used to improve developmental competence of SCNT embryos in various species include valproic acid [162], sodium butyrate [163], suberoylanilide hydroxamic acid (SAHA) [164], oxamflatin [66,165], and m-carboxycinnamic acid bishydroxamide (CBHA) [166].

The DNA demethylation agent, 5-aza-29-deoxycytidine (5-aza-dC) a derivative of the nucleoside cytidine, induced overexpression of imprinted genes in mouse embryonic fibroblast cells by lowering DNA methylation levels [3]. It has also increased preimplantation development of cloned bovine embryos [167]. However, treatment of donor cells with 5-azacytidine prior to nuclear transfer removed epigenetic marks and improved the ability of somatic cells to be fully reprogrammed by the recipient karyoplast [37]. Unfortunately, 5-aza-dC has also reduced blastocyst formation of cloned embryos [168]. A combination of TSA and 5-aza-dC enhanced the developmental potential of treated cloned embryos both *in vitro* and full-term. It is likely that TSA and 5-aza-dC may act synergistically to modify gene expression and DNA methylation in preimplantation embryos [169,170].

Reducing methylation by knocking-down DNMT1 gene expression using siRNA technology has been applied to a bovine donor cell line with approximately a 30 to 60% decrease in global DNA methylation. Demethylated cells were used subsequently for SCNT, which doubled blastocyst rates, suggesting that demethylation prior to NT may be beneficial for NT-induced reprogramming [3,171].

Decondensation of sperm chromatin in eggs is achieved by replacement of sperm-specific histone variants with egg-type histones by the egg protein nucleoplasmin. Nucleoplasmin can also decondense chromatin in undifferentiated mouse cells without overt histone exchanges, but with specific epigenetic modifications that are relevant to open chromatin structure. These modifications included nucleus-wide multiple histone H3 phosphorylation, acetylation of Lysine 14 in histone H3, and release of heterochromatin proteins HP1beta and TIF1beta from the nuclei. At the functional level, nucleoplasmin pretreatment of mouse nuclei facilitated activation of four oocyte-specific genes [172]. Nucleoplasmin injected into bovine oocytes after nuclear transfer resulted in apparent differences in the rates of blastocyst development and pregnancy initiation. Over 200 genes were upregulated following post-nuclear transfer and nucleoplasmin injection, several of which were previously shown to be down regulated in cloned embryos when compared to bovine IVF embryos [173]. These data suggest that addition of chromatin remodeling factors, such as nucleoplasmin, to the oocyte may improve development of NT embryos by facilitating reprogramming of the somatic nucleus.

Nuclear and cytoplasmic extracts that can transform one cell type into another have been used as reprogramming factors. The procedure involves the permeabilization of one somatic cell type into another somatic "target" cell type using cytoplasmic extracts [174]. The reprogramming ability of these extracts has been evidenced by nuclear uptake and assembly of transcription factors, activation of chromatin remodeling complex, changes in chromatin composition, and expression of new genes [175]. These systems likely constitute a powerful tool to examine the process of nuclear reprogramming.

Pluripotent embryonic stem (ES) cells derived from the inner cell masses of blastocysts have an intrinsic capacity for reprogramming nuclei of somatic cells. *In vitro* hybridization of somatic cells with ES cells leads to reprogramming of somatic cells. The pluripotency of ES-somatic hybrids has been proven as the somatic cells contribute to all three primary germ layers of chimeric embryos [176,177]. The somatic pattern of DNA methylation is maintained in hybrids, indicating that ES cells only have the capacity to reset certain aspects of the somatic cell epigenome [178,179]. The use of ES cells will contribute to elucidating the mechanisms of epigenetic reprogramming involved in DNA and chromatin modifications [180]. Individual oocyte and ES cell reprogramming factors are being used in cell-free reprogramming extracts with varying success.

The recently reported use of four transcription factors (Oct4, Sox2, Klf4, Nanog and c-Myc) [181,182] to produce induced pluripotent stem (iPS) cells raises the question of whether nuclear transfer is still necessary for producing stem cells for therapeutic purposes [183]. Many cell types, including fibroblasts [181,184,185], blood cells [186], stomach and liver cells [187], keratinocytes [188,189], melanocytes [190], pancreatic β cells [191], and neuronal progenitors [192,193] have been reprogrammed into iPS cells. Like stem cells, iPS cell lines have been shown to express pluripotency genes and support differentiation into cell types of all three germ layers [185]. This differentiation potential provides fascinating possibilities for the study of genetic and developmental diseases, in addition to their potential use for drug discovery and regenerative medicine [194,195]. Pluripotent stem cells, can also be produced by fusion of somatic cells with preexisting ES cells [196,197], and can be isolated from embryos generated by nuclear transfer [198].

Opponents of stem-cell research have welcomed iPS cell technology as a method for achieving an embryonic-like state without the ethical dilemma of destroying human embryos [194]. Induced pluripotent stem cell technology could be especially attractive for re-

searchers in countries in which the use of embryonic cells is restricted, since it allows for conversion of somatic cells into pluripotent cells, without the need of embryonic cells. Uses of human iPS cells include but are not limited to: 1) disease models: the ability to create pluripotent stem cell lines from patients exhibiting specific diseases may facilitate the construction of iPS cell libraries that could be used to investigate human pathologies *in vitro* [195]; 2) generation of iPS cells from individuals with polymorphic variants of metabolic genes could contribute to the development of toxicologic assays [199]; 3) a combination of tissue engineering with iPS cells represents great potential for treatment of multiple diseases, e.g., liver diseases [194,195]; 4) iPS cells are a promising source for development of truly isogenetic grafts, as human iPS cell-derived neural and cardiomyocytes have demonstrated *in vivo* integration and function [200,201]; and finally, 5) iPS cells could represent a basic research tool for the study of DNA methylation and cellular reprogramming, to enhance the understanding of stem cell biology and facilitate therapeutic applications [195,202].

Nevertheless, it is important to consider that iPS cells seem to retain an epigenetic memory of their cell of origin that restricts their differentiation potential and is manifested in the DNA methylation patterns and in global gene expression [184,185]. In contrast, the methylation patterns and the differentiation state of nuclear-transfer-derived pluripotent stem cells, resembles more closely that of classical embryonic stem cells. These data highlight the epigenetic heterogeneity of pluripotent stem cells and the need for improved methods to ensure reprogramming of somatic cells to a "ground state" of pluripotency [203].

A recent hypothesis suggests that failure in the oocyte reprogramming mechanism to target the paternal genome of the somatic nucleus creates an unbalanced nuclear reprogramming between parental chromosomes. These authors suggest that the exogenous expression in donor somatic cells of sperm chromatin remodeling proteins, particularly the BRomo Domain Testis-specific protein (BRDT), could induce a male-like chromatin organization of the somatic genome [204]. The real advantages of such a method remain to be observed, since both the paternal and the maternal genomes, present in the somatic nucleus, need to undergo reprogramming after nuclear transfer.

In addition to the multiple proteins that participate in chromatin remodeling and DNA methylation, oocytes contain microRNAs (miRNAS) that regulate the expression of genes by inhibiting translation [205]. Several specific miRNas have been isolated from *Xenopus* [206], *Drosophila* [207], and mouse oocytes [208]. The function of miRNAs during early development is not known, but their importance in early embryo development is supported by the fact that mouse oocytes lacking miRNAs fail to cleave [208]. Although the exact role of miRNAs in nuclear reprogramming has not been explored, it has been proposed that some developmental failures of cloned embryos might be a consequence of miRNA alteration during nuclear transfer. Enucleation did not seem to remove substantial amounts of oocyte miRNAs, whereas nuclear transfer significantly increased the oocyte miRNA profile. Following their introduction to the oocyte by nuclear transfer, some miRNAs may be capable of regulating the same mRNAs they do regulate in somatic cells, or regulate other transcripts with distinct roles in embryogenesis [209].

7. Conclusions

Although a number of questions regarding the low efficiency of SCNT still remain unanswered, the central role of nuclear reprogramming on the outcome of cloning is evident. Increasing the efficiency of SCNT would have a great impact on biomedical sciences and agriculture, particularly for generation of isogenic embryonic stem cells and production of animals with desired qualities. Understanding the reprogramming process of SCNT derived embryos would be instrumental in increasing the success rate of cloning. Several strategies have been used to determine the extent of nuclear reprogramming in cloned embryos. Genomic and proteomic approaches that give a general overview of the transcriptional activity and the protein synthesis in cloned embryos have been used to determine t genes that are misregulated in embryos derived from nuclear transfer when compared to embryos produced by *in vivo* or *in vitro* fertilization.

Somatic cell nuclear transfer extensively alters the gene expression of differentiated somatic cells to more closely resemble that of embryonic nuclei. However, a combination of *in vitro* culture conditions, aggressive manipulation and insufficient reprogramming, compromises the developmental potential of SCNT embryos. Cloned embryos present varying degrees of aberrations in chromatin structure and DNA methylation, which cause inadequate expression of developmental genes or the expression of unnecessary somatic genes. Although slight alterations in DNA methylation do not seem to be

life-threatening for the cloned embryos, extensive aberrations may be fatal. The epigenetic alterations can result in different phenotypic manifestations in each embryo. The variable outcomes of SCNT from the same somatic cell line indicate that although cloned offspring have identical genomes, their phenotypes may vary greatly.

The traditional view has maintained that DNA methylation is the primary epigenetic mark responsible for repressive chromatin structure. According to this theory, DNA methylation attracts methylated cytosine binding proteins, which in turn recruit repressor complexes and histone deacetylases to further silence chromatin. An alternative model suggests that it is chromatin structure which determines the DNA methylation or demethylation [76]. Knowing the precise sequence of events leading to gene silencing will direct future research to determine the optimum approach for improving reprogramming after SCNT.

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