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Review Article

Chromatin Modifying Agents in the *In Vitro* Production of Bovine Embryos

Fabio Morato Monteiro,[1,2] Clara Slade Oliveira,[1] Letícia Zoccolaro Oliveira,[1] Naiara Zoccal Saraiva,[1] Maria Eugênia Zerlotti Mercadante,[2] Flavia Lombardi Lopes,[1] Daniel Robert Arnold,[1] and Joaquim Mansano Garcia[1]

[1] *Departamento de Reprodução Animal, FCAV-UNESP, 14884-000-Jaboticabal, SP, Brazil*
[2] *Instituto de Zootecnia, Estação Experimental de Zootecnia de Sertãozinho, Caixa Postal 63, 14.160-970-Sertãozinho, SP, Brazil*

Correspondence should be addressed to Fabio Morato Monteiro, monteiro@iz.sp.gov.br

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The low efficiency observed in cloning by nuclear transfer is related to an aberrant gene expression following errors in epigenetic reprogramming. Recent studies have focused on further understanding of the modifications that take place in the chromatin of embryos during the preimplantation period, through the use of chromatin modifying agents. The goal of these studies is to identify the factors involved in nuclear reprogramming and to adjust *in vitro* manipulations in order to better mimic *in vivo* conditions. Therefore, proper knowledge of epigenetic reprogramming is necessary to prevent possible epigenetic errors and to improve efficiency and the use of *in vitro* fertilization and cloning technologies in cattle and other species.

1. Introduction

Despite being utilized for nearly three decades [1], the production of embryos *in vitro* still has limitations, such as, lower efficiency when compared to *in vivo* production. In bovine oocytes, matured and fertilized *in vitro*, high cleavage rates are currently obtained. However, only 25%–40% of these zygotes are capable of progressing to the blastocyst stage [2]. Recent studies suggest that epigenetic alterations and consequent changes to chromatin conformation may take place during *in vitro* culture [3]. Enright et al. [4] reported elevated levels of histone H3 and H4 acetylation following long-term culture of embryos. Furthermore, *in vitro* produced bovine embryos showed altered gene expression patterns when compared to *in vivo* controls [5].

In somatic cell nuclear transfer (SCNT), perturbations in epigenetic patterning are also thought to play a role in the low efficacy seen following the use of this technology. In SCNT, the nucleus of a differentiated adult somatic cell is reprogrammed by factors in the oocyte cytoplasm in order to regain the pluripotent patterns of an embryonic cell [6–9]. However, failure in this nuclear reprogramming [10, 11], resulting in abnormal epigenetic patterns in cloned embryos, has been reported [12]. Furthermore, development to the blastocyst stage and survival to birth are significantly lower in clones in comparison to embryos produced by *in vitro* fertilization [11–14]. In cloned mice, animals do not appear to pass an abnormal trait on to their offspring produced *in vivo*. This may suggest that a perturbation to proper epigenetic patterning, rather than to genetic sequences, is responsible of the aberrant phenotypes and also of the low efficiency seen following the use of this technology [15].

In view of these concerns following the use of *in vitro* technologies for the production of embryos, recent studies aim at understanding the chromatin structure modifications during preimplantation and the improvement of *in vitro* approaches to most closely mimic the *in vivo* changes that allow the growth and survival of embryos. In the present paper, we will discuss some of the epigenetic mechanisms involved in embryo development and the use of treatments to manipulate epigenetic patterning *in vitro*.

2. Mechanisms of Epigenetic Regulation

Epigenetic refers to the control of gene function and expression without changes to the gene sequence. Such control allows for different expression patterns, from an identical genome sequence, to take place in separate cells or tissues, establishing the basis for tissue-specific gene expression [16].

Epigenetic modifications control gene expression by a variety of processes which include DNA methylation, post-translational histone modifications, noncoding RNAs [17]. The processes of DNA methylation and histone modification have gained great interest in the fields of mammalian development [16]. We will further discuss these studies and how they may offer insight into the production of embryos *in vitro*.

2.1. DNA Methylation. DNA methylation is one of the most well-described epigenetic mechanisms and plays a key role in several biological processes such as, transcriptional regulation, chromosomal organization, X-inactivation, and genomic imprinting [18–22]. A family of DNA (cytosine-5)-methyltransferases (DNMT) is responsible for the addition of a methyl group to the 5th position of the cytosine ring, and five DNMTs have been already characterized in mammals: DNMT1, 2, 3A, 3B, and 3L [23]. DNMT1 is responsible for the maintenance of methylation through the remethylation of new strands during replication if the mother strand was also methylated [22]. The DNMT3 enzymes (DNMT3a, 3b, and 3L) are involved in the acquisition of *de novo* methylation on DNA previously devoid of methylation [24]. Loss of methylation patterns through genetic ablation of these enzymes is lethal at early embryonic or postnatal stages in mice [24, 25].

Amongst all the biological processes involving DNA methylation, genomic imprinting is one of the most intriguing, as it involves the formation of an epigenetic "mark" at certain loci in a parent-of-origin-specific manner such that genes are expressed monoallelically [26]. Biallelic expression or silencing of imprinted genes is detrimental to fetal growth, phenotype, and survival, considering that the accurate maintenance of inherited methylation patterns on imprinted genes in preimplantation is critically important for the success of both *in vivo* and *in vitro* embryonic developments.

Patterns of DNA methylation are erased in the primordial germ cells during embryonic life and re-established in a sex-specific manner in the gametes during germ cell development. However, for the purpose of the review, we will discuss another important wave of demethylation that occurs postfertilization. Genome-wide methylation is erased, except for methylation of imprint genes and certain repeat sequences, with reacquisition of methylation in mice taking place during the peri-implantation stage in the expanded blastocyst (review in [26]).

2.2. Histone Modifications. Another important epigenetic process is histone modification. Histones are proteins bound to DNA forming the highly conserved structural polymer known as chromatin. Nucleosomes are the fundamental repeating units of chromatin. A nucleosome consists of 146-base pairs of DNA wrapped around a core of histone proteins. The histone proteins that make up this core are 2 copies each of H2A, H2B, H3, and H4. A linker histone, H1, is bound to the DNA between nucleosomes, allowing for the solenoid helical fiber structure of DNA in the nucleus. These core histones are highly conserved across species. Histone proteins have a globular carboxy-terminal domain that binds the DNA and a flexible amino-terminal tail that extends out of the nucleosome structure. Modifications of histones occur on amino residues, primarily on the amino-terminal tail (reviewed in [27]). These covalent modifications have fundamental functions on chromatin condensation, DNA replication, DNA repair, and gene regulation. Depending on the type of modification that occurs, the nucleosome will either open up to allow for transcriptional factors to bind or remain tightly wound.

Histone acetylation is commonly associated with activated transcription, whereas deacetylation is associated with transcriptional repression [27–29]. Research has shown that acetylation of histone H4 is reduced on the inactive X chromosome in female mammals, suggesting that absence of acetyl groups is be a prerequisite for a more condensed and inactive chromatin stage [30]. Histone amino groups can also be methylated, phosphorylated, and ubiquitinated [27, 31].

Examples of some modifications that are commonly associated with euchromatin, regulating gene expression are acetylation of histone H3 and H4, as well as di- and trimethylation of lysine 4 on histone H3 [31]. The region of DNA on the chromosome that is constitutively silenced (telomere, centromeres, and heterochromatin) is hypoacetylated [31–33]. In addition, these regions are highly methylated on particular amino acid residues (lysine 9 and lysine 27 of histone H3) [31, 34]. Among the histone modifications, acetylation and methylation have been the most studied. Unlike histone acetylation, histone methylation is more complex, with gene expression being affected differently depending on which residue is modified [35]. Loci-specific modifications of histones, and the combination of these modifications, have been described as a "histone code" that defines the state of a cell's transcriptional potential [36].

Several enzymes have been reported to control histone modification. Two proteins involved with controlling the acetyl groups are histone acetyltransferase (HAT; adds acetyl groups) and histone deacetylase (HDAC; removes acetyls groups) [37]. Several of these enzymes (HDACs 1, 2, 3, 7, and HAT1) have been detected in bovine embryos [38]. As more research is conducted, it becomes apparent that crosstalk between enzymes is a common feature. For example in *Schizsaccharomyces pombe,* HDAC is required for deacetylating the histone 3 lysine 9 residue in order for histone methyltransferase to act on that particular residue [34]. The HAT enzymes not only affect chromatin, but also act as coactivators on certain transcription factors [39]. Interactions also occur between HDACs and DNMTs [40–42].

3. Epigenetic Modifications in Mammalian Embryos

Embryonic development involves a wide array of epigenetic modifications, including DNA methylation and histone modifications, which are fundamental for genomic imprinting and X-chromosome inactivation in female embryos [43]. In mammals, levels of histone methylation are higher and histone acetylation levels are lower in male gametes compared to female gametes [12, 44–47], resulting in minimal gene expression at this stage.

During normal bovine embryo development, histone demethylation occurs soon after fertilization [12]. Histone methylation is reduced in 2 to 4 cell embryos, and starts to increase at the 8- to 16- cell stage, concurrently with zygotic genome activation [48]. Histone acetylation levels peak at the time of zygotic genome activation, corresponding to the time of increased gene expression, then diminish during the morula stage [49]. The first cellular differentiation of the developing embryo occurs at the blastocyst stage giving rise to the inner cell mass (ICM) and trophectoderm [48]. At the blastocyst stage, DNA and histone methylation is elevated in the ICM, whereas DNA and histones are hypomethylated in the trophectoderm. Thus, blastocyst formation establishes the beginning of epigenetic differences between the two cell lineages [50].

Another important event in normal mammalian embryo development is X-chromosome inactivation (XCI). Gene expression from one X chromosome is sufficient to allow for normal embryonic development, as seen in male embryos [51]. Therefore, in female embryos, the extra X chromosome must be silenced [12]. Establishment and maintenance of XCI are regulated by epigenetic mechanisms. Research has shown XCI imprinting in the bovine [52] and murine [53, 54] placentas. In mice, imprinting of XCI occurs during preimplantation development, with the paternal chromosome preferentially silenced [52–54]. The paternal X chromosome is silenced at the 4-cell stage, shortly after zygotic genome activation [55]. This pattern persists in the trophectoderm linage, where the active X chromosome in the placenta is of maternal origin. However, in the ICM of developing embryo, the paternal X chromosome is reactivated and forthwith both maternal and paternal X chromosomes are randomly selected for inactivation [55, 56].

Regards to DNA methylation, during the early postfertilization stages there is an intense wave of DNA demethylation in both pronuclei, albeit at different speeds, followed by a global remethylation that takes place at peri-implantation stages in the mouse. Further, not all sequences have their methylation patterns removed, as this is the case for imprinted sequences, which maintain their methylation marks throughout life except in the germ cell line [43].

3.1. Epigenetic Modifications in Cloned Embryos. In cloning by somatic cell nuclear transfer, the epigenetic patterns of the adult somatic cell must be erased and reprogrammed to those of a totipotent embryonic cell. During the development of embryos *in vivo*, patterns carried by both gametes are each reprogrammed in a very specific and timely manner and embryonic patterns of the totipotent cell must be established to support the embryonic genome activation that ensues [57–59]. Failure is the re-establishment of correct epigenetic marks that can affect totipotency and proper differentiation and development of embryos [60, 61]. Initial epigenetic patterns of the adult somatic cell used for cloning can affect reprogramming following nuclear transfer [62]. In fact, differences in embryo development have been described following the use of cells of different tissue origins for SCNT [63–65].

Several studies have demonstrated abnormal DNA and histone methylation patterns in SCNT bovine embryos compared with *in vivo* and *in vitro* produced embryos [43, 48, 66–68]. Also, global transcriptome profile experiments revealed down regulation of genes involved in chromatin remodeling in cloned embryos compared with *in vitro* fertilized embryos [69].

As mentioned above, early embryos present an intense and very well-orchestrated reprogramming of DNA methylation, and questions arise as to whether the nucleus of the donor cell in SCNT embryos, carrying a somatic cell pattern of methylation, can be manipulated in order to mimic this intense reprogramming taking place during early embryonic stages [43]. Unlike normal embryos, SCNT embryos have incomplete demethylation after the one cell stage, regardless of species [43]. In addition, these SCNT embryos started to undergo *de novo* methylation prematurely (4-cell stage versus 8- to 16- cell stage for *in vivo* embryos), so by the morula stage, methylation in the blastomeres resembled that of the donor cells [43]. The methylation status of ICM in SCNT bovine blastocysts is similar to *in vivo* blastocyst embryos. However, the trophectoderm of SCNT blastocsyts is abnormally hypermethylated [12, 43]. These changes in the normal timing of methylation result in potentially serious consequences in epigenetic reprogramming and further development of the trophectoderm, turning mammalian cloning to an impractical reproductive technology until the timing of events taking place during *in vitro* production can be controlled by scientists [70].

In addition to abnormal DNA methylation, histone modifications are also altered in SCNT embryos. Santos and coauthors [48] demonstrated that SCNT bovine embryos had hypermethylation histone H3-K9 associated with genome-wide hypermethylation. A study also reported that acetylation of lysine 5 on histone H4 (H4-K5ac) appears to change dramatically during early embryo development of IVF produced embryos, but remains consistently elevated in SCNT produced bovine embryos [71]. Compared to *in vitro* fertilized embryos, SCNT bovine embryos have elevated heterochromatic histone methylation (H3K9me2) and H3K9-acetylation in the trophectoderm layer [48]. These and other modifications could explain the altered expression of vital developmental genes later in development.

Another complication with nuclear transfer is the reprogramming of imprinted genes. Abnormalities generally associated with cloned animals resemble those observed in mice with imprinted gene mutations [72]. Analysis of bovine H19 demonstrated that SCNT animals that died shortly after birth had biallelic expression [73]. The placenta of SCNT animals

appears to be especially vulnerable to abnormal expression of imprinted genes [12]. Inoue et al. [74] observed abnormally low levels of expression of both imprinted and nonimprinted genes in the placentas of SCNT mice, whereas expression in the embryo was not altered. Similarly, Yang et al. [75] observed abnormal expression of the imprinted gene IGF2R in the placenta but not in the organs of SCNT calves. Smith et al. [76] found reduced expression of the placenta-specific gene *Cd81* between SCNT and *in vivo* bovine blastocysts. In early pregnancy, expression of the imprinted placental genes Ascl2 has been shown to be altered in SCNT bovine embryos and placental tissues [77]. In SCNT embryos, Ascl2 was overexpressed.

Nuclear reprogramming also affects X chromosome inactivation, particularly in the placenta. Reports have shown that the ICM of SCNT embryos had normal XCI (i.e., random paternal/maternal inactivation), whereas the placenta had altered gene expression from X-linked genes [12]. However, in day 8 blastocysts, Smith et al. [76] found no evidence of abnormal expression of X-linked genes from SCNT bovine embryos. It must be noted that complete XCI in bovine embryos has been reported to occur at day 14-15 [78]. At later stages, reports of bi-allelic expression of X-linked genes in the placenta of deceased clones indicate the absence of the paternal XCI that is observed in non-SCNT calves [52]. Live SCNT calves had one active X-chromosome, similar to non-SCNT calves, suggesting that abnormal XCI could contribute to fetal loss [52].

These results provide further evidence towards the incomplete epigenetic reprogramming of SCNT embryos and the trophectoderm cell lineages appear to be highly vulnerable to these defects.

4. The Use of Histone Deacetylase Inhibitors for *In Vitro* Production of Bovine Embryos

The first reports of utilizing HDAC inhibitors were in cancer therapies. Inhibitors of HDAC promote global chromatin acetylation, which leads to excessive gene transcription. This increase in protein production in tumor cells would induce cellular differentiation, modifying their characteristics of excessive cell growth [79]. Inhibitors of HDAC have been reported to have antiproliferative and apoptotic effects, which are beneficial in cancer therapies. Mutation of the HDAC1 gene in mice caused reduced cell proliferation which led to the embryonic death at midgestation [80]. These effects were associated with increased gene expression of cell cycle inhibitors [80]. Similar antiproliferative effects were seen in embryonic stem cells [81, 82]. Crystallography studies indicated that these inhibitors act by binding the catalytic site of the enzymes, blocking therefore the access to the substrates [83]. Among the known inhibitors, some of the most used are (I) trichostatin A, (II) valproic acid, clinically used in the treatment of epilepsy, and (III) sodium butyrate [84]. These substances cause reversible inhibition of the majority of class I and II HDACs [85].

To improve SCNT efficiency, researchers have turned to stimulating donor cell reprogramming by chemical treatments. Donor cells treated with trichostatin A (TSA) have been shown to have a slight improvement on the development of cloned embryos in cows [10, 62, 86, 87], mice [49, 88–90], pigs [91–95], and rabbits [96, 97]. In pigs, live births were reported following TSA treatment [92, 95]; in rabbits, however, there was no survival of offspring to adulthood [97]. In the bovine, both treatments of donor cells [4, 10, 86] or of the reconstructed embryos [10, 87] were beneficial to the development of clones. The type of donor cell also appears to have a role on the benefits of TSA treatment. In mice, blastocyst formation was 5-fold higher in embryos treated with TSA that were produced from cumulus cells [88]. However, TSA had no effect on blastocyst development from embryos produced by embryonic stem cells. These results suggest that inhibition of HDAC is only beneficial in donor cells that are more differentiated [88]. Similar results also suggest that hypoacetylation may be a limiting factor in the development of cloned embryos [96, 98].

It is thought that TSA aids nuclear reprogramming and subsequently improves expression of embryonic genes such as, Nanog [87], SOX2, and cMyc [90], and regulates expression of genes related to chromatin structure and DNA methylation [90]. Furthermore, the use of TSA improves derivation of embryonic stem cell lines from cloned embryos [88].

The use of general HDAC inhibitors affects all chromatin, which may have negative effects when utilized at elevated concentrations for extended periods of time. Tsuji et al. [89] demonstrated that the beneficial effects of TSA treatment on cloned embryos declined after 12 hours, leading to reduced blastocyst formation and fetal loss. Li et al. [92] reported that in pigs, cloned embryos treated with TSA had a 15-hour delay to reach the stage of compacted morula. Furthermore, embryos fertilized *in vitro* had elevated apoptotic levels following TSA treatment [99]. Apoptosis, or programmed cell death, is a physiological event that has been associated with reduced viability and death of bovine embryos [100, 101]. TSA has been shown to induce expression of proapoptotic genes [102] and, by facilitating histone hyperacetylation, allows the DNA to be available for endonucleases [103].

The addition of TSA, during fertilization, promoted increased levels of histone acetylation on the sperm. Cleavage rate and blastocyst formation were not altered. However, there were more cells in the ICM of embryos that received TSA during fertilization [104]. These results suggest that alteration of histone acetylation during fertilization affects subsequent cell proliferation and differentiation [104]. Embryos cultured with TSA after fertilization, in an effort to aid the activation of embryo genome, had increased levels of histone acetylation, but blastocyst development was not affected [99]. Higher levels of apoptosis were seen in high quality embryos but not in the lower quality ones, suggesting that the use of TSA can benefit this group of embryos. Further, they reported gender differences in the response to histone hyperacetylation following TSA treatment, with female embryos being more sensitive than males [99]. Future studies are warranted to investigate gene expression patterns in embryos following TSA treatment in order to further evaluate potential effects of this agent in early embryo development.

Other HDAC inhibitors are currently being investigated for the use in nuclear transfer in pigs: valproic acid, which resulted in higher levels of Oct4 expression [105] and *scriptaid* [94] and sodium butyrate [106], which resulted in cloned embryos with acetylation levels similar to fertilized *in vitro*. However, special attention must be paid to the fact that the use of valproic acid as an antiepiletic drug (AED) in the first trimester of pregnancy was associated with significantly increased risks of several congenital malformations [107]. Antiepileptic drugs in general are related to malformations, and it is probable that they affect development through multiple mecanisms [108]. Valproic acid has been shown to disrupt the Wnt signalling pathway. However, valproic acid analogues that do not alter Wnt signalling, do not produce teratogenic effects. Other main mechanisms have been connected to valproic acid teratogenic effects, such as, inhibition of folate metabolism and neural apoptosis induction [108], and recent studies reveal HDAC inhibition as one of the possible mechanisms for valproic acid teratogenic effects [109].

In this respect, experiments involving HDAC inhibitors supplementation must be aimed to use the minimum working concentration, in order to inhibit minimum HDAC and obtain the desired effect. Even though the dosages applied for embryo culture are lower for trichostatin A, which is supplemented at nanomolar concentrations, further studies accessing teratogenic effects of this and others HDAC inhibitors are needed.

5. Use of DNA Methylation Inhibitors

As mentioned earlier, several studies indicated that aberrant epigenetic reprogramming occurs in cloned embryos [60, 66, 68]. Partial demethylation and early methylation, at the four- to eight-cell stage in cloned embryos [110], indicate the presence of inadequate nuclear reprogramming in regards to DNA methylation [43].

Researchers aimed at preventing DNA hypermethylation in animal cloning through the use of methylation inhibitors. Pretreatment of donor cells with an inhibitor of DNA methylation, 5-aza-2′-deoxycytidine (5-aza-Dc), before nuclear transfer does not appear to improve development of cloned embryos [111, 112]. These results may indicate that inhibition of excess methylation alone fails to aid reprogramming in the donor cell nucleus, as erasure and reacquisition of methylation during the preimplantation period in embryos is a very complex procedure and may require the use of a combination of agents to try and mimic this process *in vitro*.

6. Conclusions and Future Directions

As discussed here and in the work of others, somatic patterns of histone modifications, as well as DNA methylation, present in differentiated adult cells, must be erased and reprogrammed in a highly organized and timely manner. The acquisition of unique embryonic epigenetic marks must take place in cloned embryos in a similar manner as seen following erasure and acquisition of epigenetic marks in the female and male pronuclei. Failure during erasure and/or acquisition of these marks will affect subsequent embryonic development [60, 61].

Current studies aim at (1) understanding the factors involved in nuclear reprogramming, (2) identifying possible failures in reprogramming induced by cell manipulation and/or culture conditions, and (3) utilize inhibitors and other agents that could assist in the establishment of proper epigenetic patterning during early embryo development. All these efforts to elucidate the complex steps of epigenetic reprogramming during embryogenesis, as well as how to manipulate these steps, are necessary to improve the efficiency and applicability of *in vitro* production of embryos and cloning.

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