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Probability, odds and random chance: the difficult task of modulating the epigenetic profile of cloned embryos

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Abstract

Since the beginning of modern embryology, scientists have wondered about how a small number of totipotent embryonic cells can become an individual with a wide variety of organs and tissues with distinct functions. Also, the idea of generating a cloned animal using a nucleus from a donor cell is not recent. However, it has taken years of research to achieve this goal, especially regarding mechanisms of cell reprogramming required to return a differentiated cell to totipotency. Cloning by somatic cell nuclear transfer (SCNT) has been a valuable tool to understand epigenetic mechanisms related to cellular reprogramming. However, cloning efficiency is still low, with a low percentage of embryos resulting in healthy animals. The high attrition rate is associated with incomplete or abnormal epigenetic reprogramming, such that many cloned embryos have DNA methylation patterns different than controls, resulting in faulty gene expression and subsequent developmental failures. Attempts to improve genome reprogramming by modulation of oocyte quality and/or somatic cell plasticity, thereby increasing cloning efficiency and preventing detrimental effects on development, have proven ineffective. The recent development of DNA editing techniques may facilitate an improved understanding of cellular reprogramming and the role of DNA methylation in development. These novel tools may lead to new means to modulate epigenetic programming and inheritance, and hold great promise to assist in epigenetic remodeling of the donor nucleus. Such strategies are likely to improve the odds for successful cloning.

Keywords: cloning by nuclear transfer, embryo development, epigenetics, reprogramming.

Introduction

Among many questions of biological significance, one in special has intrigued embryologists for a long time: how can cells change their phenotypes without changing the genotype? The concept of genomic equivalence, in which each somatic cell in the organism has the same genetic material and, therefore, also has all the information necessary to create a complete new organism, was assumed as a fact for decades, since cells are all derived from one single cell fertilized egg. However, the ultimate evidence came only in 1996 with the birth of Dolly, the sheep, cloned by somatic cell nuclear transfer (SCNT) procedures using somatic cells from an adult ewe (Wilmut *et al*., 1997). At last, animal cloning demonstrated that a differentiated somatic cell nucleus could be re-directed (or reprogrammed) for the development of an entire new organism. Since then, more than 20 vertebrate species have been cloned, from a variety of somatic cells, for a multitude of purposes, at various levels of success, demonstrating the usefulness of the procedure for applications ranging from science to the industry, animal conservation to production, genetic selection to transgenesis.

Twenty years after Dolly has passed, and cloning efficiency still remains low. Despite efforts to improve it, less than 5% of the animals come to term and remain healthy afterwards (Selokar *et al.*, 2014, 2015). Even though the precise factors are yet to be determined, such low efficiency is undoubtedly associated with incomplete or abnormal chromatin remodeling and genomic reprogramming after cloning, which is related to nonphysiological epigenetic modifications dependent on (i) the quality of the recipient oocyte or cytoplast where the donor nucleus need to be reprogrammed (Wells *et al.*, 2010; Aguiar *et al.*, 2016), (ii) the origin, quality, and plasticity of the donor nucleus or karyoplast *per se* prior and after its transfer into a cytoplast (Campbell *et al*., 2005), and (iii) the level of technical expertise and biological variation in all steps related to cloning (Bertolini *et al.*, 2012). For normal embryo development and survival, success in cloning depends on correctly reversing specific markers of cellular differentiation to reactivate or resume processes needed during development, all associated with proper specific and global gene expression (Niemann, 2016).

Even with the concept of genomic equivalence, the ideia that the genome can be strongly affected by external or environmental influences lay the ground basis for what is referred as epigenetics, and what such a field is about: how genes are regulated, what factors can influence them, and how genes control and are controlled in the course of development throughout life, under physiological or pathological conditions, and in health and disease. In that regard, animal cloning still is a useful model to dedifferentiate specialized somatic cell nuclei to a totipotent state, in a process referred as epigenetic reprogramming. Thus, this review aims to briefly discuss some concepts and views on the role of epigenetic and reprogramming mechanisms in growth

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and development, especially after cloning by SCNT, including attempts to epigenetically modulate gene function and normal development.

Chromatin remodeling, epigenetic reprogramming, and early events in development

Nuclear remodeling after cloning or remodeling of chromosomal architecture is the designation for a series of molecular events involving modification and expression of nuclear laminins (Kubiak *et al*., 1993), formation of the nuclear envelope (Szollosi and Szollosi, 1988), and changes in protein synthesis (Fulka *et al.,* 1996). The cytoplasmatic kinase named Mitosis or Meiosis Promoter Factor (MPF) has great importance in the nuclear remodeling (Campbell, 2002, 2013). The kinase activation triggers the onset of mitosis or meiosis, resulting in nuclear envelope break (NEBD), chromatin condensation, reorganization of the cytoskeleton and changes in cell morphology (Nurse, 1990; Campbell, 2002, 2013).

It has not been determined if the nuclear remodeling is an absolute necessity for the normal development of cloned embryos. However, it has been demonstrated for quite some time now that nuclear MPF facilitates remodeling after cloning (Fulka *et al.,* 1996). In metaphase II (MII) oocytes containing high levels of MPF active kinase, the maintenance of the cytostatic factor (CSF) that keeps MPF active remains until oocyte fertilization or activation (Fulka *et al*., 1996; Marteill *et al*., 2009). After the steps of fusion/activation in conventional cloning procedures, the kinase activity of MPF phosphorylates numerous cellular proteins leading to the completion of meiosis II, the remodeling of the maternal and paternal chromatin, also contributing to the embryonic activation process (Sirard, 2011).

In cloning, among the most notorious events of the MPF action in the MII oocyte on the donor nucleus, usually at the G0/G1 stages of the cell cycle, are the breakdown of the nuclear envelope, the premature chromatin condensation (PCC) and the formation of the achromatic spindle, which culminates in the extrusion of the second polar body (Campbell, 2013). Conversely, after fertilization, embryonic activation follows, when CSF is degraded and the MPF activity decreases in the first hours, coinciding with the nuclear envelope reconstruction in the form of male and female pronuclei and chromatin decondensation, initiating an interphase (Szollosi *et al*., 1988; Nakai, 2016). In cloned embryos, which are artificially activated by electrical or chemical processes, MPF activity remains high for a longer period of time (Kubiak *et al*., 1993; Sterthaus, 2009). Currently, researchers are still trying to identify factors and their importance in the success or failure of epigenetic reprogramming after cloning, seeking the understanding of how to improve protocols and processes to attain more reliable results.

Intrinsic and extrinsic modulators of epigenetic reprogramming and early development after cloning

Animal cloning by SCNT, although simple in concept, involves multiple steps that make the process complex, a fact responsible for its success or failure in subsequent development (Bertolini *et al*., 2012). Some steps, e.g., oocyte *in vitro* maturation (IVM) or *in vitro* culture (IVC) of embryos, are common to other *in vitro* embryo production (IVP) systems, such as *in vitro* fertilization (IVF) procedures. However, other steps are exclusive to cloning, such as those that replace fertilization by SCNT.

The cloning success is directly associated with the source, quality and preparation of enucleated recipient cytoplasms (cytoplasts), the donor cell nucleus (karyoplasts), and also cloning procedures *per se*, including embryo reconstruction and activation (Campbell *et al*., 2005; Wells, 2010). Therefore, the intrinsic features of cytoplasts and karyoplasts are modulated by physical and chemical factors, including manipulation and *ex corpore* exposure, which is always a suboptimal environment and biologically less suitable for development (Bertolini *et al*., 2012).

Cytoplast source and cell cycle synchrony/asynchrony

The oocyte source is a limiting factor for many species, in terms of availability, quality and biological competence, also being one of the main sources of variation in programs of *in vitro* embryo production (Galli *et al*., 2003; Wells, 2010). *In vivo*-matured oocytes are routinely used for cloning in many animal species, as such oocytes are presumably more competent for nuclear reprogramming and to subsequent embryonic development (Campbell *et al*., 2005; Ribeiro *et al*, 2009; Wells, 2010; Mezzalira *et al*., 2011; Aguiar *et al.*, 2016).

The cytoplast appears to be one of the key elements for cloning success as everything associated with the oocyte is essential for embryonic and subsequent fetal development, still being one of the most efficient *milieu* for the induction of epigenetic reprogramming of the genome (Campbell *et al*., 2005). Reprogramming factors, for example, must be abundant and sufficient present to effectively reprogram a genome (Campbell *et al*., 2001; Oback and Wells, 2003; Wen *et al.*, 2014). It is believed that nuclear reprogramming capacity reaches its highpoint from fertilization/reconstruction up to the embryonic genome activation, as the oocyte cytoplasm contains chromatin remodeling factors and reprogramming factors (proteins, mRNAs, miRNAs and molecular precursors) accumulated during folliculogenesis (Alberio *et al*., 2001; Urrego *et al.*, 2015). However, factors acting in the reprogramming of the donor nucleus are still not completely identified, with the oocyte quality remaining key to successful animal cloning (Wells, 2010).

Pieces of evidence suggest that the start in embryonic genome expression occurs early in development (Flach *et al*., 1982; Graf *et al.*, 2014), leading to the hypothesis that the nucleus requires a time to adapt to a new cellular environment. Once the oocyte environment is at a state of transcription, a transferred nucleus donor would not have adequate time to adapt, negatively affecting nuclear reprogramming. This leads to another important point of discussion,

which is related to the level of synchrony between the cell cycles of the recipient cytoplasm and the donor cell.

It is known that the exposure of a donor nucleus to an environment with active MPF, such as in the MII oocyte, will lead to a series of subcellular events promoting exposure of chromatin reprogramming factors also at high levels in the cytoplasm, which possibly favors epigenetic reprogramming (Mezzalira *et al*., 2011). Thus, *in vitro* embryo development is improved when cytoplasts at MII are used in association with post-fusion activation (Heyman *et al*., 2002; Mezzalira *et al*., 2011). The time between the fusion of the cytoplast at MII with the karyoplast at G0/G1 and embryo activation is referred as reprogramming time, and such time appears to play an important role in chromatin exposure to oocyte cytoplasmic remodeling factors (Campbell *et al*., 2005). The appropriate reprogramming time usually ranges from 1 to 4 hours, but no consensus exists about the ideal exposure time under each separate condition.

MII oocytes as cytoplasts

Generally, classic cloning procedures, from Briggs and King (1952) to this day, use enucleated oocytes at MII as recipient cytoplasts. For such cloning protocols, the karyoplast cell cycle can be used either at the G1 phase, which is the interval between mitosis (M phase) and onset of DNA replication (S phase), or at the G0 phase (Wilmut *et al.,* 1997; Kato *et al.,* 1998; Heyman *et al.,* 2002), with cell remaining metabolically active but mitotically quiescent (Oback and Wells, 2002). Then, the somatic cell nucleus must always adopt the parameters of the cell cycle of the MII recipient cytoplast (Kikyo and Wolffe, 2000). As non-activated MII cytoplasts have high MPF levels, donor nuclei are forced into the M phase, with cells at G0/G1 remaining diploid, whereas cells at the G2 or at the S phases result in aberrant chromosome configurations (Oback and Wells, 2002). In such cases, if the cloning protocol does not allow the extrusion of the second polar body, the resulting embryo may become tetraploid or, at its best, the rupture of the nuclear envelope and premature chromatin condensation (if DNA synthesis is not complete) can lead to aneuploidy and chromosome loss (Barnes *et al*., 1993, Østrup, 2009). Then, cell cycle synchronization into G0/G1 is usually attained by many procedures to ascertain proper embryo development after cloning (Gerger *et al.*, 2010).

Universal cytoplast

Pre-activated oocytes are referred as universal cytoplasts when used for cloning after the decrease in MPF activity, as such cytoplast is compatible with development using cells at any stage of the cell cycle. Under all situations, resolution of ploidy will occur, as there is neither the breakdown of the cell nuclear envelope at the interphase nor a premature chromatin condensation (Campbell *et al*., 1996). Nevertheless, the use of such oocytes usually results in lower rates of development. Similarly, aged oocytes have lowering CSF levels due to gradual decay, destabilizing MPF, leading to an increase in spontaneous activation by physicochemical environmental events (Wakayama *et al*., 2003). However, despite being easier to activate, aged oocytes have lower potential for further embryo development (Chian *et al*., 1992; Takahashi, 2013).

Late TII oocytes as cytoplasts

As an additional oocyte source for cloning, also related to the cell cycle, the embryo reconstruction using oocytes at late telophase II (Bordignon and Smith, 1998) results in proper embryo development, but at slightly lower rates than MII oocytes, possibly due to MPF levels still being abundant to trigger events observed with MII oocytes, some associated with reprogramming and chromatin remodeling (Mezzalira *et al*., 2011). Such system is successfully in use in commercial cloning operations in Brazil and abroad.

Karyoplast or nuclear donor cell type

The choice of the karyoplast or nuclear donor cell type has a significant biological impact on cloning and accounts for large variations in the success rates (Campbell *et al*., 2005, Liu *et al.*, 2015). Cellular reprogramming efficiency after SCNT seems to depend on a multitude of factors, including the genotype (species, breed, gender, age, individual factor, etc.), the stage of development (embryonic, fetal, adult animal), tissue origin, cell type, degree of differentiation, cell cycle, and overall features when in culture (Batchelder *et al*., 2005; Wells, 2010). As mentioned above, the current understanding is that most of the cloning efficiency is associated with the use of synchronized somatic cells at G0 or G1 of the cell cycle (Wilmut *et al*., 1997; Wakayama *et al*, 1998; Wells, 2010). In addition, cells of embryonic origin seem to present a higher potential for further development and lower level of abnormalities than cells from fetal origin, which in turn seem to be more efficient than adult cells (Heyman *et al*., 2002; Batchelder *et al*., 2005). Cells from the same individual but from different tissue origins at distinct differentiation levels have also been described to favor cells of lower degree of differentiation (Batchelder *et al*., 2005). Likewise, the isolation method, the type of cell culture and the number of cell passages have an important effect on the results (Wells, 2010; Martins *et al.*, 2016), whereas cells with few passages in culture appear to exhibit greater potential for epigenetic reprogramming after cloning (Li *et al.*, 2014). All these aspects should be carefully controlled, as it is known that the culture conditions can cause genomic instability and consequent failures after cloning (Humpherys *et al*., 2001). Nonetheless, the small number of reported births, the absence of extensive controlled studies, and the low technical efficiency make it a difficult task to adequately compare cell types in cloning by SCNT (Tecirlioglu *et al*., 2005).

Embryo reconstruction procedures

The embryo reconstruction itself can be accomplished by micromanipulation with (Wilmut *et*

al., 1997) or without (Oback and Wells, 2003) zona pellucida, or manually by handmade cloning (HMC) without zona pellucida (Vajta *et al.*, 2003), with no apparent differences in the final efficiency between procedures (Tecirlioglu *et al.*, 2005). Usually, embryo reconstruction by SCNT involves the steps of oocyte enucleation, nucleus transfer by membrane fusion with the mix of different cytoplasms, oocyte activation and embryo culture. Oocyte enucleation may result in loss of cytoplasm and, when excessive, can compromise subsequent embryo development (Ribeiro *et al*., 2009). On the other hand, the fusion of cell membranes leads to mosaicism and cytoplasmic heteroplasmy by mixing different cytoplasts, and it is still not clear which are the possible biological consequences on subsequent development (Ribeiro *et al*., 2009; Mezzalira *et al*., 2011). In addition, fusion aims to introduce a donor nucleus, typically at the G0/G1 phases of the cell cycle, into a MII oocyte. Such process exposes the chromatin to reprogramming factors and to heterologous proteins and miRNAs, all likely important for genome reprogramming (Alberio *et al*., 2001).

Oocyte or embryo activation also appears to be related to the possible development problems since, physiologically, it occurs by the action of factors released by the sperm cell, causing intracellular periodic oscillations in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in the oocyte for a certain period of time, which depolarizes the cell membrane and induces a chain of events that leads to embryo activation and the first embryo cleavage (Galli *et al*., 2003). In cloning by SCNT, activation of the reconstructed embryo is usually induced artificially by electric or chemical procedures, leading to elevated $[Ca^{2+}]_i$ in a single peak. Moreover, the use of inhibitors of protein kinases (targeting MPF) or protein synthesis (blocking cyclin translation), especially for cells at G0/G1, is deemed necessary for further embryo development (Campbell *et al*., 2005). Finally, the *in vitro* embryo culture system has also been implicated with epigenetic changes, resulting in distinct gene expression patterns in embryos and in embryonic stem cells (Dean *et al,* 1998; Wrenzycki *et al.,* 2001).

Altogether, the summation of intrinsic and extrinsic factors related to the cytoplast source and karyoplast type, along with the biological and technical aspects in all steps involved in cloning procedures *per se*, ultimately will determine the overall cloning efficiency, which is by far and large dependent on the level of genome reprogramming at early embryo stages, making animal cloning a process of survival of the epigenetically fittest (Bertolini *et al.*, 2012).

Improving genome reprogramming: is it feasible?

Overall, and despite such variety in the idiosyncrasies and peculiarities to reach success after cloning by SCNT, once a minimal understanding of the methodology, certain levels of skill and experise, and the control of some basic biological factors are attained, obtaining cloned blastocysts is a relatively easy outcome. Still, blastocyst yield does not necessarily reflect success. An example is the possibility of achieving high blastocyst rates by parthenogenesis, a procedure commonly used to infer oocyte quality (Ribeiro *et al.,* 2009). Thus, although blastocyst yield is a rather easy pitfall to overcome, proof of success after cloning can only be certified when there is subsequent *in vivo* development after the embryo transfer (ET) to recipient females, culminating with the birth of viable offspring (Bertolini *et al.,* 2012).

Pre- and postnatal abnormalities observed after cloning by SCNT are directly related to the efficiency of each step in embryo reconstruction. In the process, the functional and molecular asynchrony between donor nucleus and ooplasm components leads to inadequate genomic reprogramming. This fact is well demonstrated by several groups in which mammalian cloned embryos have an abnormal genome epigenetic configuration associated with phenotypic and physiological changes during development (Dean *et al.*, 2001; Wrenzycki *et al*., 2001; Beaujean *et al*, 2004). Such features support the hypothesis that failures at the level of gene expression and embryonic development are caused by improper reprogramming of the donor genome during and after SCNT, due to many of the factors and components already discussed above, affecting the pattern of DNA methylation, or histone acetylation, methylation, phosphorylation or ubiquitinization during the first rounds of embryo development, compromising proper gene function and development afterwards.

Role of DNA methylation in development

DNA methylation in mammals is an important regulator of gene transcription, being a mechanism often used to silence and regulate genes without changing the original DNA sequence; this is one of the most important strategies for epigenetic modifications of the genome (Jaenisch and Bird, 2003). Biochemically, DNA methylation is a process involving the addition of a methyl group at position 5 of cytosine residues (5mC) in the DNA. In adult somatic cells, the DNA methylation typically occurs in dinucleotide sequences called CpG islands, which are in guanine- and cytosine-rich regions. Between 60 and 90% of all CpGs are methylated in mammals (Tucker, 2001), and these islands are usually also present in the 5' regulatory regions of many genes, which are also associated with differentially methylated regions (DMR), directly related to genomic imprinting.

DNA methylation is essential for normal development and performs a variety of biological functions, being associated with very important processes, including repression of gene expression, genomic imprinting, X-chromosome inactivation, suppression of repetitive elements and carcinogenesis. Thus, the relationship between DNA methylation and chromatin structure is very important because this epigenetic modification affects cell function, allowing cells with the same genetic material to differentiate, yielding multiple organs, or perform various functions (Jaenisch and Bird, 2003). DNA methylation can also suppress the expression of endogenous retroviral genes and other deleterious DNA sequences that may have

been incorporated into the host genome over time. The methylation can also protect DNA from enzymatic cleavage because most restriction enzymes are incapable of recognizing and binding to sequences (epigenetically) modified externally.

As one of the most important functions, DNA methylations at CpGs have the specific effect of reducing gene expression, being found in all vertebrates, with extensive evidence that genes with high levels 5-methylcytosine in their promoter region are transcriptionally silenced (Jaenisch and Bird, 2003), as shown in Fig. 1. DNA methylation can affect gene transcription in at least two ways: 1) physically preventing the binding of transcription proteins; and/or 2) binding proteins known as MBDs (methyl-CpG-binding domain proteins). These proteins may recruit additional proteins to the site, such as histone deacetylases and other chromatin remodeling proteins that can modify histones, thereby forming a compact and inactive chromatin called heterochromatin (Nan *et al.,* 1993).

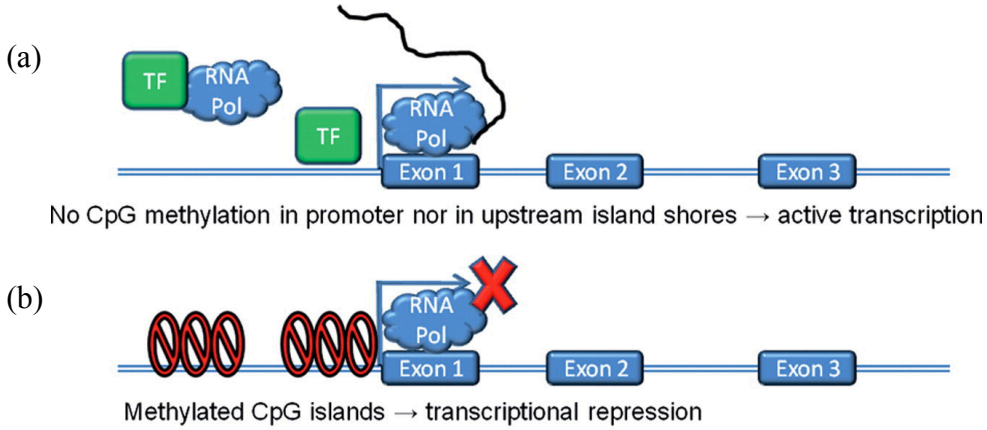

Figure 1. Regulation of gene transcription by DNA methylation. (a) Active transcription: unmethylated CpG islands at promoters of genes leads to active transcription. (b) Inactive transcription: hypermethylated CpG islands at promoters of genes leads to transcriptional repression. TF: transcription factors. RNA Pol: RNA polymerase. From Turunen and Ylä-Herttuala (2011).

Interestingly, no significant methylation pattern is seen in embryonic stem cells (Lister *et al.,* 2009), but in somatic cells there are high levels of DNA methylation generally transmitted to daughter cells with high fidelity. Therefore, the resulting epigenetic changes are normally stable, continuous and unidirectional, preventing an organism from reverting any level of cell differentiation or the conversion of a tissue to another.

Role of DNA Methyltransferases in development

The pattern of global and specific DNA methylation in mammalian cells appears to be propagated during the development of the conceptus after each DNA replication cycle by an action of either *de novo* or maintenance DNA methyltransferases (Jackson-Grusby *et al.,* 2001; Golding and Westhusin, 2003). DNA methyltransferases (DNMTs) are part of a family of enzymes that catalyze the transfer of a methyl group to the DNA using S-adenosyl-methionine (SAM) as a methyl donor (Smith *et al*., 1992). Like restriction enzymes, methyltransferases have specific target DNA sequences. In mammalian cells, DNMTs methylate DNA sequences in CpG islands, located generally closer to or within the promoter regions, and exhibit different patterns of DNA methylation, also determining the imprinting pattern. In such cells, DNA methylation that occurs mainly at the C5 position in CpG dinucleotides is done by two general classes of DNA methyltransferases: maintenance and *de novo* DNMTs (Smith *et al*., 1992). Three active DNMTs have been identified in mammals, being called (maintenance) DNMT1, and (*de novo*) DNMT3a, DNMT3b and DNMT3L (Golding and Westhusin, 2003).

DNMT1

The DNMT1 is the most abundant DNA methyltransferase in mammalian cells and is considered the main maintenance enzyme, predominantly methylating CpG di-nucleotides in the hemimetilated genome (Kho *et al.,* 1998). The maintenance of methylation activity is necessary to preserve DNA methylation responsible for the copy of the methylation patterns for "daughter" strands after each cellular DNA replication cycle. *In vitro*, the enzyme is 7 to 100 times more active in hemimetilated DNA compared to non-methylated substrate and is more active in the *de novo* methylation than other DNMTs. DNMT1 has several isoforms: the somatic form, the most common in the organisms and always present in the nucleus of somatic cells; variant DNMT1b isoform; and DNMT1o, an oocyte-specific isoform, which is synthesized and stored in the cytoplasm of oocytes, being allocated in the nucleus during specific stages of early embryo development (Golding and Westhusin, 2003). Abundant levels of transcripts to the main isoforms of DNA methyltransferases are found in oocytes and embryos at preimplantation stages (from 1-cell to blastocyst) in cattle (Golding and Westhusin, 2003) and humans (Huntriss *et al.,* 2004). The DNMT1o variant, present in

murine and human oocytes and embryos, is unidentified in cattle (Golding and Westhusin, 2003).

DNMT3

The DNMT3 is a DNA methyltransferase family that may also methylate the CpG sites of unmethylated and hemimetilated DNA. The structure is similar to DNMT1, with a regulatory region linked to a catalytic domain, with three known members of the family: DNMT3a, DNMT3b, and DNMT3L. The DNMT3a and DNMT3b can mediate methylation-dependent gene repression, with these enzymes able to establish DNA methylation patterns in early embryonic development and to promote cell differentiation. The DNMT3L is a protein homologous to the other DNMT3s, but with no catalytic activity. Such protein interacts with DNMT3a and DNMT3b, being co-located in the nucleus, aiding the DNA binding to the *de novo* methyltransferases, stimulating their activity (Rhee *et al*., 2002).

Differentially methylated regions (DMRs) and genomic imprinting

The DMRs are genomic regions with different states of methylation, with functional regions involved in regulating gene transcription, and a strong association with genomic imprinting, including imprinting control regions (ICRs) into DMRs. The identification of DMRs from various tissues may show epigenetic differences among them, as well as cancer DMRs can show aberrant methylation patterns when compared with normal samples (Irizarry *et al*., 2009). It is well known that DNA methylation is associated with cell differentiation and proliferation (Reik *et al*., 2001), also strictly related to distinct DMRs in development and through the reprogramming process (Meissner *et al.,* 2008; Doi *et al.,* 2009). In addition, there are intra- and inter-individual DMRs with longitudinal changes in the global methylation, increasing with age in a given individual or between individuals (Bjornsson *et al.,* 2008; Bock *et al*., 2008).

The expression of imprinted genes crucial to development is also regulated at the epigenetic level by imprinting/methylation within DMRs, which regulate gene expression either from paternal or maternal alleles. One of the classical examples in imprinted gene regulation by imprinting/methylation within DMRs is played by the Igf2-H19 *locus* that encodes the pleitropic growth factor IGF2 and the H19 gene, which results in a non-coding RNA precursor of several microRNAs that have tumor suppressor effects (Reik and Murrel, 2000; Ratajczak, 2012; Singh *et al.*, 2012), as illustrated in Fig. 2. Under normal imprinting pattern, a DMR positioned between the Igf2 and H19 coding regions is unmethylated on the maternal allele, and methylated on the paternal counterpart, with the expression of both genes being under the regulation of a downstream 3'-distal enhancer (Fig. 2a). The methylation of the paternal DMR prevents binding of the CTCF insulator protein, allowing the activation of the paternal Igf2 promoter by the distal enhancer. Conversely, as the maternal DMR is unmethylated, CTCF insulator protein binds to it, preventing the activation of the maternal Igf2 promoter, with the maternal H19 being transcribed. Then, the proper somatic imprint of the DMR at the Igf2-H19 *locus*, with a lack of methylation on the maternal allele and a methylation pattern of the paternal allele, ensures that Igf2 is transcribed only from the paternal chromosome, and H19 only from the maternal chromosome, under a balanced expression pattern (Reik and Murrel, 2000; Ratajczak, 2012; Singh *et al.*, 2012).

However, under certain epigenetic conditions, such as after faulty nuclear reprogramming following cloning by SCNT or in primordial germ cells (PGCs), the pattern of expression may change significantly, affecting pre-natal growth, development and survival to term. In the erasure of imprinting, when both DMRs at the Igf2-H19 locus are unmethylated, only H19 is transcribed from both parental alleles (Ratajczak, 2012). Conversely, in the loss of imprinting, when both DMRs at the Igf2-H19 *locus* are methylated, as seen in cancer cells in several cancer types, only Igf2 is expressed from both parental chromossomes (Fig. 2b and c). The imprinting process appears to be more susceptible to environmental effects and faulty reprogramming, with deregulations of ICRs/DMRs and loss of imprinting, generally resulting in abnormal phenotypes (Rancourt *et al.,* 2013). Consequently, embryonic manipulations, such as IVF and cloning by SCNT, should deregulate genomic imprinting (Moore, 2001).

Physiological epigenetic changes in the course of development

DNA methylation appears to be critical to the course of normal embryonic development in mammals, as chromatin remodeling after fertilization is closely related to a fast demethylation of the parental genome (Reik *et al.,* 2001; Dean *et al.,* 2003). As illustrated in Fig. 3, DNA methylation pattern in gametes is typically removed during early embryo stages, to be restored during the successive cell divisions in development, altering the stable gene expression during cell division and inducing cell differentiation. In fact, global genomic reprogramming occurs rapidly after fertilization, with the degree of DNA methylation decreasing in about 30% of the average level observed in somatic cells (Bird, 2002). This fact is interesting because when demethylation was experimentally induced below such values, a negative interference in embryonic development was observed, showing that a certain degree of methylation needs to be maintained in the genome (Giraldo *et al.*, 2009). Following the reduction in DNA methylation during the first cleavages, a *de novo* methylation pattern is promoted by DNMT3a, 3b and 3L (Fig. 3), beginning at the 8-cell and at the blastocyst stages in cattle and mice, respectively (Dean *et al.,* 2003).

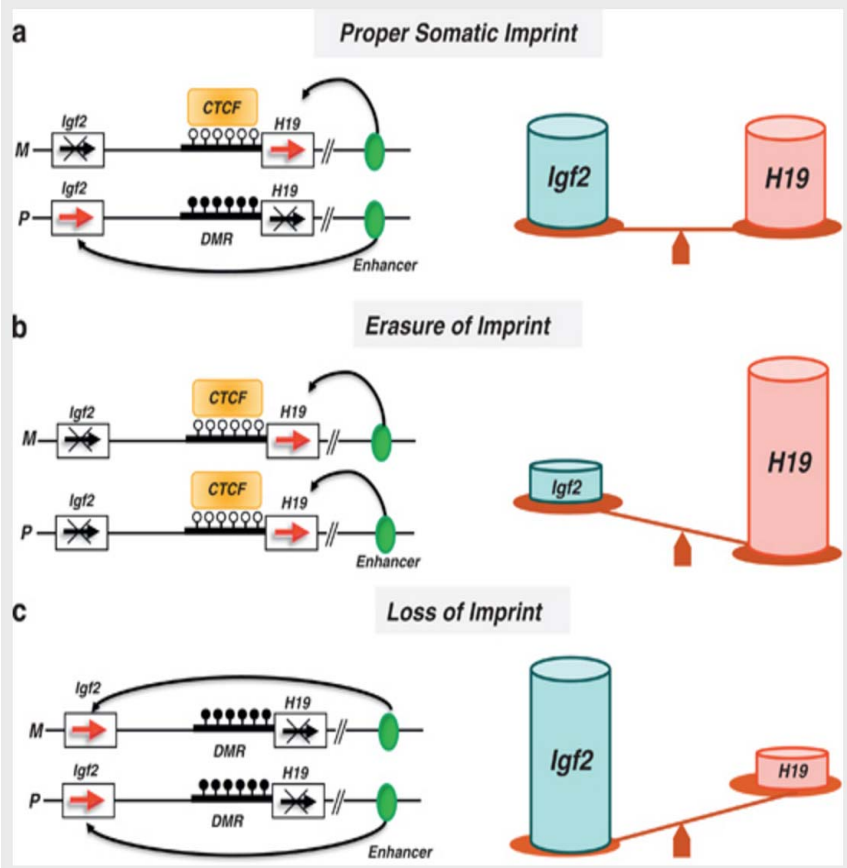

Figure 2. Regulation of expression pattern in the imprinted genes Igf-2 and H19 by changes in the methylation state in the DMR within the Igf-2-H19 locus, and expression balance (proportion) between genes. (a) Proper somatic imprint. (b) Erasure of imprint. (c) Loss of imprint. See text for details. M: maternal chromosome. P: paternal chromosome. CTCF: CTCF insulator protein. DMR: differentially methylated region. From Ratajczak (2012).

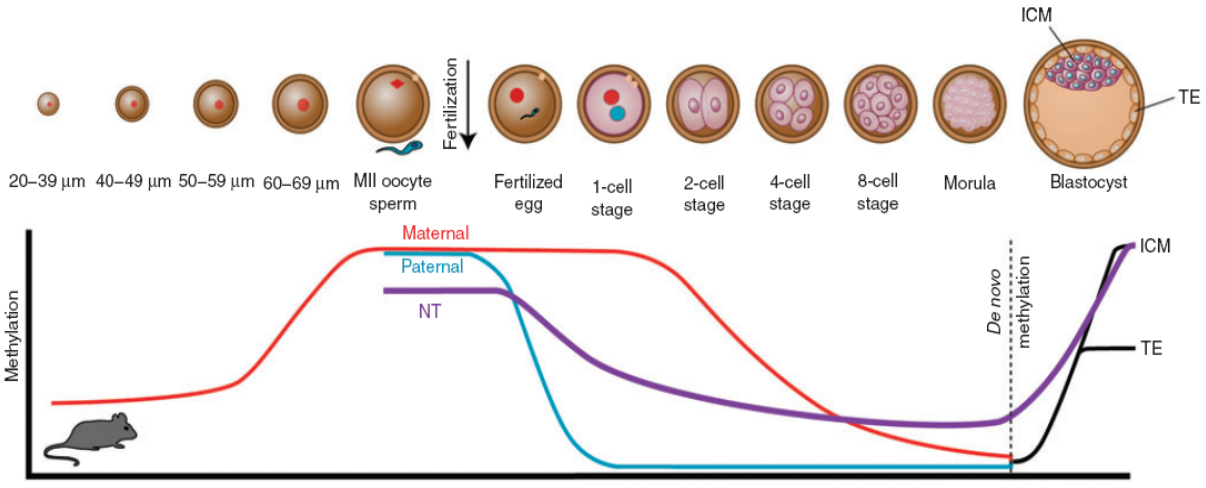

Figure 3. Methylation dynamics during normal early embryonic development and in clones. In the mouse, methylation of the germinal vesicle nucleus increases with size of the oocyte. After fertilization, a quick hydroxymethylation of the paternal genome (blue), previously thought to occur as an active demethylation process, and a passive demethylation of the maternal genome (red) occurs. *De novo* methylation occurs at the blastocyst stage, with a differential methylation pattern between the inner cell mass (ICM) and the trophectoderm (TE). In cloned embryos (purple), some demethylation takes place by the blastocyst stage, after nuclear transfer but before *de novo* methylation, with aberrant methylation (hypermethylation) of the TE. From Yang *et al.* (2007).

Hydroxymethylation

Until recently, the demethylation of the paternal genome was considered as part of an active process (replication independent), whereas in the maternal genome, the demethylation occurs passively

during DNA replication (replication dependent) in each cell division (Yang *et al.*, 2007). Pieces of evidence suggest that in fact a passive process also occurs in the paternal genome, by the convertion of 5'-methylcytosine (5mC) into 5'-hydroximethylcytosine (5hmC) prior to demethylation *per se* (Wossidlo *et al.*, 2011). Enzymes previously linked to chromosomal translocations in leukemia (Lorsbach *et al.*, 2003), the ten-eleven translocation proteins (TET1, 2 and 3), have been recently implicated as responsible for the oxidation of 5mC into 5hmC (Kriaucionis and Heintz, 2009; Tahiliani *et al.*, 2009). Then, the final demethylation process is resolved by enzymes of the DNA repair machinery in the cell (Fig. 4).

The drastic drop in paternal DNA methylation after fertilization (Mayer *et al*., 2000), attributed earlier to a fast and active demethylation, occurs concurrently with an increase in conversion from 5mC to 5hmC (Wossidlo *et al.*, 2011). Conversely, at the maternal genome, an aparent partial "protection" against TET3 prevents the addition of hydroxyl groups in the DNA, leading to a passive demethylation pattern (Nakamura *et al.*, 2012). The mechanism used to control the methylation assumes that 5hmC interferes with the recognition of methylated cytosines by DNMT1, allowing a passive demethylation pattern to be established (Fig. 4a). Another possible path is explained by the low affinity of the 5hmC to methyl-CpG binding proteins, which specifically recognize the hydroxymethyl group, e.g., DNA repair proteins as 5hmC-specific DNA glycosylase (5hmC-DG), as shown in Fig. 4b (Dahl *et al*., 2011).

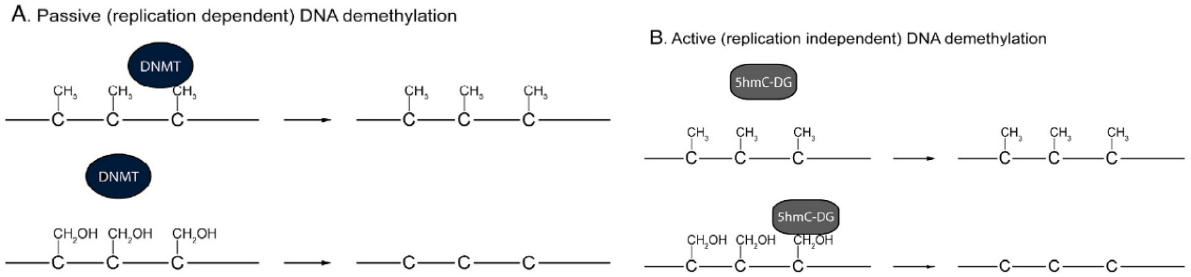

Figure 4. (A) Top: DNA methyltransferase 1 (DNMT) recognizes 5mC and maintain methylation during DNA replication. Bottom: 5hmC is not recognized by DNMT, which will prevent maintenance methylation during DNA replication, resulting in passive (replication dependent) DNA demethylation. (B) Top: 5mC is not recognized by DNA repair proteins, maintaining methylation during DNA replication. Bottom: 5hmC may be recognized by DNA repair proteins, e.g., a 5hmC-specific DNA glycosylase (5hmC-DG), which will convert 5hmC to cytosine, leading to active (replication independent) DNA demethylation. Adapted from Dahl *et al*. (2011).

Abnormal patterns of DNA methylation and consequences for development

Chromatin remodeling after fertilization, associated with the reprogramming of the DNA methylation, appears to occur similarly in embryos produced both *in vivo* and *in vitro* (Han *et al.,* 2003). However, several SCNT cloning studies showed that the introduction of the somatic donor nucleus (highly methylated) in a recipient cytoplasm (oocyte) filled with chromatin remodeling components is not sufficient to reprogram, modify or delete certain stable genomic epigenetic markers of differentiation, leading to faulty gene expression patterns (Bird, 2002). This reprogramming failure is associated with the hypermethylation status of DNA in murine and bovine embryos produced by SCNT, which may have similar levels of DNA methylation of the somatic cell donor nucleus (Dean *et al.*, 2001, 2003; Beaujean *et al.,* 2004). This fact can be observed in Fig. 3 above, where a high amount of methylated DNA is maintained after nuclear transfer in mice, while *in vivo*-derived embryos go through normal processes of replication dependent and independent demethylation.

Due to its role in gene expression, epigenetic modification is considered essential to the memory of a specific cellular function during development (Bird, 2002) and regulation of nuclear reprogramming (Han *et al*., 2003). The efficiency of epigenetic reprogramming after cloning by SCNT seems to be strongly dependent on the type and state of differentiation of the somatic cell donor used (Wells *et al.,* 2003). In rats and cattle, the type of cell lineage in culture, a cell line or even subclone populations may reveal different developmental capabilities prior to and after cloning (Humpherys *et al*, 2001; Vichera *et al*., 2013).

An apparent relationship exists between the process of chromatin remodeling, genome reprogramming, and the profile of DNA methylation. In mice, a significantly higher percentage of blastocysts are obtained when cloned embryos are reconstructed with rather unmethylated cells, such as murine embryonic stem cells (ESC) or primordial germ cells (PGC), than other somatic cell types (Humpherys *et al.,* 2001). In the absence of DNMT1 in the nucleus and after fertilization, the replication generates only unmethylated DNA strands which, over time, leads to passive DNA demethylation, as occurs with zygote DNA from maternal origin. Interestingly, higher amounts of transcripts for DNMT1 were observed in bovine blastocysts derived by cloning SCNT (Wrenzycki *et al.,* 2001), which coincides with the detection of a more methylated state of cloned embryos. Thus, it is possible that the hypermethylated state of

cloned bovine embryos is associated not only from poor demethylation of nuclear DNA, but also through the presence of active DNMT1 of somatic origin in the nucleus during early development, contributing to hypermethylation.

Genomic imprinting appears to be more susceptible to epigenetic changes, with deregulation of ICRs or DMRs generally resulting in abnormal phenotypes (Rancourt *et al.,* 2013). Consequently, embryonic manipulations, such as IVF and SCNT, seem to deregulate imprinting (Moore, 2001). On the other hand, when DNMT1 activity is reduced, the DNA methylation in ICRs or DMRs associated with genomic imprinting was generally refractory to epigenetic changes, maintaining their methylation patterns in cells in culture (McGraw *et al*., 2013). However, the permanent loss of DNMT1 by gene deletion leads to a reduction in monoallelic expression of several imprinting genes (McGraw *et al*., 2013). Interestingly, homozygous DNMT1 mutant mouse embryos null by deletion died between days 9-11 of gestation (Jackson-Grusby *et al*., 2001). The loss of DNMT1, however, had no effect on the proliferative potential of mouse ESC in culture, but the induction of differentiation in these cells also lead to cell death (Rhee *et al.*, 2002). On the other hand, DNMT1 overexpression induces a progressive DNA hypermethylation associated with transcriptional inactivation of several imprinting genes, leading to embryonic lethality (Biniszkiewicz *et al*., 2002).

It is not surprising that the set of distinct epigenetic features and changes at the transcript levels observed in cloned bovine blastocysts (Wrenzycki *et al*., 2001) should cause a significant effect in the expression of a variety of imprinting and nonimprinting genes after cloning (Wrenzycki *et al*., 2001; Bertolini *et al*., 2002, 2004). Thus, an inadequate activation or inactivation of important developmentally important genes can predispose animals to different degrees of abnormalities after cloning by SCNT, depending on the interaction between qualitative and quantitative discrepancies, subsequent to the level or degree of genome reprogramming obtained after embryo reconstruction (Bertolini *et al*., 2012). For instance, cells with changes in imprinted genes may be directed to the trophectoderm of cloned embryos, potentially generating defective fetal membranes, as frequently observed after cloning (Wei *et al.*, 2011).

The modified genome configuration of cloned embryos by aberrant DNA methylation patterns may be closely associated with reprogramming failures, altered gene expression profiles, and abnormal embryo development. In cattle, abnormal DNA methylation patterns have been linked to abnormalities after cloning, which in turn can be phenotypically expressed by fetal and placental disorders, increased pre-natal losses and lower postnatal survival (Bertolini and Anderson, 2002; Han *et al*., 2003; Salilew-Wondim *et al*., 2013). However, a certain degree of variation in gene expression pattern after cloning, generally attributed to epigenetic features transmitted by the donor cell nucleus, is totally acceptable and compatible with subsequent normal growth, development and survival after birth (Humpherys *et al*., 2001; Wells *et al*., 2003).

Manipulation of the epigenetic profile in the somatic cell or the early embryo: there is no silver bullet

Epigenetic therapy

Currently, the manipulation of cellular epigenetic profiles, such as DNA methylation levels or histone modifications, has been applied in epigenetic therapy (Daura-Oller *et al*., 2009). Since the birth of Dolly, the scientific community and the commercial users have been coping with the low efficiency observed after cloning by SCNT, along with the appearance of abnormalities in the course of development, usually referred as the Abnormal Offspring Syndrome (AOS). As abnormal gene expression patterns appear to be a consequence of faulty epigenetic reprogramming after cloning, several potential strategies for improving cloning outcome have been endeavored for use in gametes, somatic cells and/or early developing embryos of several animal especies, as illustrated in Fig. 5 and Tables 1 and 2. Efforts have been dedicated to modulate key cellular events to mold or mimic physiological processes to obtain more suitable cytoplasts and karyoplasts for epigenetic reprogramming, chromatin remodeling, and related biological processes aiming to support proper development pre- and post-natal development after cloning by SCNT. Such attempts to modify epigenetic features of somatic cells or embryos, either at specific targets (DNA methylation, histone modifications, X chromosome inactivation, Table 1) or for broader alternative epigenetic modifications and/or on processes to enhance embryo activation and development (Table 2) have shown variable results. The basis for the most applied or promising strategies for the epigenetic modulation of mammalian cell nuclei is discussed below.

DNA demethylating strategies

In oncology, many tumor suppressor genes are silenced by DNA methylation and several chemotherapeutic treatments have been developed to reduce genome methylation in an attempt at re-expressing suppressor genes. Thus, to reduce the DNA methylation levels in cancer cells, demethylating agents, such as the cytidine analogues 5'-aza-cytidine (5-Aza-C) or 5'-aza-2'-deoxycytidine (5-Aza-dC), have been used in chemotherapy to inhibit DNA methylation by DNMTs, as the cytidine has a nitrogen at the position 5, instead of a carbon. However, to work, such agents must be incorporated into the genome, which can cause mutations in daughter cells. Some of such strategies using 5-Aza-C and 5-Aza-dC have also been attempted for cloning, with wide-ranging results. Generally, an adverse effect on embryo development is seen after cloning due to exposure of donor cells or embryos to relatively high doses of the cytidine analogues for rather long periods. Therefore, even if used with some success as chemotherapeutic agents in oncology, the demethylating chemicals above are unstable and may

promote toxic and irreversible long-term effects. These treatments may adversely affect the period of chromatin remodeling of the embryonic genome, which includes the remethylation after the 8-cell stage in cattle (Dean *et al*., 2003). Other demethylating agents, such as adenosyl homocysteine (SAHA) and zebularine, more stable and less toxic DNMT inhibitors than the previous (Yoo *et al*., 2004), in addition to other demethylating strategies, including the RNAi-induced depletion of DNMT1 or the overexpression of cytidine deaminase, have been also applied to cells and embryos with promising results.

Increasing histone acetylation

When acetylation occurs in the histone structure, it becomes less compact, being more permissive to DNA transcription. The use of some chemical agents have been rather successful to increase histone acetylation, mainly by reduce the effects of deacetylase enzymes, aiming at improving cloning efficiency (Table 1). Trichostatin A (TSA) was demonstrated as a potent histone deacetylase inhibitor (HDACi), with greater specificity than other reagents previously used (Yoshida *et al*., 1990). Since then, TSA has been used to enhance embryo production efficiency by SCNT, as its use increases the amount of acetylated histones and was also related to a decreased histone methylation pattern. Scriptaid is a HDACi structurally similar to TSA, although it is considered more effective and less toxic (Su *et al*., 2000). Novel HDACi, such as oxamflatin, has been also used in pig embryos, but more studies are needed to verify its efficacy after cloning (Hou *et al.*, 2014).

Histone demethylating strategies.

Histone 3 methylation of lysines at positions 9 (H3K9) and 27 (H3K27) is associated with transcriptional repression, with H3K9me3 being highly correlated with constitutive heterochromatin, which is the condensed, transcriptionally inactive state of chromatin. Recently, histone demethylating modulators, such as BIX-01294 or histone methylases, have been successfully used to increase the overall SCNT cloning efficiency. BIX-01294 inhibits the G9A histone lysine methyltransferase (HMTase), playing a role in H3K9 methylation, regulating gene expression and chromatin organization (Kubicek *et al*., 2007).

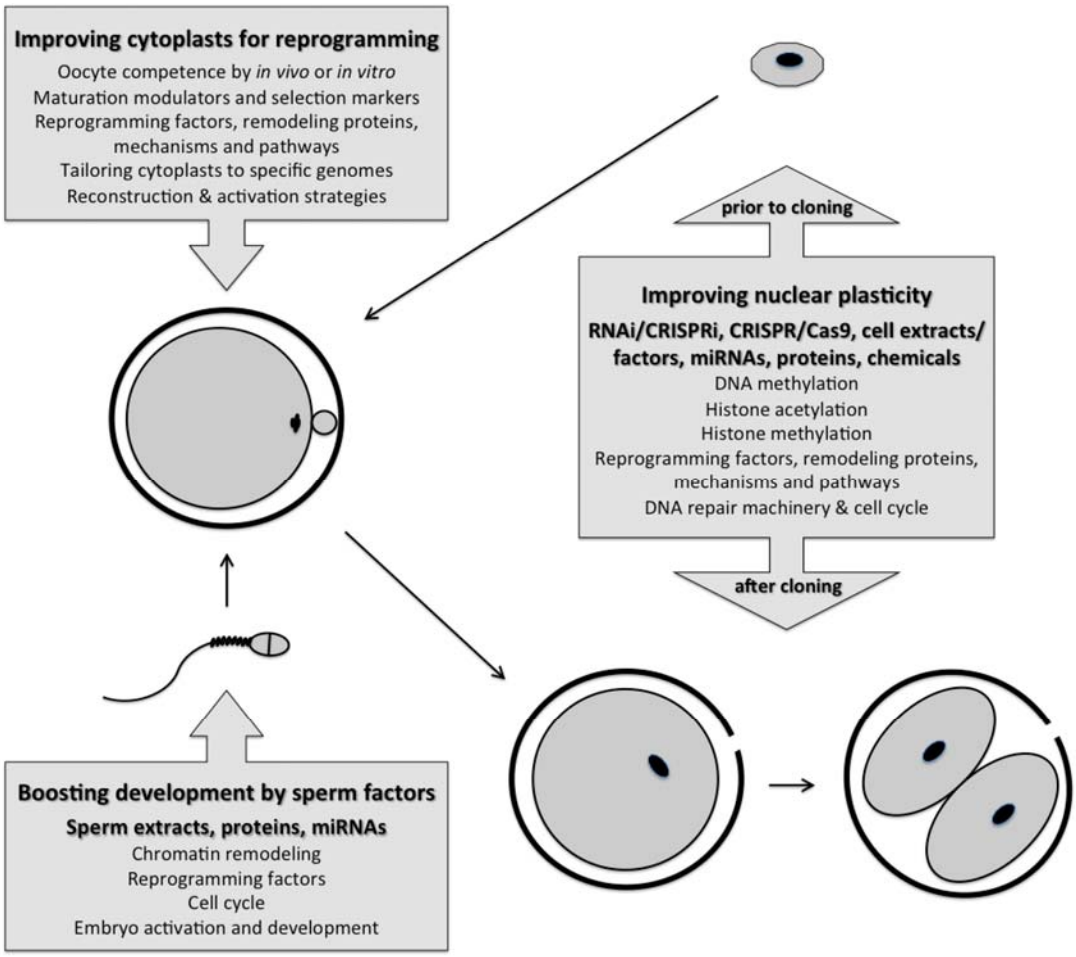

Figure 5. Potential strategies for improving the cloning outcome by the modulation of cellular events in gametes, somatic cells and/or early developing embryos for tailoring more suitable cytoplasts and karyoplasts for epigenetic reprogramming, chromatin remodeling, and related biological processes to support proper development pre- and post-natal development after cloning by SCNT.

Table 1. Reprogramming strategies to target specific epigenetic modifications in somatic cells or early embryos for cloning by SCNT.

Epigenetic target	Reprogramming strategy	Species and cell type	Outcome	References
DNA demethylation	DNMTi - Cytidine Analogues	Bovine somatic cells	Treated cells had decreased methylation status, but treatment affected embryo development to blastocyst stage	Enright *et al.,* 2003
		Bovine somatic cells and one-cell embryos	Use of 5-aza-dC when with TSA provided higher blastocyst rates and showed significantly lower levels of DNMT1,DNMT3b, HDAC2, and IGF2 transcripts	Wang *et al.,* 2011
		Swine somatic cells and one-cell embryos	Increased blastocyst rates and OCT4, SOX2, NANOG expression when used in fetal fibroblast cells	Huan *et al.,* 2013
		Buffalo somatic cells	Reduced global methylation and abundance of DNMT1; HDAC1 decreased when with TSA, also, the blastocyst rates were higher using cells treated with both reagents	Saini *et al.* 2014
	DNMTi - Zebularine	Yak somatic cells and one-cell embryos	Increased blastocyst rates and histone acetylation of histone 3 lysine 9 (H3K9), decreased methylation in promoter regions of OCT4 and SOX2 genes, when with Scriptaid	Xiong *et al.,* 2013
	DNMTi – SAHA	Bovine one-cell embryos	Reduced global methylation levels close to what is seen in IVF embryos when used in SCNT embryos after activation	Jafari *et al.,* 2011
		Bovine one-cell embryos	Improved development of embryos and reduced DNA global methylation, when with Scriptaid	Zhang *et al.,* 2014
	Cytidine deaminase	Bovine somatic cells	Activation-induced cytidine deaminase (AID) overexpressing cells resulted in higher blastocyst rates and expression of OCT4 and NANOG genes, while its promoter region methylation was reduced	Ao *et al.*, 2016
	DNMT1 depletion by RNAi	Bovine somatic cells	Reduced DNA global methylation in treated fibroblasts compared to control but not reached the IVF embryos level	Giraldo *et al*., 2009
		Bovine somatic cells	Stable DNMT1 fibroblast lineage was able to develop to blastocyst stage but failed to carry to term	Golding *et al*., 2011
		Bovine somatic cells	Reduced DNA global methylation, improving *in vitro* development of SCNT embryos	Yamanaka *et al*., 2011
		Bubaline one-cell embryos	High development level and lower DNMT expression and protein abundance	Selokar *et al.,* 2015
		Bovine one-cell embryos	Reduced DNA global methylation levels without compromising embryo development	Zhang *et al.,* 2015

Histone acetylation	HDACis – TSA	Bovine somatic cells	Histone H3 acetylation and blastocyst rates were increased causing morphological changes to the cells	Enright *et al.,* 2003
		Mouse somatic cells	Successful mouse cloning of outbred strain using cumulus and fibroblast cells	Kishigami *et al.*, 2006
		Swine one-cell embryos	Increased blastocyst rates independently of different cell lines	Zhang *et al.,* 2007
		Bovine somatic cells and one-cell embryos	Improved development to blastocyst; OCT4 and SOX2 expression when with 5-aza-dC; likely, TSA act synergistically with 5-aza-dC	Wang *et al*., 2011
		Bovine one-cell embryos	Cloned embryos had increased acetylation but not in pre- and post-implantation development	Sangalli *et al.,* 2012
		Swine one-cell embryos	Rescued disrupted IGF2/H19 imprinting in piglets; enhanced efficiency producing animals with fewer abnormalities.	Huan *et al.,* 2015
		Swine one-cell embryos	Increased overall cloning efficiency and increased acetylation to a pattern similar to IVF embryos	Zhao *et al.,* 2010
	HDACis - Scriptaid	Bubaline embryos	*In vitro* but not *in vivo* survival rates were achieved when Scriptaid in handmade cloned embryos	Panda *et al.,* 2012
		Swine one-cell embryos	Increased blastocyst rates of SCNT embryos derived of MII cytoplasts but not TII, showing that positive effect depends on cell cycle interactions between cytoplast and nuclear donor cells	Rissi *et al.*, 2016
	HDACis - TSA and Sciptaid	Rabbit embryos	Increased OCT4 expression and morula and blastocyst rates	Chen *et al.,* 2013
	HDACis - Oxamflatin	Swine embryos	Increased blastocyst rates with reduced HDAC activity, also downregulating DNMT1 expression	Hou *et al.,* 2014
	Valproic acid	Bovine somatic cells	Improved blastocyst rate, decreased H3K9me3 levels, enhancing reprogramming in donor cells	Selokar *et al*., 2013
Histone demethylation (H3K9me3)	Methylase	Mouse somatic cells	Inducing histone H3K9me3 demethylase ectopically expressed, and using cells with H3K9 methyltransferase depletion improved SCNT efficiency but did not reactivated the reprogramming resistant regions	Matoba *et al.*, 2014
	G9A HMTase inhibitor - BIX-01294	Swine one-cell embryos	Enhanced *in vitro* and *in vivo* development of embryos, increased OCT4, SOX2, NANOG expression and decreased histone acetylation levels of H3K9, H4K8 and H4K12	Huang *et al.,* 2016
X chromosome inactivation	Xist depletion by RNAi	Mouse one-cell embryos	Treatment efficiently corrected SCNT-specific aberrant Xist expression at the morula stage and increased survival and overall efficiency to term	Matoba *et al.*, 2011

5-aza-dC: 5'-aza-2'-deoxycytidine; HDACi: Histone deacetylase inhibitors; TSA: trichostatin A; DNMTi: DNA methyltransferase (DNMT) inhibitors; SAHA: Adenosyl homocysteine; G9 HMTase: G9 histone methyltransferase; RNAi: RNA interference.

Table 2. Examples of strategies for the treatment of mammalian somatic cells for cloning by SCNT or early embryos for broader epigenetic modifications or to enhance embryo activation and development.

Target response	Reprogramming strategy	Species and cell type	Outcome	References
Chromatin remodeling	Protaminization of somatic cell nuclei	Ovine cells expressing human protamine	Protaminized nuclei injected into enucleated oocytes efficiently underwent protamine to maternal histone exchange and developed into normal blastocysts	Iuso *et al.*, 2015
	Cell fusion	Mouse embryonic/thymic-lymphocyte hybrid cells	Demethylation of imprinting and nonimprinting genes and epigenetic modifications compared to reprogramming events during germ cell development	Tada *et al.*, 1997
		Biparental and parthenogenetic neural stem cells	Expression of pluripotency markers in fused cells and methylation of some imprinted genes compared to ESC cells	Jang *et al.*, 2016
Chromatin remodeling, epigenetic reprogramming	iPSC cell extract	Swine cell and embryos	iPSC extract affected histone modification and gene expression but did not improve blastocyst rates of SCNT embryos	No *et al.,* 2015
	Oocyte extract	Bovine somatic cells	Components of *Xenopus leavis* oocytes and egg extracts modified the nuclear lamina of bovine fibroblasts inducing a nuclear structural change contributing to reprogramming	Alberio *et al.*, 2005
		Porcine and bovine somatic cells	Induction of pluripotency markers and reactivation of OCT4 gene in fibroblasts showing a partial reprogramming to a embryonic state using *Xenopus leavis* extract	Miyamoto *et al.*, 2007
		Ovine somatic cells	*Xenopus leavis* extract treatment increased birth rates after SCNT and slightly increased lamb survival to adulthood	Rathbone *et al*., 2010
Chromatin remodeling, epigenetic reprogramming, embryo activation	Sperm extract	Equine/Bovine oocytes	Stallion sperm cytosolic extract activated equine and bovine oocytes after injection and developed to cleavage stage	Choi *et al*., 2002
		Bovine oocytes	Injection lead to activation of oocyte mechanism, including the completion of the meiotic cell cycle, pronuclear development and anti-polyspermy defense	Sutovsky *et al.,* 2003
		Equine oocytes	Sperm extract associated with 6-DMAP provided higher cleavage rates of equine SCNT embryos and higher cleavage rates with normal nucleus in equine parthenogenetic embryos	Choi *et al.,* 2004

			Injection of sperm extract into ICSI oocytes that received non-motile sperm resulted in a significant decrease in blastocyst development compared with injection of non-motile sperm alone	Choi *et al*., 2006
			Injection of sperm extract previous ionomycin treatment obtained higher blastocyst rates and groups of sperm extract injection obtained higher number of live foals, however this result was not statistically significant	Hinrichs *et al*., 2007
			Injection of sperm extract for 0,1 sec. using Femtojet obtained higher blastocyst rates in equine SCNT	Choi *et al.*, 2009
			Horse ICSI oocytes injected with lyophilized sperm diluted in sperm extract obtained high blastocysts rates	Choi *et al.,* 2011
Embryo activation	Sperm proteins	Mouse oocytes	Mouse PLCz of a single sperm activated mouse oocytes leading to blastocyst stage; the protein was identified as the trigger of an oocyte to an embryo	Saunders *et al.,* 2002
		Equine oocytes	Mouse PLCz injection was proven suitable to activate equine oocytes leading to high cleavage rates	Bedford-Guaus *et al*., 2008
		Bovine oocytes	Mouse and bovine PLCz injection caused oocyte parthenogenetic activation	Ross *et al.,* 2008
		Mouse oocytes	Human PLCz triggered activation of mouse oocytes leading to blastocyst stage when used in a specific range	Yu *et al.,* 2008
		Equine oocytes	Mouse PLCz cRNA injection did not increase SCNT blastocyst development when compared to sperm extract	Choi *et al*., 2009

iPSC: induced pluripotent stem cells; ICSI: intracytoplasmic sperm injection; PLCz: phospholipase C zeta.

Alternative strategies.

The use of cell-free systems allows the manipulation of cell programs, by the exchange of nuclear factors between distinct cell types. Fractionated cell homogenates (cell-free extracts) can maintain a biological function and are an important tool for the study of cellular and molecular processes (Alberts *et al.*, 2002), providing a valuable means for examining biological mechanisms associated with nuclear and chromatin remodeling (Kanka, 2003). In fact, the ability of somatic cell extracts to modulate epigenetic reprogramming and chromatin remodeling, activating gene expression in differentiated somatic nuclei have been shown for years. Using this technology, Hakelien *et al.* (2002) and Landsverk *et al.* (2002) developed a somatic-cell-free system (stimulated human T cell extracts) that remodeled chromatin (hyperacetylation of a specific repressed gene promoter) and activated the expression of repressed genes in heterologous differentiated nuclei (293T fibroblasts and resting human T cell nuclei). Such studies paved the way for further studies on strategies using cell fusion, cell extracts or specific cell factors for the treatment of somatic cells or early embryos for broader-spectrum epigenetic modifications or to enhance embryo activation and development, as illustrated in Table 2. Overall, such systems also have variable results, lacking simplicity and effectivity. However, the identification of factors with biological functions usually become natural candidate molecules to target specific epigenetic modifications in somatic cells or early embryos for cloning by SCNT.

The present and the future: RNA-guided epigenetic regulators

RNA interference (RNAi)

The process of RNAi is an important post-transcriptional silencing mechanism, conserved in mammalian cells, oocytes and embryos, also significant for the maintenance of genome stability and early development (Svoboda *et al*., 2004). The RNAi technology has been effectively used for specific mRNA depletion from both mother and zygotic source (Svoboda *et al*., 2004), providing an efficient approach to induce a transient downregulation of developmentally important molecules, such as DNMTs, either in somatic cells or embryos, without inducing residual effects on the subsequent development (Table 1). Since this technology is potentially less toxic and without prolonged direct residual effect, RNAi can be used as an alternative to the chemical processes used to modulate embryonic reprogramming.

DNA editing and DNA regulation tools

Programmable nucleases, such as Zinc Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/ CRISPR-associated protein (Cas9), have been developed for effective gene editing, and have been broadly used in mammalian organisms (Gaj *et al.*, 2013; Doudna and Charpentier, 2014; Kim and Kim, 2014). The CRISPR/Cas9 system is the most powerful technology and the main gene editor available, due to its versatility and design simplicity (Whitelaw *et al.*, 2016). Yang (2015) has recently demonstrated the power of such tool by performing a one-shot genome-wide inactivation of 62 porcine endogenous retroviruses (PERVs) using a multiplex system. In such way, cells or embryos can be genetically modified either by site-directed mutations or by gene insertions, generating transgenic founders potentially at a greater efficiency than using traditional methods. In fact, generation of specific double strand breaks (DSBs) by CRISPR/Cas9 has been exploited to modify the genome of livestock species, either through the non-homologous end joining (NHEJ) repair machinery, mostly as gene repressor to disrupt functional alleles (Hai *et al.*, 2014; Ni *et al.*, 2014; Crispo *et al.*, 2015; Wang *et al.*, 2015, Bevacqua *et al.*, 2016), or through the homologous recombination (HR) pathway, for precise gene insertion (Ruan *et al.*, 2015; Jeong *et al.*, 2016; Wu *et al.*, 2016). One example of the usefulness of the CRISPR/Cas9 system in epigenetic reprogramming out of a multitude of possibilities is the knockout of genes coding for histone methyltransferases associated with repressive states of the chromatin or heterochromatin, which will favor gene expression. However, in addition to the gene-editing role, the system CRISPR/Cas9 can also be used as epigenetic repressor or activator without editing the genome, which promises to become the Holy Grail in epigenetic reprogramming of cells, tissues or whole organisms in the near future.

CRISPR interference (CRISPRi)

Recently, the CRISPR/Cas9 system has gained a new version, the nuclease deactivated Cas9 (dCas9), that blocks transcription in prokaryotic and eukaryotic cells, known as CRISPR interference (CRISPRi), which can be used for the purpose of gene regulation instead of genome editing. As this catalytically dead Cas9 lacks endonuclease activity, it can be used to knock down multiple target genes simultaneously, with reversible effects (Qi *et al.*, 2013). When coexpressed with a guide RNA, CRISPR/dCas9 generates a DNA recognition complex that can specifically interfere with transcriptional elongation, RNA polymerase binding, or transcription factor binding. This new system presents some advantages over the use of RNAi, mainly because the latter causes incomplete silencing of the desired gene (Jackson *et al.*, 2003, Krueger *et al*., 2007), becoming an excellent alternative to transcriptionally repress genes, moving toward the study of different metabolic pathways in cells and embryos of different mammalian species.

CRISPR activator (CRISPRa)

In addition to the CRISPRi, the CRISPR/Cas9

system can be tailored to epigenetically modify the chromatin at specific target sites in the genome, activating silenced genes (CRISPR activating system, or CRISPRa). Such application can benefit the treatment of human epigenetic diseases, such as the Angelman Syndrome, in which a functional parental allele is silenced due to genomic imprinting, whereas the other allele is mutant. In such cases, it is possible to use the CRISPR/Cas9 system as an epigenetic activator of silenced genes, allowing expression of an intact copy of a missing gene (Vora *et al*., 2016). For instance, Hilton *et al.* (2015) fused Cas9 to the core catalytic domain of the histone acetyltransferase (HAT) of the human E1A-associated protein p300, which allowed the acetylation of the lysine residue at position 27 of Histone 3 (H3K27), activating gene expression. Such tool will soon be also available for other epigenetic applications, as for instance, the use of fused Cas9-demethylases to perform precise and specific DNA demethylation in the genome.

Final considerations and perspectives

In summary, no silver bullet is available for the precise and complete epigenetic reprogramming of cell nuclei for any given purpose or application. Also, a solid relationship between *in vitro* treatments and *in vivo* efficiency is yet to be established (Sangalli *et al*., 2012; Kim *et al*., 2015). Moreover, no consensus exists on the usefulness of such epigenetic modulating systems mainly due to differences between species, cell types, doses, combinations, and treatment times between studies, among biological and technical factors, which makes such strategies of limited application.

It is apparent that we are just beginning to understand the epigenetic mechanisms in cell biology, which is the basis for improving genome reprogramming and cloning success. We are also only scratching the surface for the effective and widespread use of novel technologies, especially the RNA-guided epigenetic modulators. Irrespective of the cloning outcome, and due to the low efficiency of the process *per se*, progress in the field depends and will rely on the development of effective ways to reversibly modulate the epigenetic marks in the donor nucleus by inducing more permissive chromatin states suitable for reprogramming and/or to improve the reprogramming internal milieu in cytoplasts prior and after cloning.

As mankind aquires more knowledge into epigenetics, and as procedures to manipulate gametes, cells and embryos evolve to mimic nature into more homeostatic and homeorhetic physiologic processes, all fields in biology and medicine will advance towards the resolution of problems related to epigenetic programming and inheritance, minimizing random chance, thus improving the odds ratio for successful cloning.

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