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Achievements and perspectives in cloned and transgenic cattle production by nuclear transfer: influence of cell type, epigenetic status and new technology

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Abstract

Genetically modified cattle production is motivated by many factors, including recombinant protein production for therapeutic purposes, disease models and animals presenting improved production traits. Nuclear transfer (NT), combined with efficient cultivation methods, genetic modification and donor cell selection is important for transgenic cattle production. Studies have found that adult cells (such as fibroblasts and *cumulus* cells, among others) used as nuclear donors achieved results similar to those of fetal cells, with the advantages of easier collection and a known genotype/phenotype. However, no consensus has been reached on the influence of cell type on transgene expression levels and post-reprogramming capacity after nuclear transfer, and these factors appear to be related to epigenetic factors. The development of new strategies, such as chromatin-modifying agents (CMAs), *in vitro* generation of induced pluripotent cells (iPS cells) and precise genome editing techniques are being explored and may influence nuclear reprogramming success for efficiently producing genetically modified bovine clones.

Keywords: bovine, cloning, epigenetic, nuclear reprogramming, transgenesis.

Introduction

Commercial and scientific interest in transgenic animal production has grown worldwide. This is due to the possibility of using animals to produce recombinant proteins for therapeutic purposes (An *et al*., 2012), develop *in vivo* gene function models to study organogenesis and development (Berg *et al*., 2011), understand important diseases and produce animals with improved production characteristics (Wu *et al*., 2015).

DNA pronuclear injection was the first and most common method for producing transgenic animal for years, but low transgenesis rates limited its use. Murakami *et al*. (1999) reported that only 2.9% of bovine embryos produced after DNA pronuclear injections were genetically modified, and 70.3% of those were chimeras. This is due to the random insertion of exogenous DNA into the host cell genome (Murakami *et al*., 1999).

Establishing nuclear transfer (NT) (Wilmut *et al*., 1997) in conjunction with cell culturing and efficient genetic modification methods provides new strategies for producing transgenic livestock due to the possibility of selecting cells prior their use as a nucleus donor, ensuring a transgenesis efficiency of near 100%.

Initially, nuclear reprogramming by NT was reported possible using embryonic cells (blastomeres) as nuclear donors for both laboratory and farm animals (Illmensee and Hoppe, 1981; Willadsen, 1986), and it was believed that pluripotent cells were responsible for nuclear reprogramming success and proper embryo development (McGrath and Solter, 1983; Prather *et al*., 1987). This technique limited livestock production because deriving and maintaining embryonic stem cell (ES) cultures *in vitro* is not possible as pluripotent cells cultured *in vitro* are not yet fully characterized or reproducible in domestic animals (Brevini *et al*., 2008; Nowak-Imialek *et al*., 2011). The birth of the first farm animal from nuclear transfer using *in vitro* cultured fetal cell lines was reported in 1996 (Campbell *et al*., 1996), and the birth of Dolly "the sheep" (Wilmut *et al*., 1997) revolutionized the reprogramming concept at that time by demonstrating that differentiated adult somatic cells could be nuclear donors to produce cloned animals.

A short time after Dolly's achievement, Schnieke *et al*. 1997, demonstrated the first use of NT to produce the first transgenic animal. They produced a transgenic sheep that secreted factor IX in its milk (Schnieke *et al*., 1997). Thereafter, other cell types have been used to clone and produce transgenic embryos with different transgene incorporation efficiency rates (Arat *et al*., 2001; Feng *et al*., 2015; Gong *et al*., 2004).

Recently developed precise techniques for genome editing have enhanced the safety and efficiency of producing transgenic farm animals desirable in both agriculture and biomedicine (Petersen and Niemann, 2015; Rémy *et al*., 2010). TALENs (transcription activator-like effector nucleases) technology has allowed genetic modification to become more precise and less time-consuming. More recently, CRISPR technology revolutionized the animal transgenic field, bringing several advantages such as more precise targeting, fewer off-targets, and faster technology (Carlson *et al*., 2012; Menchaca *et al*., 2016; Lotti *et al*., 2017). It is important to highlight, however, that efficient TALEN or CRISPR technology protocols for producing transgenic farm animals rely on NT

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procedures to assure high efficiency in terms of transgenic offspring birth. Therefore, evaluating the cell phenotype for the precise genetic modification to be performed remains a concern.

Although NT uses different cells types, the influence of the cellular differentiation stage on both cloning and transgene integration efficiency are of interest. This relationship is not well-defined, and more studies are needed to fully understand it; however, it is clear that epigenetic factors are involved, affecting primarily post-nuclear transfer reprogramming efficiency (Smith *et al*., 2012, 2015).

This review (i) presents a brief history of the cell types used in cloning and transgenic cattle production, (ii) addresses the epigenetic issues that may affect transgenic and cloning cattle production efficiency and (iii) describes current strategies, such as chromatin-modifying agents (CMA), iPS cells and endonucleases as means to innovate and improve results.

Use of somatic cells by nuclear transfer in transgenic cattle production

The term "transgenic" refers to an organism whose genome was permanently altered by insertion, modification or inactivation of DNA, with the genetic modification being transmitted to its offspring (Rülicke *et al*., 2007). In livestock production, transgenic animals have been developed primarily for use as bioreactors to produce high quality medicinal proteins on a large scale, with lower costs and higher efficiency compared to other production methods such as bacterial, yeast and cells culturing (Houdebine, 2009; Bagle *et al*., 2012).

NT is the most common method for transgenic cattle production (Jeong *et al*., 2016; Lu *et al*., 2016; Ren *et al*., 2017; Gao *et al*., 2017;). This is primarily due to the possibility of identifying the transgene in the cell genome before using it as a nuclear donor, which may avoid chimera production, genetic anomalies in the offspring, homogenous offspring production or an unintentional knock-out due to transgene localization in the non-coding DNA (Bressan *et al*., 2008, 2011).

The NT technique fuses one diploid cell (embryonic, fetal or adult), with an enucleated oocyte (recipient cytoplast) and is chemically activated to trigger embryo development. Thus, the recipient oocyte must induce nuclear reprogramming and support embryonic development, while the donor cell nucleus must be totipotent (Wilmut *et al*., 2015).

Many cell types and culture and manipulation conditions (presence or absence of bovine fetal serum, cell passage number, oxygen tension and others) have been studied for their effects on transgene expression levels in the donor cell nucleus and the cloned calves (Cho *et al*., 2004; Gong *et al*., 2004; He *et al*., 2016). As previously discussed, initial studies used embryonic stem cells from embryos in the morula or blastocyst stage for their ability to generate any embryonic tissue (Puri and Nagy, 2012). In mice, embryonic stem cells efficiently produce genetically modified individuals, but their use in producing transgenic farm animals is challenging as was previously described regarding ES cells from domestic animals (Blomberg and Telugu, 2012; Gandolfi *et al*., 2012). A brief summary of important NT achievements is shown in Figure 1.

After the NT of a differentiated adult cell (mammary gland cell) to an enucleated oocyte, the birth of Dolly the sheep in 1996 confirmed speculation that even after having reached a certain stage of differentiation, differentiated somatic cells could be reprogrammed if aided by a cytoplasmic apparatus (Wilmut *et al*., 1997).

Therefore, the possibility of using others cell types was investigated for producing both clones and transgenic cattle by NT. In 1998, Kato *et al*., reported the birth of eight heifers produced by nuclear transfer using cumulus cells and oviduct epithelial cells from an adult animal (Kato *et al*., 1998). In 2000, the same group compared clone production efficiency using adult female cells (cumulus, oviduct and uterine cells) and ear and skin cells from neonates and adult male cattle. These authors observed that clones from adult cells often died in the final stages of gestation, and those that survived often had abnormalities (Kato *et al*., 2000). The authors attributed these findings to donor cell mutations and telomere shortening, factors that could occur in aging animals. Today, it is known that these modifications are also caused by epigenetic failures in donor cell reprogramming (Yang *et al*., 2007; Song *et al*., 2014).

Arat *et al*. (2001) demonstrated the first use of adult cells (granulosa cells) for transgenic embryo production in cattle. Cho *et al*. (2004) reported that the embryo development rate post nuclear transfer, as assessed by green fluorescent protein (GFP) expression, was greater when smaller cumulus cells from early passages were used compared to adult and fetal fibroblasts. According to the authors, using small cumulus cells allows for better nucleus-cytoplasmic interaction in the recipient cytoplast. Moreover, when NT is performed with metaphase II oocytes, synchronized cells in the G0/G1 phase of the cell cycle (smaller cells) are more effective in embryonic development (Campbell, 1999). Concerning passage number, prolonged culturing periods can potentially result in genetic alterations. Genetic alteration is a major factor in cloned and transgenic production. It is not fully understood; however, interestingly, embryos resulting from NT failed to express some genes related to nuclear reprogramming (e.g., *Oct-4* gene) and placentation (Yang *et al*., 2007).

Higher production rates of transgenic bovine blastocysts were also obtained using cumulus (44.6%) and oviduct fetal epithelial cells (49.1%) than with fetal fibroblasts (32.7%) (Gong *et al*., 2004). This indicates that adult cells can efficiently produce transgenic cattle. In some situations, adult cells may be more suitable than embryonic or fetal cells due to easier collection and knowing the genotype/phenotype.

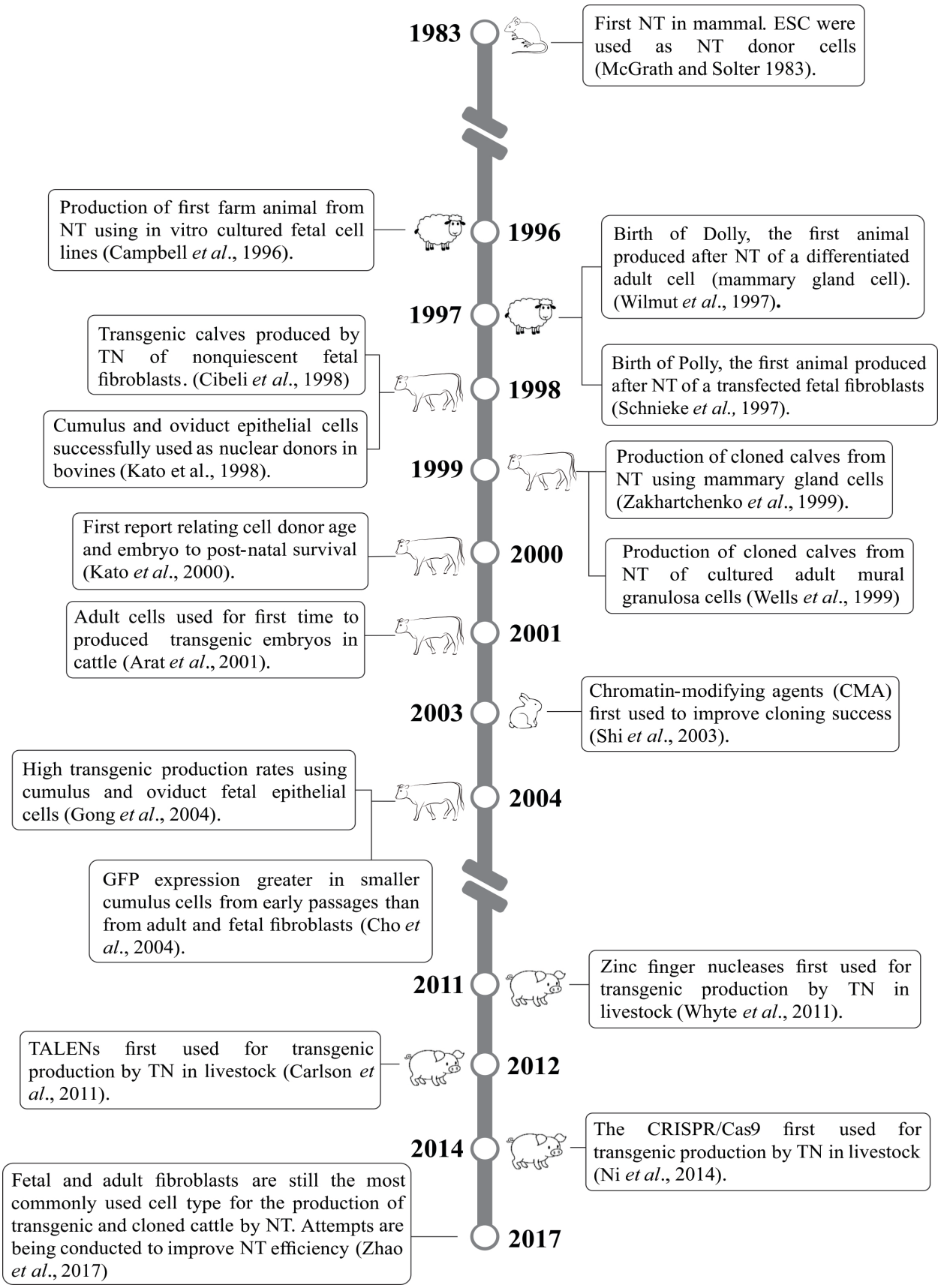

Figure 1. Timetable of a brief summary of important NT achievements.

In 2006, Sung *et al*. tested hematopoietic mouse cells at different differentiation stages (hematopoietic stem cells (HSC), progenitor cells and granulocytes) and found that cloning efficiency increases with the donor cell's differentiation state, and granulocytes were the best cell type for cloning. The authors concluded that hematopoietic cells appeared to be an exception to the hypothesis that "undifferentiated cells are more efficient than differentiated somatic cells for cloning production". Inoue *et al*. (2006) demonstrated that cloned embryos derived from HSC failed to express five of the six important embryonic genes examined, including *Hdac1* (encodes histone deacetylase 1), a key zygotic gene activation regulator. These results were attributed to less plasticity of hematopoietic stem cells (Inoue *et al*., 2006).

Mammary gland epithelial cell use in transgenic animal production has grown due to its applicability (Zhan *et al*., 2017). Regarding recombinant protein production, Feng *et al*. (2015) used mammary gland cells to produce transgenic sheep expressing the alpha-lactoferrin gene. After transfection with plasmid encoding alpha-lactoferrin, cells secreted recombinant protein at detectable levels in the culture media. Similarly, our group reported generating live offspring after donor cell NT from developing mammary glands in cattle. We described constructing specific vectors to encode the B-casein promoter and the hFIX gene, as well as integrating them into cattle donor cells and generating offspring (Monzani *et al*., 2011, 2013). This improvement represents another effective method for selecting cells prior to their use as nuclear donors and analyzing tissue-specific promoter activity.

Fetal and adult fibroblasts remain the most common cell type for producing transgenic cattle by NT. This may be due to the ease of collection and *in vitro* culturing and that the cells divide several times before reaching senescence. In theory, different cell types could be used to produce clones and transgenic animals by NT with different success rates, but nuclear reprogramming efficiency appears to be related to donor cell nucleus plasticity and the capacity to undergo nuclear programming before NT. The role of the nucleus donor cell's epigenetic status on reprogramming efficiency is thus gaining interest, and this relationship will be further discussed below.

Epigenetic factors related to animal production

Epigenetic modifications are heritable changes in DNA structure and organization that are unrelated to changes in the nucleotide base sequence. The term was first proposed to explain how cells carrying identical DNA could express different genes (García *et al*., 2012).

This concept is now well-recognized, and these modifications occur through changes in the chromatin that alter gene transcriptional activity; however, the effect of these alterations remains unclear. Herein we will focus on epigenetic modifications that primarily occur by two distinct mechanisms: (i) histone modification and (ii) cytosine methylation in CpG islands in double-stranded DNA (Cheng and Blumenthal, 2010; Reik *et al*., 2001). Changes in the chromatin, such as methylation and histone modifications, are highly correlated and alter gene transcriptional activity by leaving chromatin exposed or protected from transcription machinery (Cheng and Blumenthal, 2010).

DNA is a long molecule that must be packaged for nuclear containment. Thus, the chromatin is wrapped around a histone octamer, constituting the nucleosome, the DNA's basic organizational unit. Histones are proteins with a carboxy-terminal domain and amino-terminal tail where epigenetic modifications occur. The main histone modifications include methylation of lysine and arginine residues, acetylation of lysine residues, and ubiquitination, sumoylation and phosphorylation of serine and threonine residues. Of these, lysine residue acetylation and methylation are the most well-known and studied (García *et al*., 2012).

Histone acetyltransferase (HAT) and histone deacetylase (HDAC) are the enzymes that control histone acetylation levels. Histone hyperacetylation is associated with chromatin regions with high transcriptional activity, while hypoacetylation is found in low activity regions (Schmittwolf *et al*., 2005). Histone methylation may be related to non-transcribed or transcribed regions, depending on which residue is methylated. For example, histone H3 lysine residue 9 methylation (H3K9me) and histone H3 lysine residue 27 methylation (H3K27me) are related to non-transcribed regions, while histone H3 lysine residue 4 (H3K4me), 36 (H3K36me) and 79 (H3K79me) methylation are related to transcribed regions. These events are controlled by histone methyltransferase enzyme (HMTs).

DNA CpG island methylation is a primary epigenetic mechanism that directly affects gene expression as some transcription factors only bind to unmethylated DNA sequences; thus, methylation may induce gene sequence expression loss (Salozhin *et al*., 2005). Methyltransferases are enzymes that control DNA methylation levels. DNA (cytosine-5)-methyltransferase 1 (DNMT1) maintains methylation levels during DNA replication. DNMT2 is related to RNA methylation to enhance tRNA stability, and DNMT3A and DNMT3B are responsible for *de novo* methylation (Iager *et al*., 2008).

Proper DNA methylation reprogramming is essential during gametogenesis and early embryogenesis to generate healthy offspring; therefore, its disruption or incomplete reprogramming may directly affect other processes such as NT. Male and female gametes have a hypermethylated genome due to gametogenesis, and after fertilization, the pro-nuclei undergo a process called "demethylation". In the male pro-nucleus, this is a fast and active process, whereas in females, it occurs slowly and progressively (Yang *et al*., 2007). At the 8-16 cell stage of concurrent embryonic genome activation, epigenetic parameter reprogramming occurs, known as *de novo* methylation. These processes are usually coordinated, culminating in successful embryo implantation and embryonic and fetal development. During early embryonic development and cell differentiation, epigenetic markers are gradually

established, forming different heritable epigenetic patterns that are important for maintaining the identity of differentiated cells (Schmittwolf *et al*., 2005).

In nuclear transfer, nuclear reprogramming involves two complex epigenetic steps: (i) dedifferentiation of a differentiated somatic cell to a totipotent stage by epigenetic marker removal in the donor nucleus and (ii) redifferentiation of totipotent embryonic cells into all tissue types of a new animal (Yang *et al*., 2007). Such events occur quickly, whereas in naturally fertilized embryos, such epigenetic markers are established during gametogenesis and fertilization, and thus may diminish NT efficiency.

Despite current efforts and findings in this field, embryo production by NT remains low at approximately 1-5%, and embryos derived from NT typically present implantation failures, fetal abnormalities, and gestational, placentation and calving problems. Many of these abnormalities are due to nuclear reprogramming failure, caused by changes in histone parameters, DNA methylation and gene expression related to early embryogenesis and placentation (Bressan *et al*., 2009; Smith *et al*., 2015; Suzuki *et al*., 2009; Yang *et al*., 2007).

Several studies report that the more advanced the developmental stage of the nuclear donor cell, the lower the efficiency of the embryo production by NT. In mice, for example, reduced clone production occurs when blastomeres at a more advanced developmental stage are used as donor cells (22% - 2 cells, 14% - 4 cells and 8% - 8 cells). In cattle, NT blastocyst production rates were reported at 28% when blastomeres were used as nuclear donors. When fetal and adult fibroblasts were used, efficiency was significantly reduced (13% and 5%, respectively) (Wilmut *et al*., 2002).

Blelloch *et al*. (2006) demonstrated that stem cell production efficiency in mice was higher when neural stem cells were used as NT donors compared to differentiated neuronal cells. They also demonstrated that hypomethylation of differentiated cell genomes increased clone production (Blelloch *et al*., 2006).

Matoba *et al*. (2014) identified mouse reprogramming resistant regions (RRRs) that were well-expressed in 2-cell *in vitro* fertilized embryos, but not in cloned embryos. These RRRs are enriched for H3K9me3 (trimethylation of histone H3 on lysine residue 9 H3K9me3) on the donor cell genome. According to these authors, this region is a major barrier to efficient nuclear reprogramming in mice and its removal by ectopically expressed H3K9me3 demethylase, Kdm4d, significantly improved SCNT efficiency (Matoba *et al*., 2014).

Thus, more recent approaches have focused on the possibility that open chromatin configurations (already found in stem cells, embryonic and fetal cells) could benefit nuclear reprogramming (Song *et al*., 2014). Because the use of embryonic stem cells in farm animals is still incipient, new strategies have been developed to optimize clones and transgenic animals produced by NT. Among these strategies, using chromatin-modifying agents (CMAs) in donor cells prior to NT, as well as the possibility for using non-embryonic but pluripotent stem cells (the induced pluripotent stem cells or iPS cells) as donor cells have shown significant potential and are further discussed herein.

Modeling cellular epigenetic status for nuclear transfer

Due to growing evidence of epigenetic reprogramming failures in NT embryos, chromatin-modifying agents (CMAs) are being studied in several species such as cattle, sheep, buffalo, mice, swine and rabbits (Cervera *et al*., 2009; Hu *et al*., 2012; Iager *et al*., 2008; Kishigami *et al*., 2006; Luo *et al*., 2013; Shi *et al*., 2008). CMAs are chemical substances that change cellular epigenetic patterns. Trichostatin A (Kishigami *et al*., 2006), sodium butyrate (NaB) (Shi *et al*., 2003), m-carboxycinnamic acid bishydroxamide (CBHA) (Song *et al*., 2014), Scriptaid (Wang *et al*., 2011) and valproic acid (Xu *et al*., 2012) are examples of substances used for NT procedures, as shown in Figure 2.

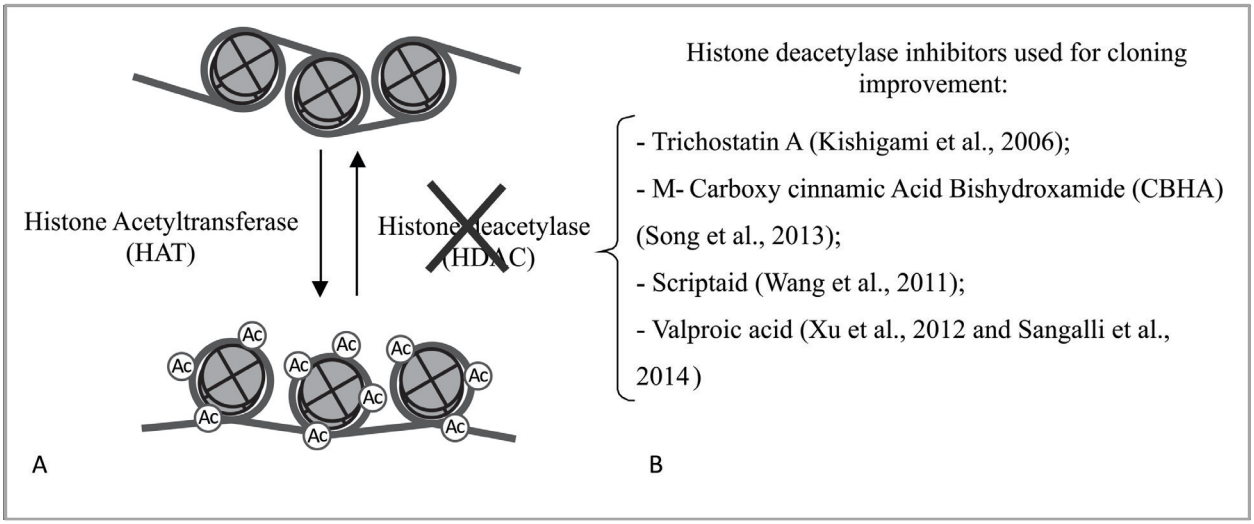

Figure 2. (A) Schematic drawing of histone acetyltransferase and histone deacetylase activity. Note that using histone deacetylase inhibitors may improve histone acetylation levels, which could increase chromatin exposure to transcription machinery. (B) Examples of histone deacetylase inhibitors.

In cattle, use of trichostatin A (TSA), a histone deacetylase inhibitor, led to production of 8-cell cloned bovine embryos with histone H4 acetylation levels at lysine 5 (H4K5ac) similar to their fertilized counterparts and significantly greater than the control group. This treatment has also improved *in vitro* embryo production, reaching levels close to those of *in vitro* fertilization (IVF) embryos (Iager *et al*., 2008). According to Kong *et al*. (2014), TSA also increases telomere length and may be a mechanism by which this substance improves cloned embryo development (Kong *et al*., 2014).

In 2003, Shi *et al*. analyzed the effect of treating nucleus donor cells with different chromatin-modifying agents: Trichostatin A; 5-aza-2-deoxycytidine (Aza-C, DNA methylation inhibitor); 5-bromodeoxyuridine; and sodium butyrate (NaB) on embryo development. Interestingly, NaB, another histone deacetylase inhibitor, improved the proportion of cloned embryos that developed to blastocyst stage (59%) compared to the untreated group (26%) (Shi *et al*., 2003).

In swine, the use of CBHA increased blastocyst formation as well as the levels of histone 3 acetylation at residue 9 (H3K9ac) and residue 18 (H3K18ac) and histone 4 at residue 16 (H4K16c). It also increased development-related gene expression, such as POU5F1, CDX2 and the imprinted IGF2 gene (Song *et al*., 2014).

Valproic acid (VPA) is a fatty acid used to treat central nervous system diseases. Studies have shown it induces differentiated cell reprogramming. In miniature pigs, embryos treated with VPA showed enhanced *Oct-3/4* expression and *in vitro* development after cell nuclear transfer (Miyoshi *et al*., 2010). In bovines, Xu *et al*. (2012) tested different VPA concentrations at different time periods for activation. They found that 4 nM of VPA over 24 hours increased cleavage and blastocyst rates, reduced apoptosis in blastocysts and improved immunofluorescent signals for H3K9ac in a pattern similar to that of *in vitro* fertilized (IVF) embryos (Xu *et al*., 2012).

Using scriptaid 14 hours after activation increased NT bovine embryo production *in vitro* and immunofluorescent signals for H3K9ac, decreased fluorescent signals for H3K9m2 in all analyzed embryonic stages (two-cell, eight-cell, and blastocyst stages) and increased expression levels of two developmentally important genes, *Interferon tau (IFN-t)* and *Oct4* (Wang *et al*., 2011). A recent work also demonstrated that Scriptaid increased *in vitro* development of NT mini-pig embryos and improved acetylation levels on H3K14 and development-related gene expression *(AKT, Oct4 and PGC-1α)* (Zhang *et al*., 2017).

Interestingly, less than twenty genes are typically deregulated in these embryos, and it is important to identify which gene networks are disrupted (Beyhan *et al*., 2007). A recent microarray study used Trichostatin A to evaluate the effects of assisted epigenetic modification of NT bovine blastocyst transcriptional profiles. Despite TSA treatment (TSA-NT) and improved epigenetic reprogramming parameters, *in vitro* embryonic development yield and quality compared to the untreated control (CTR-NT) revealed few genes were differentially expressed in TSA-NT embryos (1907 = 5.1% versus CTR-NT 4.3%). According to the authors, this suggests that the imperfect expression of a few genes can result in a defective phenotype due to a ripple effect, which is not completely responsive to TSA treatment (Hosseini *et al*., 2016).

Although much has been reported on cloned embryo production, minimal and controversial data are available regarding the offspring, especially from domestic animals. Our group has studied whether CMAs targeting chromatin acetylation and DNA methylation can alter chromatin configuration to improve nuclear reprogramming and enhance offspring results. Thus, bovine fibroblasts were treated with either 5-aza-2'-deoxycytidine (AZA) plus trichostatin (TSA), hydralazine (HH) plus valproic acid (VPA) or VPA alone and used as NT donor cells. Cloned bovine zygotes were also treated with TSA to test whether the effect would be more pronounced in cells or embryos. Although live offspring were born from both the control and treated groups, no evidence that either nuclear donor cells or cloned zygotes with CMAs positively affected pre- and post-implantation development of cloned cattle (Sangalli *et al*., 2012, 2014). Thus, more studies are needed to identify potentially important deregulated bovine genes to find strategies to correct their expression and to identify possible targets of disrupted maternal recognition before the embryos are transferred to recipients.

iPS cell use for cloned animal production by nuclear transfer

iPS cells are adult cells that have been genetically induced into a pluripotent state similar to embryonic stem cell stages by introducing the specific genes, *Oct4*, *Sox2*, *KLF4 and C-MYC*, known as OSKM factors (Takahashi and Yamanaka, 2006; Takahashi *et al*., 2007). The role of other factors, such as *Nanog*, *Lin-28, TCL-1A*, has also been evaluated (Yu *et al*., 2007; Picanço-Castro *et al*., 2011).

iPS cells are a new type of pluripotent stem cell that are highly proliferative and can form different tissues. iPS cells are similar to embryonic stem cells in morphology, pluripotent gene expression, promoter methylation level, teratoma formation capacity and ability to differentiate into all cell types (Cao *et al*., 2012).

In vitro pluripotent cell production technology plays an important and unique role in animal production because “true” stem cells cannot be efficiently produced or maintained *in vitro*, as previously discussed (Muñoz *et al*., 2008; Nowak-Imialek *et al*., 2011). Conversely, iPS cells have already been reported in several species, including both domestic (e.g., cattle, dog, sheep, swine, horses) and endangered animals (e.g., felids) (Gonçalves *et al*., 2014; Koh and Piedrahita, 2014; Ezashi *et al*., 2016).

iPS cells, as well as embryonic stem cells, allow accurate genetic manipulation (Park and Telugu,

2014). In cattle, their use accelerates improvement of desirable genetic traits in livestock, which has been the aim of different research groups (Cao *et al*., 2012). Recently, studies using iPS cells as donor nuclei for NT have been reported in mice (Zhou *et al*., 2010; Liu *et al*., 2012) and swine (Cheng *et al*., 2012). Although these studies have reported that cloned embryos were generated after NT using iPS cells, no major improvement on embryo production was reported until now. Therefore, despite the advances that combining these two biotechniques may bring to the reproductive and biomedical fields, more solid and reliable studies are soon expected.

Perspectives in animal transgenesis and conclusions

Efforts are underway in the cloning and transgenesis fields, as the efficiency of the animals that are born remains low. The use of transgenic iPS cells to enhance TN efficiency is promising but has not currently been tested or validated on a large scale. The use of CMAs, however largely studied, still presents controversial results regarding its efficiency in improving offspring number and health. However, transgenesis techniques have evolved into safer and simpler techniques. Thus, endonucleases appear to have a key role in facilitating mammalian genome engineering.

Endonucleases are restriction enzymes that cleave DNA in a specific manner. Meganucleases, zinc finger nucleases (ZFNs) and transcription activator-life effector nucleases (TALENs) were the first three classes of customizable DNA-binding proteins developed, but their low-specificity limits their use (Peng *et al*., 2016).

Recently, a defense system against foreign nucleic acids, such as viruses or plasmids, initially described in prokaryotes, has been used to improve transgene efficiency (Mojica *et al*., 2000). The CRISPR (clustered regulatory interspaced short palindromic repeats) technology uses repeated short sequences interspaced by non-transcribed regions (spacers) that are closely associated with gene regions (CRISPR associated genes – Cas) that codify nucleases, polymerases and helicases, which are fundamental to the system's function (Jansen *et al*., 2002).

Therefore the CRISPR-Cas 9 system is a powerful, precise and relatively simple tool developed for gene editing, based on the bacterial CRISPR-Cas defense system (Jinek *et al*., 2012; Cong *et al*., 2013). The technique refers to RNA guides (CRISPR) that orient the endonuclease, Cas 9, to cleave the DNA sequence in a specific manner. Using CRISPR-Cas 9 makes it possible to introduce a sequence of exogenous DNA (knock-in) at a specific site to prevent deleterious effects from random integration into the genome, which is important for transgenic animal production (Hsu *et al*., 2014).

A recent study produced cattle with increased resistance to tuberculosis, by inserting natural resistance-associated macrophage protein-1 (NRAMP1), using the single Cas9 nickase (Cas9n)-mediated single-strand break (SSB) for the first time, with the potential to generate a non-homologous end-joining (NHEJ) repair pathway. This technique reduced off-target effects and both transgenic bovine fetal fibroblasts and cattle were efficiently produced (Gao *et al*., 2017). Bevacqua *et al*. (2016) reported using the CRISPR/Cas9 system to induce knockout and knock-in alleles of the bovine PRNP gene responsible for mad cow disease, both in bovine fetal fibroblasts and in IVF embryos (Bevacqua *et al*., 2016).

The CRISPR-Cas 9 system has also been used to produce knockout genes. Choi *et al*. (2015) reported disrupting chromosomally integrated exogenous eGFP genes using an RNA-guided endonuclease in bovine transgenic somatic cells. The fibroblasts were efficiently used for NT and developed in the blastocyst stage. This system may also be used for DNA labeling, regulating gene expression, RNA cleavage, gene mapping and RNA screening (Hale *et al*., 2009; Choi *et al*., 2015; Deng *et al*., 2015). Recently, Jeong *et al*. (2016) reported the first production of pharmaceutical products in cattle using the CRISPR-Cas9 system. They produced human fibroblast growth factor 2 (FGF2) transgenic fibroblasts using CRISPR-Cas9-mediated homologous recombination. According to the authors, FGF2 was secreted in culture medium and when used as a nucleus donor in NT, the blastocysts produced were also transgenic (Jeong *et al*., 2016).

In brief, precise genome editing technologies are widely used in cattle and other domestic animals to improve transgenesis (Menchaca *et al*., 2016; Niu *et al*., 2017; Wu *et al*., 2017), and most of these studies rely on TN technology to produce live offspring. It is possible that the knowledge gained through the CRISPR-Cas 9 system may soon be used in cloning production. The system should be used to identify genes that do not work well after nuclear reprogramming and to correct the problems associated with the SCNT technique.

In conclusion, (i) different cell types can theoretically be used for cloning and transgenic production with different rates of nuclear reprogramming success and transgene incorporation. (ii) Nuclear reprogramming efficiency appears to be closely related to the differentiation stage of the donor cell nucleus, and its success rate is higher when using less differentiated cells in SCNT, such as stem and fetal cells. (iii) These results are likely due to epigenetic marks, and (iv) studies on CMAs and iPS cells have shown promising results in this field. Finally, (v) the use of RNA-guided endonucleases should improve transgene expression in offspring. The knowledge gained in this field could be used to improve nuclear transfer efficiency.

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