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NUCLEAR REPROGRAMMING AND ITS EPIGENETIC CONSEQUENCES IN SOMATIC CELL NUCLEAR TRANSFER DERIVED ANIMALS

Reprogramación nuclear y sus consecuencias epigenéticas en transferencia nuclear de células somáticas en animales

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RESUMEN

La transferencia nuclear de células somáticas (SCNT) es una herramienta poderosa para entender los mecanismos que conducen la reprogramación celular, así como direccionar interronagntes fundamentales en la biología del desarrollo. Cientos de animales se han generado a través de esta tecnología, sin embargo, su uso a gran escala todavía se limita por la baja eficiencia y alteraciones en el fenotipo de recien nacidos reportados por el uso SCNT. Por lo tanto, esta revisión tiene como objetivo poner en consideración algunas de las consecuencias que a menudo se observan en los animales clonados, discutir y proponer algunas posibilidades y perspectivas para evitar estas alteraciones epigenéticas durante la reprogramación.

Palabras clave: *Clonación, bovino, transferencia nuclear, epigenética.*

ABSTRACT.

Somatic cell nuclear transfer (SCNT) is a powerful tool to understand the mechanisms driving the cellular reprogramming, as well as addressing fundamental questions on developmental biology. Hundreds of animals have already been generated through this technology, however its large-scale use is still hampered by the low efficiency and disrupted phenotypes reported in SCNT derived newborns. Therefore, this review aims to expose some of the consequences often seen in cloned animals, as well as to discuss and propose some possibilities and perspectives to avoid epigenetic failures during reprogramming.

Keywords: *Cloning, cattle, nuclear transfer, epigenetics.*

INTRODUCTION.

On a biotechnology perspective, SCNT arose as a tool to generate patient-specific stem cells lineage (Tachibana *et al.,* 2013), production of valuable proteins in the milk of transgenic animals (Monzani *et al.,* 2015), cloning of endangered species and high genetic merit animals in livestock industry (Galli *et al.,* 2014). However, almost two decades has passed since the first report of a cloned mammal birth, and SCNT efficiency remains low (Wilmut *et al.,* 2002).

Among the factors affecting SCNT efficiency, the epigenetic modification that somatic cells acquired during the cellular differentiation process are blamed to be the responsible (Pasque *et al.,* 2011). The reason is because the oocyte evolved its machinery to reprogram both its own chromatin and the spermatozoon, which possess chromatin with poor histone retention and packed with protamines (Carone *et al.,* 2014). These gamete-derived chromatin posses epigenetic marks that seems to drive the oocyte machinery to a correct reprogramming. Contrarily, somatic cells have a lot of repressive marks, such as DNA and histone methylation that maintains genes related to pluripotency silenced in these cells (Le *et al.,* 2014). Previous studies showed that cloned embryos retain the methylation patterns of somatic cells used as donor nuclei (Dean *et al.,* 2001). For this reason, approaches aiming to target the epigenetic marks using chromatin-modifying drugs (i.e DNA and histone methyltransferase/ deacetylase inhibitors) have been utilized (Kelly *et al.,* 2010). Successful results were achieved first in mouse, using the histone deacetylase inhibitor (HDACi) Trichostatin A (TSA) (Kishigami *et al.,* 2006), and later in other species such as pigs and rabbits (Zhao *et al.,* 2009; Yang *et al.,* 2007). However, the beneficial effect of this drugs improving embryonic development remains controversial, especially in bovines (Akagi *et al.,* 2013). So far our lab has tested a couple of these epigenetic modulating compounds, and we did not observe difference throughout pregnancy in cattle (Sangalli *et al.,* 2012; Sangalli *et al.,* 2014).

Since it is well established that less differentiated cells are easily reprogrammed compared with terminally ones, utilization of cells induced to pluripotency using Yamanaka's factors emerged as an alternative strategy to improve cloning efficiency (Yanamaka and Blau, 2010). A recent study produced SCNT embryos using ovine IPSs cells, but surprisingly the authors observed that these cells offer resistance to reprogramming (German *et al.,* 2015). Our lab has attempted use bovine iPS cells as nuclear donors for SCNT and we have observed similar effects (unpublished data- personal communication). It's still unclear the reasons for this low efficiency, but the difficulty to synchronize the cell cycle in pluripotent cells contribute to this observed behavior.

In this paper, we describe the consequences of SCNT in animal production, the possible ways to prevent some of the hurdles faced by SCNT and also some perspectives for its use.

Outcomes of SCNT in animal production

Several assisted reproductive technologies (ARTs) including in vitro embryo production, intracytoplasmic sperm injection (ICSI) and embryo transfer are widely being used for reproductive purposes (i.e., correction of inherited or acquired infertilities) in both veterinary and human medicine, for creation of biomedical models, and also to improve animal production through breeding strategies (Hall *et al.,* 2013).

The SCNT technology has been also considered for the derivation of stem cells from immunocompatible embryos (therapeutic cloning), however the advent of induced pluripotent stem cells generation has hindered its use (Yamanaka, 2007; Fulka *et al.,* 2013).

The improvement of animal production, therefore, is undoubtedly one of the main applications of SCNT. The Food and Drug Agency of the USA government (FDA) has already reported that food products from cloned cows, pigs or goats or from their offspring are safe and not different from naturally bred animals. The cloning of breeders on a large scale could accelerate the genetic gain of the herd (Meirelles *et al.,* 2010). Also, SCNT has reported to enhance the efficiency of transgenic animals production by ensuring the presence of the gene construct in the offspring, thus creating models of major importance in recent advances in biotechnology (Bressan *et al.,* 2011; Houdebine, 2005).

Indeed, after Dolly's birth reprogramming the somatic nuclei by SCNT has been showed reproducible in several species. The efficiency of producing healthy animals, however, has not improved as expected. The efficiency rate of healthy cloned cattle production is often less than 5% and such low survival of cloned offspring is certainly a major drawback for large-scale commercial applications for SCNT (Smith *et al.,* 2012).

Abnormalities during pre- and post-natal periods have been described worldwide in SCNT offspring (Bertolini and Anderson, 2002; Heyman *et al.,* 2002; Wells *et al.,* 2004). The early development is often the period when the majority of losses are reported, however abnormal placentation and therefore impaired fetal–maternal interaction, together with neonatal cardio-respiratory and hepatic complications, the large-offspring syndrome are also common phenotypes (Heyman *et al.,* 2002; Hill *et al.,* 1999).

High abnormalities rates during development and birth of animals derived from ARTs have been considered consequences of in vitro manipulation of gametes or early embryos, mainly because this early stage of mammalian development goes through a global change in the epigenetic regulation of the newly created embryo, i.e., the initial embryo fails to establish a typical embryonic pattern of its chromatin modifications, as discussed further in this review (Santos and Dean, 2004; Eilertsen *et al.,* 2007; Kohda, 2013).

The source of the problem: The epigenetic barrier

During embryonic development, the zygote after fertilization changes the transcriptional program for an embryonic pattern. This process is called maternal-to-embryonic transition (MET), and it is responsible for activating the pluripotent genes required for embryo development.

In bovine, the major MET occur on 8-cell stage, and pluripotent genes like NANOG, SOX2, STAT3, and OCT-4 are reactivated at this time (Jhonson *et al.,* 2006). Furthermore, more than 300 transcripts were identified at the MET, and the majority is involved in gene transcription, RNA processing, or protein biosynthesis and some are potentially involved in the maintenance of pluripotency observed in embryos (Vigneault *et al.,* 2009).

Nonetheless, differently from what happens during fertilization, the MET during SCNT has been show as critical point for nuclear reprogramming, since at this moment the epigenetic memory from the donor cell must be erased (Ng and Gurdon, 2005). This resistance to reprogramming the nuclear donor cell, that lead to an incomplete transcriptional activation, is caused, in part, by incomplete chromatin decondensation, and abnormal removal of differentiation chromatin marks (Pasque *et al.,* 2011).

Gene expression analysis comparing IVF and cloned embryos at the MET reveled an aberrant pattern of transcription. Some regular gene expression on IVF embryos, instead exhibited increase and, in some case, silencing gene expression (Susuki *et al.,* 2005; Vassena *et al.,* 2007). This abnormal activation at the MET may be explained with the persistent epigenetic memory of somatic cell nuclei (Ng and Gurdon, 2005).

The most studied mechanisms responsible to maintain this epigenetic memory are DNA methylation and histone modifications. For instance, Santos et al has shown that abnormal cloned embryos exhibited a hypermethylated pattern; both DNA and histone, suggesting this marks are refractory to the reprogramming events (Matoba *et al.,* 2014).

Recently, Matoba et al indicated that tri-methylated histone 3 lysine 9 (H3K9me3) as a critical epigenetic barrier in SCNT. They showed that important genes for development remain silenced after MET, due the H3k9me3 hypermethylation from the nuclear donor. Therefore, the authors pointed the H3K9me3 as the key to keep the epigenetic somatic memory in SCNT (Matoba *et al.,* 2014). In induced pluripotent stem cells (iPS), H3K9 methylation has also been shown as epigenetic barrier (Chen *et al.,* 2013). Recently, Mizutani and coworkers showed were capable to clone adult neuronal cells from mice using donor cell with low levels of H3K9me2, and thereafter treatment of zygotes with TSA, a histone inhibitor deacetylase (HDAC) (Mizutani *et al.,* 2015).

Several works has shown improvement on SCNT efficiency using HDACs to modifying the epigenome, mainly the acetylation of histones (Kishigami *et al.,* 2006; Akagi *et al.,* 2013). However, these results are controversial when different species are compared. The adoption of HDACs in porcine (Zhao *et al.,* 2009) and mice (Kishigami *et al.,* 2006) cloning has significantly improvement. However, in our previous results, we showed that HDACs treatment has not improved the nuclear transfer efficiency in bovine (Sangalli *et al.,* 2012).

Besides the histone modification, DNA methylation is broadly related to epigenetic failure on nuclear reprogramming (Dean *et al.,* 2001). As consequence of this failure, cloned dead calves can show abnormal gene expression in comparison to fertilization derived animals (Lin *et al.,* 2008).

Different methods can assist to reverse the DNA hypermethylation, and supposedly improve efficiency; like drugs, as 5-aza-2'-deoxycytidine, a methyltransferase inhibitor (Kumar *et al.,* 2013), or through genetic manipulation (Blelloch *et al.,* 2006). Nevertheless, low levels of DNA methylation does not ensure improvements in nuclear reprogramming (Enright *et al.,* 2003), on the contrary, essential genes, as the group of imprinted genes, has been shown hypo instead hypermethylation in cloned cattle (Smith *et al.,* 2012; Bertolini and Anderson, 2002).

Imprinted genes have their regulation by parental DNA methylation and are often deregulated on cloned cattle (Smith *et al.,* 2012). Besides, not only cloning presents this deregulation, others assisted reproductive technologies (ART) has also been associated, in both bovines (Smith *et al.,* 2015) and humans (Nelissen *et al.,* 2014). Epigenetic syndromes in humans are associated to the use of ARTs, as Beckwith-Wiedemann, Prader-Willi,

Russell-Silver, and Angelman (Amor and Halliday, 2008). As stated before, in vitro production of bovine embryos, mainly through SCNT, are also related to an epigenetic syndrome called Large Offspring Syndrome, offering a great model of study to human disorders.

The epigenetic barrier is only a part of the wide spectrum of problems that are related to cloning inefficiency. In fact, many efforts are still needed to discover and understating all the process to reprogramming the somatic nucleus to a pluripotent state efficiently.

New perspectives to improve nuclear reprograming

Despite all the tentative to improve nuclear reprogramming the epigenetic barriers are still the major pitfalls. Strategies already tested to overcome these obstacles include the use of global epigenetic modulators and the induction of pluripotency in donor cells. New perspectives to this field comprise the possibilities of inducing epigenetic reprogramming through cell-secreted vesicles or the use of tools able to promote site-specific epigenetic modifications.

One of the approaches to lead to regulated epigenetic modifications are the use of Histone Deacetylase inhibitors (HDACis), which have been widely applied in biological studies ranging from clinical oncology to epigenetics (Federation *et al.,* 2014). These compounds have also been used in studies involving stem cells and nuclear reprogramming (Huangfu *et al.,* 2008). The most extensively studied HDAC inhibitors in the context of reprogramming are trichostatin A (TSA) and valproic acid (VPA). Recently, the effects of these compounds on SCNT in cattle were addressed in order to analyze the embryo development throughout gestation. According to the results treatment with HDACis such as TSA or VPA did not improved the full-term development in cattle clones. Surprisingly, treatment with HDACis did not affect pregnancy establishment, neither the rate of fetal loss (60 to 270 day) or development to term. Also treatment did not increase the level of abnormalities when compared to control animals (Sangalli *et al.,* 2012; Sangalli *et al.,* 2014).

One alternative approach is the induction to pluripotency in somatic cells, which has been achieved through the use of pluripotency factors (OCT4, Sox2, c-Myc and Klf4), resulting in induced pluripotent stem (iPS) cells. In order to understand the effects of these pluripotency factors; iPS cells lines were generated using fetal fibroblasts from F1 hybrid (B. taurus ⇥ B. indicus). Interestingly, our results indicate that the H19 and SNRPN DMRs are hypomethylated in some iPS lines suggesting the stochastic characteristics of the nuclear reprograming events. Also, gene expression analyses revealed bi-allelic expression of H19 and decreased global expression of both H19 and IGF2 in most iPS lines. Interestingly, SNRPN transcripts were exclusively monoallelic regardless of a significant increase in global expression of SNRPN. All together these results demonstrate the need to understand the complexity of nuclear reprograming (Smith *et al.,* 2015; Bressan *et al.,* 2014).

Recently, part of the nuclear reprograming process started to be addressed using RNA-seq and bisulfite sequencing of human germ cells. Results from three different experiments demonstrated that the histone modifications play an important role and can fluctuate according to the fetal age. While H3K9me2 and H3K27me3 are depleted during PGCs reprograming, H3K9me3 appears to present a constant pattern preventing constitutive heterochromatin in PGCs (52). Curiously, the expected global demethylation did not occur since the poor repeat sequences presented persistent methylation. Most of these poor repeat sequences are young retrotransposons. Additionally, these poor repeat sequences were associated to genes involved with transgenerational diseases such as obesity-related traits, schizophrenia, and multiple sclerosis (Von Meyenn *et al.,* 2015).

Based on these results we can conclude that the complexity of nuclear reprograming is coordinated by several factors, thus suggesting the need to develop a new approach to improve nuclear program. A recent study hypothesized that cell-secreted vesicles (new signaling mechanism) containing proteins, mRNAs and non-coding RNAs could play a role modulating gametes and embryos, thus improving pregnancy rates (Barkalina *et al.,* 2015; Da Silveira *et al.,* 2015). Extracellular vesicles are present in several body fluids including semen, follicular fluid and uterine fluid and are capable of transferring bioactive molecules among reproductive cells generating a niche to nurture the gametes or embryos (Da Silveira *et al.,* 2014; Al-Dossary *et al.,* 2015; Sullivan, 2015). Furthermore, extracellular vesicles were able to improve porcine SCNT embryo production, in a co-culture system using parthenogenetic (PA) embryos in the bottom of the well with NT embryos on top separated by a filter membrane. In this experiment the co-cultured system of PA/NT embryos demonstrated to improve cleavage rate and blastocyst rate compared to NT/NT embryo system. Also, transcripts for OCT4, KLF4 and NANOG were identified in extracellular vesicles isolated from the cell culture media of PA embryos; additionally, levels of these transcripts were increased in NT embryos following the co-culture with PA embryos. Additionally was demonstrated that these extracellular vesicles could be up take by NT embryos independent of the zona pellucida presence, demonstrating that these vesicles are capable of cross this structure (Saadeldin *et al.,* 2014), opening new perspectives to improve nuclear reprograming based on the comprehension of the natural niche were cells can reprogram.

Recently, new tools able to promote gene-specific epigenetic modification arose, and open the perspective of precisely targeting epigenetic deregulations. These epigenetic editing tools are based on the use of TALE (transcription activator-like effector) systems associated with epigenetic writers (DNMTs and HACs) and erasers (TETs and HDADs); or with light-sensitive protein cryptochome 2 (Cry2) with promote light-triggered epigenetic modification. Such proof-of-principles were already demonstrated in some biological situations (Li *et al.,* 2015; Day *et al.,* 2014), however, its ability to improve reprogramming in SCNT systems is yet to be tested.

CONCLUSIONS

Thousands of animals were produced worldwide using SCNT technique, but despite an apparent success, the development to term of health animals is still hindered by the lack of proper epigenetic reprogramming. It has been extensively reported that in vitro culture systems lead to epigenetic disturbances in embryos and offspring. Cloned embryos and animals are more prone to epigenome abnormalities probably due to the persistence of epigenetic memory of somatic cells impairing the regain of pluripotency. Attempts to overcome this epigenetic barrier included the use of broad-spectrum chromatin modifiers in donor cell or reconstructed embryos or the use of donor cells previously induced to pluripotency, without any major breakthrough in cloning efficiency. New tools that allows for selective modifications of the epigenome arises as new strategies to investigate the effects of epigenetic marks on development.

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