

## Reprogramming somatic cells: pluripotency through gene induction and nuclear transfer

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### ABSTRACT

**Background:** The understanding of nuclear reprogramming pathways provides important contributions to applied and basic sciences such as the development of autologous cellular therapies for the treatment of numerous diseases, the improved efficiency of animal-based biotechnology or the generation of functional gametes *in vitro*. Strategies such as nuclear transfer and induced reprogramming have been used to induce somatic cells into an embryonic-like pluripotent state. Both techniques have been routinely performed worldwide, and live offspring have been successfully derived from them, resulting in a proof of efficacy of both techniques. Detailed studies on cellular and molecular mechanisms that mediate reprogramming, however, still require further investigation to develop practical applications in veterinary and human medicine.

**Review:** Studies on cell reprogramming, differentiation and proliferation have revealed that a core of transcription factors, as for example, OCT4, SOX2 and NANOG, act together promoting cell commitment or pluripotency. Mechanisms of induced reprogramming by pluripotency-related transcription factors forced expression or nuclear transfer seems to be mediated by the same pathways observed in fertilization, eliciting nuclear remodeling and modulating gene expression. However, abnormal chromatin conformation, often leading to disrupted imprinting and atypical gene expression patterns are frequently observed on *in vitro* reprogramming. Strategies used to facilitate nuclear remodeling, such as chromatin modifying agents, as for example, histone deacetylases inhibitors or DNA methyltransferases; or chemicals responsible for the inhibition of development-related pathways, as for example, MEK and GSK3 inhibitors, when used in the *in vitro* culture of cells or embryos, have proved to favor transcriptional regulation and improve reprogramming. Such alternatives are highly prone to enable the routine use of *in vitro* reprogramming in animal production and medical sciences, for example, by promoting the generation of functional male and female functional gametes capable of producing viable offspring. Thus, the properties, deficiencies and implications of induced reprogramming and nuclear transfer techniques in somatic cells were discussed in this review, as well as its probable outcomes.

**Conclusions:** The combination of both reprogramming techniques - induced reprogramming and nuclear transfer, may be essential to clarify the mechanisms of gene expression that are responsible for induced pluripotency. As discussed here, the mechanisms responsible for triggering the pluripotency status of a somatic cell are probably closely related to the epigenetic changes and gene expression profiles present in early development following fertilization. We report here that the nuclear transfer of SOX2 expressing donor cells resulted in similar rates of embryo production when compared to control cells. A better understanding of the contribution of each reprogramming factor used in induced reprogramming may result in the establishment of strategies aiming to enhance *in vitro* reprogramming performance. Such knowledge will contribute to *in vitro* animal production by increasing the cloning efficiency and regenerative medicine through the derivation and adequate culture of reprogrammed embryonic stem cells.

**Keywords:** bovine, pluripotency, reprogramming, nuclear transfer.

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## I. INTRODUCTION

Increased efficiency and control of nuclear reprogramming is desirable for developing therapeutics to allow the derivation of autologous stem cells in cellular therapies and for animal production because increased efficiency and control are indispensable for the success of several assisted reproductive technologies (ARTs). Several studies and reviews have attempted to elucidate the molecular mechanisms involved in cellular pluripotency. However, the exact mechanism of cellular reprogramming is not completely understood.

The process of reprogramming into an undifferentiated stage similar to the embryonic pluripotency has been performed through the transfer of a somatic nucleus into an oocyte (somatic cell nuclear transfer (SCNT, or cloning [126]). Another process that is capable of reprogramming a cell is cellular fusion. In this technique, one somatic cell is fused with an embryonic cell, which reprograms the somatic cell into a pluripotent stage similar to the embryonic pluripotent stage [22]. More recently, the direct reprogramming of the somatic cell has been achieved through a revolutionary experiment that was based on the introduction and expression of known transcription factors, which have been designated as pluripotency induction factors [112].

## II. REPROGRAMMING AND PLURIPOTENCY

In mammals, early embryonic development is characterized by the induction of totipotency, which reprograms male and female nuclei into an undifferentiated state, resulting in the formation of the zygote and the initiation of embryonic development. Consecutively, the process of embryonic development leads to the differentiation of cells into two different cell lineages, the inner cell

mass and the trophectoderm, which are visually distinguishable at the blastocyst stage. The trophoblast originates part of extra-embryonic tissues, whereas cells from inner cell mass, which are classified as pluripotent cells, exhibit the ability to differentiate into one of the three following germ layers: endoderm, mesoderm and ectoderm. These cells will give rise to more than 200 different cellular types that constitute an organism [85]. Because no genetic modifications are involved in the developmental determination and cellular differentiation, these events depend on epigenetic modifications to drive the expression of specific genes [5].

Initial experiments on the mechanisms of cell differentiation and reprogramming have revealed that pluripotent cells express a group of genes that are responsible for the dedifferentiation or pluripotency status. *OCT4* and *NANOG* were first identified as fundamental transcription factors for early embryonic development and the maintenance of stem cell pluripotency. *SOX2*, which is another transcription factor, heterodimerizes with *OCT4* and regulates several genes in pluripotent cells [12]. *OCT4*, *SOX2* and *NANOG* are essential but not unique transcription factors to regulate the pluripotent status in embryonic cells. Aside from the triad *OCT4*, *SOX2* and *NANOG*, many pluripotent factors have been identified such as *SALL4*, *DAX1*, *ESSRB*, *TBX3*, *TCL1*, *RIF1* and *NAC1* [51]. A connected network of regulatory genes is responsible for the development or maintenance of the pluripotency status of embryos in a complex and probably species-dependent manner [6]. Even in primordial germ cells, several pluripotent genes, including *OCT4*, *SOX2* and *NANOG*, are expressed in embryonic cells. The expression of these genes is reduced with the progression of gonadal cell differentiation into gametes [2].

Not only the transcription factors bind their DNA target sites, the proteins interact with each other and also with other chromatin remodeling factors and enzymes that regulate histone modifications to modulate chromatin structure and, consequently, gene expression [17,124].

Transcriptional regulation is an essential mechanism for the differentiation, dedifferentiation or maintenance of pluripotency and acts on normal or induced processes of reprogramming such as those

mediated by the fertilization of the oocyte by spermatozoa, the cytoplasm in SCNT or the fusion of somatic and stem cells and in directly induced pluripotency.

### III. REPROGRAMMING THROUGH SOMATIC CELL NUCLEAR TRANSFER

The factors that determine the ability of the oocyte cytoplasm to reprogram the somatic cell nucleus have been under investigation for a long time. The first experiment on nuclear transfer was performed over 50 years ago and demonstrated that the development of embryos or adult individuals may be obtained after the injection of cells in the embryonic stage or the injection of differentiated cells into enucleated oocytes of amphibians, respectively [14].

Notably, the reprogramming ability of the oocytes is not restricted to the embryonic nucleus. The birth of Dolly, which was the first viable mammal that was produced by SCNT, has shown that the oocyte can reprogram a highly differentiated nucleus into a pluripotent state and facilitate the development of viable animals.

The reprogramming of differentiated cells into pluripotent embryonic cells using SCNT has been successfully applied in several species, including humans [31]. Moreover, several farm, pet and laboratory animals have already been cloned, e.g., bovine, caprine, ovine, swine, equine, rabbits, mice, ferrets, camels, cats, dogs and wolves [3,53].

Although SCNT reprograms a differentiated nucleus, it remains an inefficient technique. Commonly, less than 5% of produced embryos generate healthy adult animals [19,125]. Several studies have demonstrated nuclear reprogramming deficiencies in cloned embryos [11,25] leading to problems such as placental dysfunctions, large offspring syndrome, and hepatic and respiratory complications [39,72].

Upon the formation of pluripotent cells after fertilization, chromatin that is derived from male and female gametes is independently demethylated. The paternal genome is actively demethylated, probably through the oxidation of 5-methylcytosine followed by the substitution for non-methylated cytosine [50], whereas the maternal genome is passively demethylated during first embryonic cleavages. For

normal embryonic development, these processes are followed by *de novo* methylation, which is essential for establishing embryonic patterns of gene expression, X chromosome inactivation in females and the maintenance of genomic imprinting, which is established in germinal lineages [83].

Remodeled global patterns of chromatin methylation are designated as nuclear remodeling and are incomplete in SCNT-derived embryos. The nucleus frequently fails to reestablish an embryonic pattern of chromatin modifications, resulting in abnormal expression patterns of genes that are related to early development [7]. Cloned bovine embryos show an incomplete wave of demethylation followed by a precocious *de novo* methylation [128]. Therefore, the reprogramming of global methylation in cloned embryos is different from *in vivo*-derived embryos. The methylation patterns of cloned embryos are similar to somatic cells indicating that cloned embryos undergo only a partial remodeling of the somatic genome [9]. These abnormal patterns are frequently associated with the loss of monoallelic expression of imprinted genes, thus compromising the normal development, growth and placental function of these embryos.

### IV. FACILITATING NUCLEAR REMODELING IN SCNT

Numerous factors play a role in SCNT efficiency, and the failure of the donor nucleus to promote epigenetic reprogramming seems to be a central cause of low SCNT efficiency [25,]. Because nuclear remodeling is not complete, strategies that facilitate the relaxation of chromatin may be useful to enable nuclear reprogramming. Several approaches that are mainly based on the effects of proteins and transcription factors on chromatin have been described. Such approaches include the potential transfer of chromosomes and chromatin by increasing the probability of cytoplasmic proteins that are freely exchanged from the oocyte to the introduced genetic material, reducing the amount of proteins from somatic origin. It is also highlighted the relaxation of chromatin chromatin-modifying agents (CMAs). CMAs such as histone deacetylases inhibitors (HDACi) and DNA methyltransferases have emerged as important tools to study nuclear reprogramming mechanisms, to correct epigenetic failures and

improve the production of cloned animals [48].

The inhibition of histone deacetylases in nuclei donor cells or reconstructed embryos with CMAs such as trichostatin A (TSA) or *Scriptaid* 6-(1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide) have been effective to increase the global acetylation of histones, which facilitates transcription. In addition, modifying DNA methylation using chemicals such as 5-aza-2 $\beta$ -deoxycytidine is a possible approach to facilitate reprogramming in cultured donor cells or reconstructed embryos.

Numerous studies have reported the use of CMAs in cultured donor cells before SCNT or cloned embryos after activation. Some groups have reported increased blastocyst production [1,21,59], quality and *in vivo* development [120]. Adverse effects have been observed with high doses of CMAs.

The effects of these drugs on pregnancy rates and the development to term are rare in bovine. Reasons include the extended length of gestation and the high cost of recipients, which hinder further studies that evaluate the impact of these drugs on development in bovine. To our knowledge, one unique study has described a positive effect of CMAs on the development of viable cloned calves.

Similarly, it is postulated that cloning efficiency is inversely correlated to the differentiation status of the donor cell, suggesting that an undifferentiated nucleus is more likely to be remodeled and reprogrammed [36]. SCNT procedures using embryonic cells [47] or differentiated somatic cells such as lymphocytes and fibroblasts [35] have demonstrated that the use of undifferentiated cells results in higher efficiency [40], suggesting that using a compromised nucleus reduces success rates [8,47].

Data from our laboratory has indicated that efficient cloning is required for the production of transgenic animals with high and homogeneous transgene expression and it is improved by a second round of nuclear transfer. These data corroborated earlier studies, and we speculated that this effect was caused by the use of a cell lineage that has previously been successfully reprogrammed by SCNT [32,57].

Therefore, the selection of cell populations that are, somehow, amenable to reprogramming using the ooplasm may be important to increase the cloning efficiency [105].

#### V. GENETICALLY INDUCED PLURIPOTENCY – NEW POSSIBILITIES

In 2006, directly induced dedifferentiation was established through the incorporation of known transcription factors into the mouse and human genomes. Takahashi and Yamanaka have shown that the expression of four transcription factors, *OCT3/4*, *SOX2*, *KLF4* and *C-MYC* (represented by OSKM), is sufficient for the induction of fibroblasts into pluripotency. Induced pluripotent stem cells (iPSCs) retain most of the characteristics of embryonic stem cells, including a high proliferating rate and the ability to form many tissues from the three germinal layers *in vitro* and *in vivo*.

Hundreds of studies have been published since 2006 to confirm the consistency of induced pluripotency in mice and human somatic cells and, more recently, in rats, rabbits, dogs, pigs, non-human primates, sheep and cattle through the expression of the *OSKM* factors in several combinations, in addition to *NANOG*, *LIN28* and *TCL-1A*, which are genes that belong to a connected network of embryonic pluripotency as previously described [38,44,45,64,86,108,130].

Interestingly, iPSCs have been characterized in some species such as bovine, ovine and equine, in which “true” embryonic stem cells had not been derived. The lack of defined characteristics in these species has been reported by several studies. Because these species do not show the same characteristics that are used to classify embryonic stem cells [114], the pluripotent cells are designated “stem cell-like”. After the derivation of iPSCs, seems that the main mechanisms of pluripotency in mammals use a constitutive pathway independent of peculiarities within each species.

The iPS cells are extremely promising for the study and therapeutic development for human diseases [89] because their similarities to embryonic stem cells, regarding self-renewal and the development of originating tissues from the three germ layers. However, its clinical usage is limited for several reasons, including a low reprogramming efficiency and genomic alterations arising from viral integration. The derivation of these cells must be improved to allow its therapeutic use without restrictions [65]. On the other hand, iPSCs are a unique model to elucidate the mechanisms of the genetic induction into pluripotency and

differentiation as well as cellular commitment, which is usually represented by C. H. Waddington's developmental potential model (*epigenetic landscape model*; Waddington, 1957; [43]).

The induction of pluripotency without transgene integration in human and mouse genomes has been obtained through the continuous transfection of non-integrating OSKM cDNA in the genome of the cell, e.g., utilizing non-integrative adenovirus [107] or expression plasmids and episomes [87]. Recent strategies such as mRNA and protein approaches have also been effective [62] and represent an important step toward its use in cell therapy. Nonetheless, these strategies are less efficient when compared with lentiviral transduction.

#### VI. FACILITATING INDUCED PLURIPOTENCY

The generation efficiency of cells that are completely pluripotent after genetic induction is usually lower at 0.01% to 0.1% than that related to SCNT at less than 5% (reviewed by [43]). Microarray-based gene expression analysis has revealed that the pattern of the global gene expression of iPS cells is similar to those of embryonic stem cells (ESCs) and not those from fibroblasts. However, differences between iPSCs and ESCs are evident [112,134].

SCNT has a low efficiency that is mostly credited to the failure of donor cell epigenetic reprogramming. Unsurprisingly, the epigenetic reprogramming of iPSCs is considered abnormal. Methylation abnormalities of genes such as *OCT3/4* and *NANOG* have been reported. Studies on the imprinting of these cells have shown that, similarly to cloned embryos, a significant number of lineages exhibits an abnormal expression of imprinted genes, including *H19*, *IGF2R*, *PEG3* and *MEG3* [93]. However, similar to SCNT, the ability of these cells to generate mice to term has been demonstrated.

The "partially reprogrammed" status has been reported and characterized in iPSCs. In 2009, Chan and collaborators reported that the expression of commonly used stem-cell markers as follows: alkaline phosphatase, SSEA-4, GDF3, hTERT and NANOG. These markers do not distinguish between partially and fully reprogrammed colonies. In contrast, transgene silencing, TRA-1-60, DNMT3B and REX1 expression, are found in colonies that are considered "true" (completely reprogrammed colonies [16]).

The treatment of these partially reprogrammed cells with signaling cascade inhibitors, e.g., inhibitors against extracellular signal-related kinase (ERK) and glycogen synthase kinase 3 (GSK3) signaling pathways, have enabled the conversion of partially reprogrammed colonies into fully reprogrammed cells [104,129].

Interestingly, the utilization of inhibitors and CMAs seems to favor the derivation of iPSCs in species with ESCs that have not been reported as "true" ESCs. Two interesting examples occur in rat and bovine, in which probably the same factors that support induced pluripotency may exert favorable roles in nuclear transfer by becoming collaborators in reprogramming events or by increasing the success rates. These factors, then, could enable the routine use of inhibitors and CMAs on animal production. In rats, the derivation of "true" ESCs was not reported until 2008 [15], when the use of inhibitors against the FGF receptor, MEK (MEK*i*) and GSK3 pathways (GSK3*i*, designated 3*i*) or only MEK*i* and GSK3*i* (designated 2*i*) enabled the derivation. Therefore, the use of these and other chemicals has been tested on the derivation of iPSCs from unconventional species [79].

#### VII. WHEN REPROGRAMMING CLOSES THE CYCLE – BACK TO THE GAMETES

Male and female gametes are required for the continuation and evolution of species and are the main resource for a vast number of reproductive biotechnologies. Therefore, they are largely studied and manipulated directly in *in vitro* fertilization and culture or indirectly through hormonal stimulation in infertility treatments.

The specialization of the pluripotent inner cell mass occurs during cellular differentiation at post-blastocyst embryonic stage to form the epiblast and the hypoblast. A part of the epiblast is comprised of the cells that are highly related to the extra-embryonic ectoderm and acquires the property of contribution to the germ line [71]. This process is regulated by factors that are secreted by extra-embryonic cells (e.g., bone morphogenetic protein or BMP) which participate in a regulatory gene pathway, which will induce the development of primordial germ cells (PGCs) that are responsible for gamete generation.

Structures that are similar to male or female

gametes have been derived *in vitro* from human, mice and swine or from human and mouse stem cell populations, respectively (for a review, see). Embryonic stem cells, when cultured in monolayers or in suspension (hanging drops) without pluripotency-maintaining factors for days or weeks tend to spontaneously differentiate into cells that are compromised with one of the three germ layers. Amongst these structures, cells that are similar to PGCs may be observed [46,123]. PGCs have been derived from mouse ESC populations and the culture of swine fetal skin-derived cells that have been supplemented with follicular liquid, probably derived from a subpopulation that may be similar to other undifferentiated cells [26,].

These ESC-derived PGCs develop into structures that are similar to oogonia, which may undergo meiosis, recruit adjacent cells to form follicle-like structures, and mediate the embryonic development into blastocyst from spontaneous parthenogenesis [27]. Similar to female gametes, male germ cells have been reported to form blastocysts and viable adult animals after intracytoplasmic injections (ICSI) [33,115]. Despite the difficulties associated with the generation of human PGCs *in vitro* compared with that of mouse PGCs (revised by [90]), the possibility of using these cells as a tool for male and female infertilities and to provide autologous oocytes for many embryonic biotechnological techniques such as SCNT is invaluable.

The induction into germinal lineages may be a rare event, and the similarities between this process and the natural process requires further investigation. Interestingly, these cells seem to lack *Igf2r* and *-H19* imprints, which are representative characteristics of germ cells [33]. However, similar to studies on nuclear transfer reprogramming and the direct reprogramming by transcription factors, Nayernie and collaborators in 2006 identified remodeling events in DNA methylation with disruptions that lead to an incomplete epigenetic reprogramming of male germ cells *in vitro*.

Obtaining functional gametes *in vitro* may become as intriguing as the success of IVF babies 40 years ago. Using epigenetic studies of induced and cloned models, the ability of iPSCs to produce functional gametes may be used for the autologous treatment of several infertilities and avoid the ethical

problems that are related to embryos that are produced using SCNT.

#### VIII. SCNT AND IPS CELLS: CONCLUSIONS AND FUTURE CHALLENGES

It is known that the reprogramming events are mediated by known transcription factors, which leads to chromatin remodeling and alter gene expression to create an ESC-like status and pluripotent cells. Induced reprogramming has raised questions concerning the manner by which reprogramming factors execute alterations in gene expression. These questions apply to reprogramming events that are induced by the oocyte cytoplasm during SCNT or the cytoplasm of ESCs after cellular fusion [22].

A better understanding of the contribution of each reprogramming factor and the detailed characterization of each step of reprogramming in cloned embryos and iPSCs are required for the elucidation of the molecular basis of nuclear reprogramming.

The bovine model provides great benefits to analyze reprogramming. The characterization of ESC-like cells in these species requires further investigation because several research groups have reported conflicting characterizations [34,119]. The production of bovine iPSCs has been recently reported. In addition, bovine species shows a reasonable rate of *in vivo* development after nuclear transfer, which is indicated by the thousands of animals that are produced to term. Therefore, the bovine model is useful for understand the reprogramming events that are induced by the cytoplasm or specific reprogramming factors.

The strategies for induced pluripotency and nuclear transfer should be studied together unearthing the deficiencies of both techniques. If the mechanisms of nuclear reprogramming through oocytes are understood, this information will be used to augment the conversion efficiency of fibroblasts into iPSCs. Conversely, if the mechanisms of gene expression that are responsible for induced pluripotency are elucidated, these mechanisms can be applied in nuclear transfer experiments. Moreover, iPSCs may be the next logical step for cloning if the concept of facility in reprogramming cells more amenable to reprogramming remains valid.

In fibroblast-derived iPSCs, the integration of 10 to 20 copies of each gene in the cellular genome and the varying proportions of each OKSM factor have been reported [68,112]. These studies have suggested that different relative quantities of each factor are important for reprogramming. Such assumptions have been confirmed by Papapetrou and collaborators in 2009, which have reported that a precise stoichiometry between each factor is critical for the efficient reprogramming of iPSCs through a mechanism that involved the use of reporter genes for each of the pluripotency-inducing factors. This system has enabled the detailed study of the kinetics

of the four transgenes silencing during reprogramming [88].

Based on such mechanisms, our group studied the effect of exogenous *SOX2* expression on nuclear reprogramming through nuclear transfer (Bressan and collaborators, unpublished data). We observed that donor cells expressing exogenous *SOX2* did not differ from non-genetically modified cells in the production of cloned embryos *in vitro*. The effects of each pluripotency-related transcription factor in SCNT-produced embryos should reveal unique or common molecular mechanisms for both SCNT and iPS techniques.

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