DOI: 10.21451/1984-3143-AR915

Anim. Reprod., v.14, n.2, p.377-382, Apr./Jun. 2017

Alternative strategies for nuclear reprogramming in Somatic Cell Nuclear transfer (SCNT)

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Abstract

Twenty years passed by since the production of Dolly the sheep, but despite significant technical progress has been achieved in the manipulation procedures, the proportion of offspring following transfer of SCNT embryos has remained almost unchanged in farm animals. Remarkable progress has been obtained instead in laboratory animals, particularly by Japanese Groups, in the mouse. However, the nuclear reprogramming strategies tested in mouse do not always work in farm animals, and others are difficult to be implemented, for require complicated molecular biology tools unavailable yet in large animals. In this review we put in contest the previous work done in farm and laboratory animals with recent achievements obtained in our laboratory, and we also indicate a road map to increase the reliability of SCNT procedures.

Keywords: somatic cell nuclear transfer, protamine 1, reprogramming, large animals.

Introduction

The nuclear transfer of a somatic cell into an enucleated oocyte (SCNT) holds a great potential as a breeding tool for the making of "geno-copies" of high genetic merit, endangered and transgenic animals. However, its full application is still hampered by the low efficiency of the SCNT. The ensuing sections provide a broad view of the progress in SCNT, and highlight the unresolved problems. Finally, the latest and most promising approaches for nuclear reprogramming, including the one developed by our group, are described along with pitfalls and advantages.

Background: Large Animals

Somatic Cell Nuclear Transfer (SCNT) is an asexual reproductive tool that empowers us to "copy and paste" a selected genotype, making in theory infinite numbers of it (Wilmut *et al*., 1997). This potential is however both a strength and weakness: strength for it is effectively a revolutionary reproductive tool; weakness – for it scares the society, causing thus resistance for its acceptance at all level, decision making people and fatally research funding agencies.

Despite this discouraging scenario, our conviction is that a tactically used SCNT offers unparalleled advantages for the challenges humanity has to deal with. High performing farm animals produced through SCNT might meet the increased foodstuff demand caused by the planet's growing population. Transgenic animals, whose production is enormously facilitated by SCNT, might provide biological peptides, animal model for disease, or environmental low impact farm animals (Loi *et al*., 2016a). Finally, SCNT holds a remarkable potential to expand, or restore, animal population threated with extinction (Loi *et al*., 2001; Saragusty *et al*., 2016), with some radical fringes aiming at bringing back to live extinct animals "de-extinction" (http://www.nationalgeographic.com/deextinction/).

Leaving de-extinction in the realm of the less likely, fancy topics, SCNT for saving animals on the brink of extinction is a due undertaking human kind owns to the planet (Pimm *et al*., 2000), and an elegant path to re-establish a smooth perception about the technology by lay people. Therefore, the establishment of biobanks from threatened animals, currently pursued/carried out at several levels, it is certainly wise. In a passive perspective, the stored cells might at least tell future generations what was the genetic makeup of the extinct animals. In a more active projection however, these cells might be used for re-generating the lost animals, or to increase the number of shrank population, through SCNT, in its declination for wild and rare animals: Inter-Specific Somatic Cell Nuclear Transfer (ISCNT). ISCNT is the only way we can multiply rare animals, given the limited number of females to collect oocytes from (Loi *et al*., 2001).

However, the efficiency of SCNT in terms of offspring production is essentially the same since the original Dolly Report (Figure 1). Moreover, the occurrence of embryo/fetal losses, along with stillbirths is still a prevalent feature of SCNT (Loi *et al*., 2006). It is true that some species, like cattle, pig or horse, thrive normally once passed the crucial perinatal phase, and these clones live and age normally, as recently published (Keefer, 2015). The high developmental abnormalities might be overcome by reconstructing large numbers of SCNT embryos, as currently carried out by company commercially exploiting SCNT in cattle breeding (http://reinclonation.com/). SCNT technical simplification worked out in these decades helped in speeding up the manipulation procedures (Taylor-Robinson *et al*., 2014), but nuclear reprogramming strategies capable to reduce embryo/fetal losses and abnormalities are still missing in large animals.

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Received: October 16, 2016
Accepted: December 15, 2016

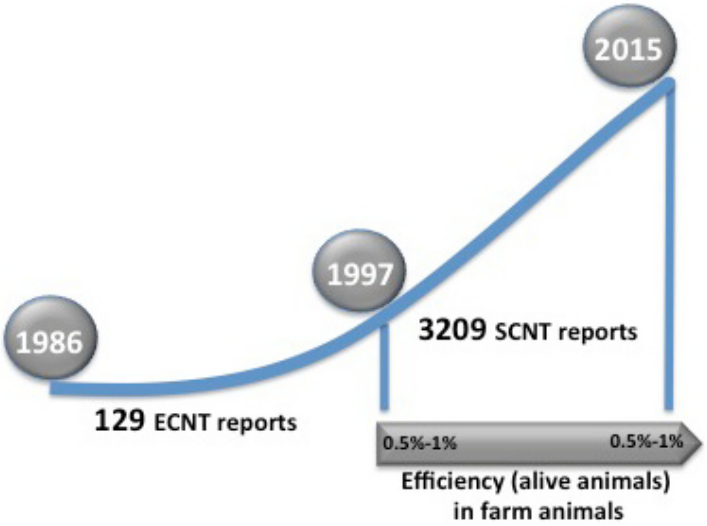

Figure 1. Number of reports of Embryonic Cells Nuclear Transfer (ECNT) and Somatic Cell Nuclear Transfer (SCNT). Note the exponential increase on the number of reports on SCNT, in contrast with the overall efficiency (From Loi P. et al., Genetic Selection Evolution 2016).

Background: Mouse

The picture changes significantly in the laboratory mouse (Ogura *et al*., 2013). Thanks to the efforts primarily from Japanese groups, the progress in nuclear reprogramming has been quite significant. The first alternative approach, targeting all genome, has been published by Wakayama's group (Kishigami *et al*., 2006). The rationale was that by providing an "open" structure to the somatic cell genome, the oocyte's reprogramming factors could attack more extensively the genome. Histone deacetylase Inhibitor, Trichostatin A (TSA), proved to be efficient in conferring an "open" state" to the somatic cell chromatin, with positive effects on nuclear reprogramming (Kishigami *et al*., 2006). The findings were also confirmed in traditionally "un-clonable" mouse strains (Kishigami *et al*., 2007) and with different classes of HDAC inhibitors (Van Thuan *et al*., 2009) (Fig. 2i). Subsequently, a more targeted strategy based on RNAi-mediated down-regulation of Xist (Matoba *et al*., 2011) (Fig. 2ii), also proved to significantly increase the proportion of offspring. The latest reprogramming efficiency, always developed for the mouse, is the depletion of H3K9me3 methylation in somatic cells before nuclear transfer (Matoba *et al*., 2014). Tri-methylated H3K9 is a epigenomic landmark conferring resistance to nuclear reprogramming, thus, its genome-wipe up through the exogenous expression of H3Kme9 demethylase increases genome accessibility to reprogramming mechanisms, enhancing in turn cloning efficiency (Matoba *et al*., 2014) (Fig. 2iii).

However, only the first of these nuclear reprogramming strategies has been repeated in large animal, but unfortunately with contradictory outcomes (Hosseini *et al*., 2016). The other two, RNAi, and H3Kme9 depletion in somatic cells have not yet repeated in large animals; the requirements of sophisticated molecular biology expertise and the lack of availability of the fine, sophisticated molecular biology tool in large animal experts renders these approached hardly manageable in large animals.

According to our opinion, a suitable nuclear reprogramming strategy should met the following requirements:

1. Transversal. SCNT is a copy and past tool that can be applied to mammals and non mammals, insects and lower vertebrate included, therefore, a nuclear reprogramming strategy must work for all of them
2. Genome wide. All the genome has to be accessible to the nuclear reprogramming machinery operative in the oocyte. The treatment with TSA, and the induced expression of the H3K9 tri-demethylase goes in this direction, for they affect the all genome.
3. Friendly user. In the perspective of a practical application of SCNT, it is mandatory to simplify the procedure and render them easily implemented by technical personnel with standard expertise.
4. Efficient. The nuclear reprogramming strategy should remove all the abnormalities that still affect the cloned offspring.

None of the strategies published so far met the above criteria. Significant advancements have been accomplished in mouse SCNT; however, the simplest approach, the treatment with TSA, does not work in large animals, or better (Sangalli et al., 2012). Of the other ones, RNAi-mediated down-regulation of Xist (Matoba *et al*., 2011) and the depletion of H3K9 methyl-transferases (Matoba et al., 2014), the first one has a sex bias, working only in female cells, while the second is technically challenging, and hardly accomplishable in large animals, where the molecular biology tool are less advanced.

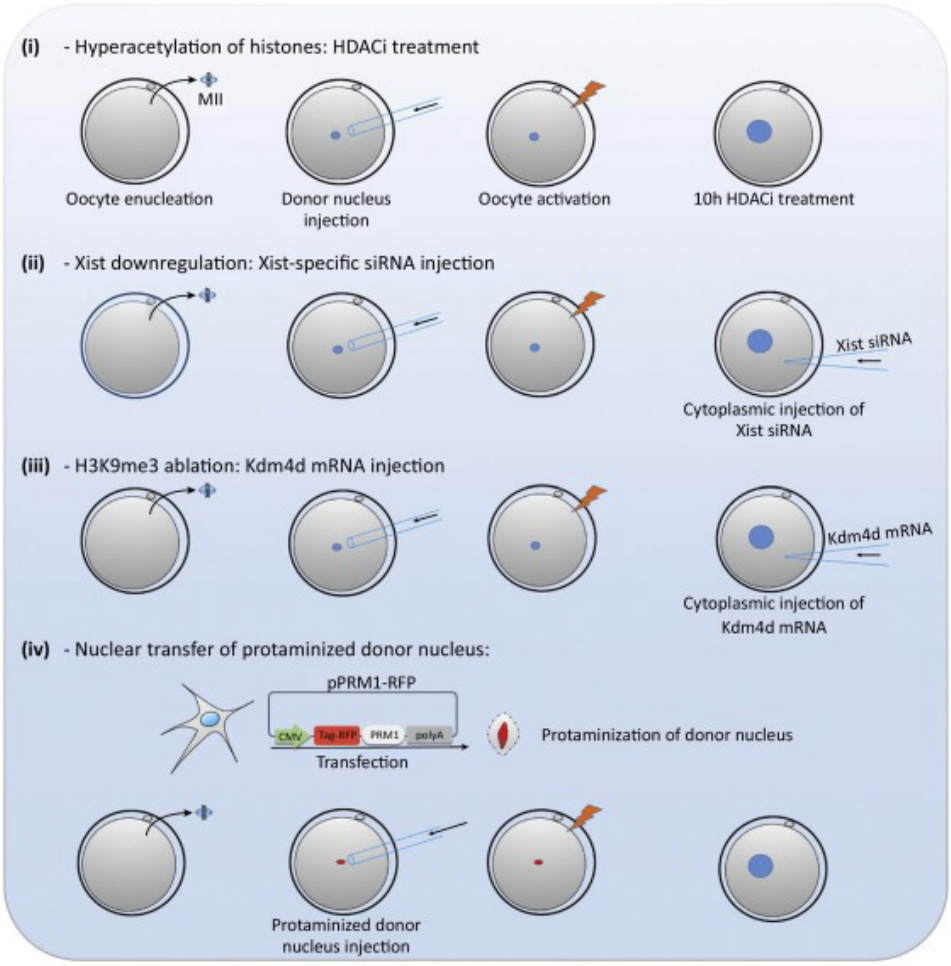

Figure 2. Diagram depicting al the major nuclear reprogramming strategies developed so far (reprinted from Loi P. *et al*., Trends in Biotechnology, 2016).

Seeking for Inspiration

The spermatozoon is the ideal nuclear transfer device Nature/God has created. Logically, if we manage to change the chromatin structure of a somatic idea into a something similar to the spermatozoa nucleus, we might have better chances to reprogram it more extensively. More than 12 years ago we started to be interested about nuclear remodelling taking place in male germ cells.

Germ cell's duty is to perpetuate the species by passing over the next generation the genetic make up of the new individual; in essence, they are repositories of the immortality of the species. The male and female gamete prepare for immortality in opposite fashion, with the oocyte that grows into a "start" cell, giving rise to all differentiated cells composing the future individual, while the spermatozoa shrinks instead into an end-line cell.

Post meiotic nuclear maturation in spermatozoa: our model to follow

The maturation of a spermatocyte into a mature spermatozoon, our object of desire, entails radical chromatin reorganization, reduces the cytoplasm leaving only the survival kit for the final journey, and enters a transcriptional silencing until activation of the zygotic genome is set up after fertilization. Spermatogenesis in its main outlines is conserved from flies to mammals. Most of the knowledge available has been gained on the laboratory mice, but also in humans, given its relevance for human infertility. The post meiotic phase is characterized by the timely translation of mRNA transcribed earlier and stored in stabilized form. Round spermatids start a complex translational program, which brings them into mature spermatozoa following the disassembly of the nucleosomes. The disassembly of nucleosomes occurs progressively, and it is promoted in first instance by the incorporation of histone variants. Testis-specific histone variants, both core and linker ones, loose the chromatin structure in preparation of the protamine incorporation. H1 variants, H1t, H1t2 and HILS1 are expressed in spermatocytes and are detectable throughout chromatin reorganization in mice paralleled by core histone variants (H2A-H2B, H3) and H4 (Brock *et al*., 1980). The incorporation of histone variants destabilizes the nucleosome conferring them an "open" configuration. A further destabilization of the nucleosome core is then induced by the post transcription modification of histones, namely acetylation (Govin and Khochbin, 2013; Chereji and Morozov, 2015). Acetylation is not however the only post-transcriptional histone modification, but other

ones, like phosphorylation, ubiquitination, 2-hydroxyisobutyrylation and crotonylation all concur to nucleosome disassembly (Govin and Khochbin, 2013). The modified histones, particularly acetylated ones, are recognized by Bromodomain, Testis specific proteins (Brdt), which operate genome wide inducing a radical reorganization of the chromatin (Pivot-Pajot *et al*., 2003). Toward the final part of spermatogenesis, ariginine and lysin rich proteins, called transition proteins (TP), bind to DNA. TP function is still debated, but the prevailing views attributes them a major role in DNA relaxing at nucleosomes (Singh and Rao, 1988), and a cooperation with topoisomerases and DNA repair enzymes (Akama *et al*., 1999) in preparation to protamine deposition. The substitution of TP by protamines (Prm) completes nuclear remodelling. Prm are small and highly basic proteins, probably derived from histone H1 from a frame-shift mutation in the tail region of the protein (Lewis *et al*., 2004). Most mammalian species express only one (Prm1) of the two Prm (Prm1&2) genes, while human and mice express both Prm1 and Prm2 (Miller *et al*., 2010). Protamines bind with high affinity to all DNA, conferring the typical toroid structure found in mature spermatozoa (Miller *et al*., 2010). A small proportion of the genome however (1%-15% according with the species) (Johnson *et al*., 2011) maintains a nucleosome organization, particularly in centromeres telomeres as well as in non-genic regions (De Vries *et al*., 2012). Prm assembly confers the shape and physical properties to the nucleus, which facilitate the trip into the female genital tract.

Upon fertilization, the remodeling is completely reversed. Paternal chromosomes exposed to the oocyte cytoplasm rapidly lose protamines and testis specific histones (Palmer *et al*., 1990; Wu *et al*., 2008) and regain a nucleosome organization built upon maternally provided histones. Hira (Loppin *et al*., 2005) and members of the nucleoplasmin/nucleophosmin family (Okuwaki *et al*., 2012) are actively involved in protamine to histone transition in the oocyte.

The remodeling of the spermatozoa head into the paternal pronucleus releases the intrinsic totipotency of its genome, leading to the development of a normal individual. When instead a somatic cell is used to "fertilize" an oocyte, as accomplished in Somatic Cell Nuclear Transfer (Wilmut *et al*., 1997), the nucleosome organization of the chromatin is a formidable obstacle for the reprogramming machinery, leading to the developmental abnormalities commonly reported in clones (Loi *et al*., 2006).

Given that the oocyte reprogramming machinery is evolutionary adapted to the chromosome configuration of the spermatozoa nucleus, an improvement in the "reprogrammibility" of the somatic cells might be induced by conferring them a protamine-based DNA organization.

Remodelling Somatic Cell Nuclei, lessons from Spermatozoa

Thus, we started to put in practice what I described in a slide presented in 2000, of course with no intention of being blasphemous "I have a Dream". The dream was to convert of interphase nuclei of a somatic cell into a spermatozoa-like one, through the transient expression of a panel of crucial testis specific remodelling factor. Crucial for the implementation of the work was the cooperation started with a leading scientist in the field of nuclear remodelling in spermatozoa, Saadi Khochbin, from Grenoble University, France. Dr. Khochbin provided the crucial remodelling proteins expressed during post meiotic nuclear maturation in spermatidis: DromoDomain-Testis Specific (BrDT); Transition Proteins 1 & 2 (TPI&II), Protamine I (Prm1, human and mouse sequences). Being our model the sheep, we opted for Prm1, expressed in ram spermatozoa. The original approach was to co-transfect all plasmodia l vectors, hoping to repeat the orchestrated events taking place during progressive nuclear compaction in spermatids. This attempt however did not fly, for multiple transfections did not work in our hands. Next, we tried a step-wise approach, starting with BrDT, followed by TPI&II, but the reduced cell viability detected as early as after BrDT transcription jeopardized each of the subsequent step. On the edge of giving up our "Dream", Domenico Iuso, then a PhD student suggested a direct transfection of the Prm1 vector (Fig. 2iv). This idea was supported by several model organisms where nuclear remodelling in spermatozoa goes directly from a nucleosomal (i.e. histonic) organization, to the compact shape of the spermatozoa without the need of intermediate remodelling factors (Martínez-Soler *et al*., 2007). The approach proved to work immediately, and it has now replicated hundreds of time in our laboratory. Twenty-four hours post transfection the Prm1, tagged by a GFP reporter sequence, started to accumulate in nuclei, first in isolated foci, then getting organized in a compact structure, with startling similarities to a spermatid nucleus, but larger, owning to their diploid nucleus.

The heterologous expressed Prm 1 displaced the linker and core histones, binding to DNA and inducing a rapid remodelling. The genome condensation resulted in a global shut of transcription, and the fully remodelled cells detach from the dish and float in the medium. Thus, fully protaminized cells are no longer viable, but their use I nuclear transfer is not precluded by this (Loi *et al*., 2002).

We next decided to test whether the nuclear protaminization is reversible by injecting the remodelled cells into enucleated sheep oocytes. To our relief, the protaminization was completely reversed, and normal pronuclear structures were evident already by 6-8 hours post activation. Therefore, our spermatid like cells can be used for nuclear transfer, and moreover, the frequency of development to blastocyst stage was twice higher in embryos reconstructed with Prm1 positive cells, comparing with control (Iuso *et al*., 2015).

We do not know exactly the proportion of the genome effectively bound to Prm1. ChIP-seq deep sequencing indicated the Prm1 binds to a large of number of domains, but if it covers all genome remains an open question. Of course, our target remains the

transformation of somatic cell nuclei into structures as close as possible to spermatozoa, thus we are concentrating our efforts in this direction (Loi *et al*., 2016b). Somatic cell conversion into male gametes structures seems to be a promising approach for improving SCNT efficiency, and hopefully removing the anomalies that still persists in cloned offspring. The final word on the efficacy of our approach will come from in vivo developmental trials.

Acknowledgements.

MIUR/CNR, Program FIRB GA B81J12002520001 (GenHome) to PL. The authors are participating in the COST action FA 1201 ''Epiconcept'' Epigenetic and Peri-conception Environment. Pl acknowledge also the support of Twinn project "EraofArt".

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