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Scientific and technological approaches to improve SCNT efficiency in farm animals and pets

Pasqualino Loi[1], Luca Palazzese[2], Pier Augusto Scapolo[1], Josef Fulka Jr[3], Helena Fulka[4] and Marta Czernik[1,2]

[1]*Laboratory of Embryology, Faculty of Veterinary Medicine, University of Teramo, Teramo, Abruzzo, Italy,* [2]*Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, Warsaw, Jastrzebiec, Poland,* [3]*Department of Biology of Reproduction, Institute of Animal Science, Prague, Pratelstvi, Czech Republic and* [4]*Department of Cell Nucleus Plasticity, Institute of Experimental Medicine of the Czech Academy of Sciences, Praha, Czech Republic*

Correspondence should be addressed to P Loi; Email: ploi@unite.it

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Abstract

The birth of Dolly through somatic cell nuclear transfer (SCNT) was a major scientific breakthrough of the last century. Yet, while significant progress has been achieved across the technics required to reconstruct and *in vitro* culture nuclear transfer embryos, SCNT outcomes in terms of offspring production rates are still limited. Here, we provide a snapshot of the practical application of SCNT in farm animals and pets. Moreover, we suggest a path to improve SCNT through alternative strategies inspired by the physiological reprogramming in male and female gametes in preparation for the totipotency required after fertilization. Almost all papers on SCNT focused on nuclear reprogramming in the somatic cells *after* nuclear transfer. We believe that this is misleading, and even if it works sometimes, it does so in an uncontrolled way. Physiologically, the oocyte cytoplasm deploys nuclear reprogramming machinery specifically designed to address the male chromosome, the maternal alleles are prepared for totipotency earlier, during oocyte nuclear maturation. Significant advances have been made in remodeling somatic nuclei *in vitro* through the expression of protamines, thanks to a plethora of data available on spermatozoa epigenetic modifications. Missing are the data on large-scale nuclear reprogramming of the oocyte chromosomes. The main message our article conveys is that the next generation nuclear reprogramming strategies should be guided by insights from in-depth studies on epigenetic modifications in the gametes in preparation for fertilization.

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Introduction

The writing of this manuscript for the Reproduction special issue revived the memories of laboratories brimming with atmosphere, just a handful at the time, working on nuclear transfer. The production of the first lambs cloned by electrofusion of blastomeres from early embryos onto enucleated oocytes (Willadsen 1986) started a race between groups to optimize nuclear transfer, then a black box from the scientific point of view. On the one hand, considerable efforts were spent trying to synchronize the cell cycle between the receiving enucleated oocyte (cytoplast) and the donor nuclei (karyoplast) (Smith *et al.* 1988, Campbell *et al.* 1994, 1996*a*); on the other hand, attempts were made to use more advanced embryonic cells to increase the number of obtainable clones (First 1990, Sims & First 1994). The core of the game at the time was played in the United Kingdom. The Bovine Embryo Multiplication Agreement (BEMA) represented a brainstorming and empirical ring in which most of the game was set. Keith Campbell's intuition was based on the initially assumed importance of inducing a G0-stage in the donor nuclei to achieve better reprogramming. However, this was later reduced to a cell-cycle synchronization method and culminated in cloned lambs from cultured embryonic cells (Campbell *et al.* 1996*b*). This was shortly followed by the production of Dolly, the sheep whose 25th anniversary is celebrated in this special issue (Wilmut *et al.* 1997).

The formidable energy deployed at that time jumped the gun: contrary to data reported in amphibians, where

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terminally differentiated cells from adult individuals did not develop past feeding tadpoles (Gurdon *et al.* 1975), the prospects of multiplying adult animals were made real. SCNT is a multistep process that relies on several factors such as oocyte quality, activation procedures, donor nuclei source and their cell-cycle stage, culture system, and the global efficiency derived from all these. A review covering all these aspects would be dispersive and unmanageable. However, the reader is referred to several authoritative reviews written on different aspects of SCNT (Narbonne *et al.* 2012, Ogura *et al.* 2013, Simmet *et al.* 2020, Wang *et al.* 2020). Here, we would like to focus on nuclear reprogramming and particularly on the strategies attempted thus far to induce global genome modifications in somatic cells. The general goal of these attempts was to induce an epigenetic asset amenable to establish a totipotent state upon nuclear transfer. We apologize for not including all valuable works published to date on other important aspects of SCNT.

State-of-the-art

Many are the published reports on SCNT. A PubMed survey launched at the time of this manuscript preparation counted 3152 publications since the original Dolly paper (Fig. 1). An in-depth data analysis revealed that while several significant technical improvements have been achieved in the reconstruction, activation, and *in vitro* culture of SCNT embryos, no major advancements in nuclear transfer efficiency in terms of offspring development have been made. However, a distinction must be made between mice and other species. The laboratory mouse (*Mus musculus domesticus*), thanks to its genetically defined strains, availability of a large volume of genetic information, and relatively easy generation of gene-modified animals are an unmatched model for SCNT research, as a companion paper published in this issue will show.

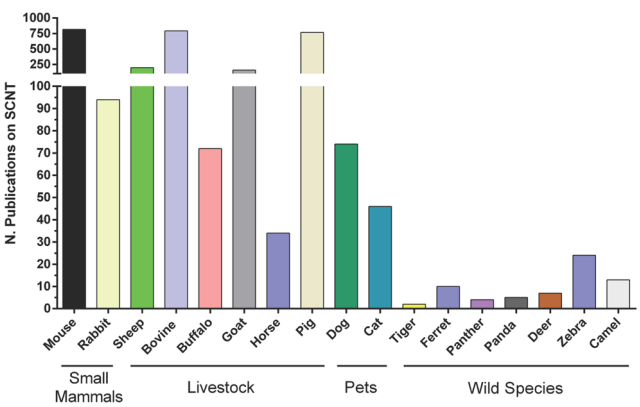

Figure 1 Number of publications since the production of Dolly (March 2017–December 2020, Pubmed survey December 2020).

Here we will briefly quote the main innovation reported in mice, as presented by Ogura (2020):

(i) Exposure of cloned embryos to histone deacetylase inhibitors (Kishigami *et al.* 2006).
(ii) Removal of Reprogramming Resistant Regions (RRRs), represented by genomic domains enriched in H3K9me3, using the ectopic expression of H3K9me3 demethylase Kdm4d (Matoba *et al.* 2014).
(iii) Correction of the Xist expression pattern in SCNT-derived embryos by Xist gene knockout or knockdown (Matoba *et al.* 2011).

Consistent data on large animal SCNT are available only for donor cell treatment and reconstructed embryos with diverse histone deacetylase inhibitors, TrichoStatin *A* (TSA) being the most common. TSA treatment was pioneered by Kishigami and Wakayama, resulting in a dramatic increase in live offspring (Kishigami *et al.* 2006). The leap in offspring production clearly resulted from an improved nuclear reprogramming of the somatic cells, although the exact mechanisms through which TSA works remain elusive. TSA 'opens' the chromatin structure in reconstructed embryos, making it more available to transcription factors during early nuclear remodeling, and facilitates the remodeling of constitutive heterochromatin. Indeed, the early embryo genome, also in cloned embryos, undergoes the most dynamic transcription phase a genome ever experiences. Chromatin de-condensation after TSA treatment and histone acetylation results in easier access of many, still unknown, remodeling factors from the ooplasm (Maalouf *et al.* 2009). Overall, it could be stated that the effects of TSA on nuclear reprogramming mechanisms in farm animal SCNT remain controversial and need further investigation, especially on the development to term (Sangalli *et al.* 2012, Sawai *et al.* 2012, Hosseini *et al.* 2016). A study in pigs claimed to have enhanced offspring production following TSA treatment, but an in-depth analysis of the data revealed that the development to term rates of TSA treated and control SCNT embryos were 0.7 and 0.4%, respectively (Huan *et al.* 2015). Less informative are the data on TSA treatment during SCNT in pets, given that almost all the reports concerned interspecific SCNT – iSCNT (Wittayarat *et al.* 2013).

If TSA does not endow significant advantages on large animal SCNT outcomes, the picture does not change much following the application of maternal *Xist* knockout in donor cells and/or *Kdm4d* mRNA injection into reconstructed embryos, mentioned earlier. In a recent cattle SCNT study, inhibition of the epigenetic writer EHMT1/2 catalytic activity markedly reduced H3K9me2 and H3K9me3 levels in cloned blastocysts but had no positive effects on the rate of cloned embryos development to term (Sampaio *et al.* 2020). Likewise, chetomin, a fungal secondary metabolite

reported to inhibit the trimethylation on histone 3 lysine 9 (H3K9me3), proved ineffective in horse SCNT embryos (Damasceno Teixeira *et al.* 2019). Lastly, a SCNT study carried out in rhesus macaque (*Macaca mulatta*) transplanting fetal and adult cells (fibroblasts and cumulus cells, respectively) into enucleated oocytes enriched with *Kdm4d* mRNA and treated with TSA after virus-induced fusion brought no major advancements. Two live offspring were delivered from fetal cells. The two infants derived from cumulus cells had to be delivered by cesarian section, and both died within 3 days because of respiratory distress. It is a great pity that no data on the placental phenotype and necropsy were reported (Liu *et al.* 2018). Therefore, a combined treatment with H3K9 demethylation and TSA brought no significant improvements when somatic cells were used for SCNT. However, it must be said that the number of available replicates is too limited to draw any definitive conclusions.

Current applications of SCNT in large animals and pets

What has been presented above about the state-of-the-art in nuclear reprogramming strategies in large animals led to our partial conclusion that no effective nuclear reprogramming strategies are available. Yet, working with large numbers of embryos allows for the application of cloning in several commercial settings, including multiplication of livestock with particular genetic characteristics, production of cloned dogs and cats, even post mortem, reproduction of castrated animals, usually horses, and production of animal models for human pathologies.

Cloning for the multiplication of livestock with particular genetic characteristics is mainly confined to cattle and swine farming. Typically, only cattle of high genetic value, mainly of beef breeds, are reproduced through SCNT. The most active companies are operating in the USA. Of these, Transova (https://transova.com) has to its credit the production of thousands of clones. In fact, the US Food and Drug Agency (FDA) gave a favorable opinion on the consumption of cloned animal products. Another strong player is China, where a private cattle and pigs cloning company, Boyalife (https://www.boyalifegroup.com), has just been founded as part of a network with Chinese universities and research centers. Boyalife is extending to Hong Kong and the USA. The situation in Europe is more difficult given that the European Parliament banned in September 2015 the import and consumption of food products derived from cloned animals, indicating a strong negative perception of cloning.

Dogs and cats were the last species to be cloned (Shin *et al.* 2002, Lee *et al.* 2005). Their importance as companion animals has fueled the emergence of cloning companies, active primarily in cases of terminally ill or even deceased animals. For that reason, these companies offer biobanking services of cells sampled from pets, to be used eventually to replace them in case of death. Again, the leading companies in this sector are in the USA, such as Viagenpets (https://viagenpets.com/) and China (e.g. Sinogene; https://www.sinogene.org).

The reproduction of castrated animals through SCNT applies primarily to sport horses. Being an asexual reproduction method, cloning offers the possibility to reproduce champions castrated before starting their sports activities. The first cloned horse was produced in Italy by Avantea (Galli *et al.* 2003). Unlike other horse breeds, such as the English thoroughbred, reproductive technologies, including cloning, are allowed in polo horses. Polo riders often change horses during the game, so the performances of the different mounts are important. A few years ago, information about an Argentinian team that regularly used cloned horses, six to be exact, all derived from a famous mare, Cuatetera, hit the news. The team's success has established cloning as a way to multiply champion racing horses, a trend that is progressively spreading to high-income countries. In addition to the Italian Avantea, other companies that deal with commercial equine cloning are Crestview Genetics and the Argentinian Kheiron, the last with approximately 200 live clones produced (http://www.kheiron-biotech.com/index_en.html).

Traditionally, the study of the onset and treatment of various human diseases use transgenic mouse models. These are transgenic mouse lines in which the gene(s) responsible for a given human pathology has been inserted/mutated. Although the advantages deriving from these models are numerous, the mouse has intrinsic limitations such as a short life span and small size. In fact, if these models are useful when striving to understand the disease onset, they are not much so in the development of treatments, including surgery, for the disease. Cloning has made it possible to produce suitable animal models of human pathologies, most often pigs butalso sheep. This was made possible thanks to the development of efficient genome editing tools, also applicable to somatic cells. Operationally speaking, it involves inserting the mutated gene of interest or modifying or removing an endogenous one. For example, the muscular dystrophy gene could be inserted through genome editing methods such as CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR-associated protein 9) into cells cultured *in vitro*. The transformed cells, selected by appropriate culture conditions, are transferred into enucleated oocytes, and the resulting embryos are transplanted into suitable foster mothers for development to term (Lee *et al.* 2020). The animals thus generated will express the mutated gene and therefore the resulting pathology. Currently, pig models are available for many degenerative and non-degenerative neurological, endocrinologic, and muscular disorders

(for review see, Holm *et al.* 2016, Whitelaw *et al.* 2016, Hoffe & Holahan 2019). Noteworthy is the production of a sheep model of cystic fibrosis (Fan *et al.* 2018).

Still in the context of animal models, pigs 'humanized' immunologically by cloning with genetically modified cells could be used to produce organs, such as liver, heart, and kidneys, for transplantation into human patients (Whitelaw *et al.* 2016). This research branch is progressing rapidly, although many are the roadblock to overcome on the way to clinical applications (Reichart *et al.* 2020). Besides immunological rejection, additional complications are represented by the risk of cross-species transmission of Porcine Endogenous RetroVirus (PERV; Yang & Wu 2018). The use of PERV knockout pig cell lines might alleviate this infectious risk (Niu *et al.* 2017). However, such an approach raised the concerns of possible cryptic mutations resulting from the massive genome editing required. Lastly, even allogenic transplant size must be finely considered (Hinrichs *et al.* 2020).

Of note, 'humanized' pigs have been recently deployed to fight the ongoing COVID-19 pandemic. Double knockout pigs, lacking CMP-N-acetylneuraminic acid hydroxylase (*CMAH*) and α1,3-galactosyl-transferase (*GGTA1*) genes that are needed to produce glyco-humanized polyclonal antibodies (GH-pAb) without the Neu5Gc and α-Gal epitopes, were immunized with the SARS-CoV-2 spike Receptor-Binding Domain (RBD). A robust hyperimmune response, with anti-SARS-CoV-2 end-titer binding dilutions of over one to a million, was found (Vanhove *et al.* 2021).

Alternative nuclear reprogramming strategies

The picture described previously, along with the verification that the numbers of SCNT reports dealing with large animals by far surpasses that in mice (Fig. 1), stresses the urgent need for an efficient and safe SCNT in large animals.

The current axiom is that, even in mice, the best protocols have not entirely eliminated the SCNT-associated placental abnormalities. An exception is SCNT with genetically modified donor cells taken from a mouse knockout (KO) for the *Sfmbt2* locus that codes for a miRNA cluster (Inoue *et al.* 2020). Moreover, 'birth rate' in mice means pup delivery by cesarian section (Ogura 2020). Clearly, the use of genetically modified cell lines or cesarian section as a routine in large animals is not an option. Therefore, in-depth studies are required to develop radical and universal nuclear reprogramming strategies.

Suitable directions for evolving nuclear reprogramming strategies

In our experience, confirmed by most of the published data, placental abnormalities are common features in cloned large animal offspring, although with a milder phenotype in cloned pigs (Constant *et al.* 2006, Loi *et al.* 2006, Palmieri *et al.* 2008, Pozor *et al.* 2016, Ao *et al.* 2019). The penetrance of the phenotype spans from a life-threatening disease of the foster mother carrying the cloned conceptus, like hydro-allantois, to milder ones, where cardiac and liver pathological signatures are correlated to compromised kidney function. Osmotic imbalances, causing an abnormal accumulation of amniotic and allantoidal fluids, ultimately impede fetal urine drainage, resulting in a systemic uremic syndrome that first affects the kidneys and then upstream organs in a domino-like fashion (Loi *et al.* 2006).

This is not surprising. The placental genes are expressed in a unique, disposable, extracorporeal organ, with a much shorter lifespan than the soma. The organ contains cells with a weird chromosomal constitution, including aneuploidy, multinucleation, and DNA endo-reduplication (Weier *et al.* 2005, Hayakawa *et al.* 2018, Bhattacharya *et al.* 2020). These properties, known as Partially Methylated Domains (PMDs), while conferring proliferative advantages to placental cells in this cancer-like organ, are epigenetic hallmarks in cancer (Decato *et al.* 2020). However, this unique organ requires a formidable safety device to hide those genes from the transcriptional machinery in somatic cells. Thus, placental genes, present in all somatic cells, as established by the Dolly's principle of 'genome totipotency,' must be 'double-locked' and made inaccessible for transcription. As a result, placental genes are only superficially affected by the nuclear reprogramming machinery when using the approaches so far reported.

There is an urgent need to radically change our vision about nuclear reprogramming and seek inspiration from the most efficient and universally adopted nuclear transfer device – the spermatozoa and its natural 'recipient,' the oocyte.

Paternal-specific nuclear reprogramming

The preparation for immortality, ensured by establishing a totipotency state in the male and female germlines, takes several months or years, depending on the species. Our view is that we need to mimic, even partially, the epigenetic/chromosome organization in these two highly specialized cells if we wish to find innovative solutions for nuclear reprogramming. Following this line of thought, we started a decade ago to explore the conversion of somatic cell nuclei into a spermatid-like structure. The approach we initially followed was a progressive expression of testis-specific proteins in somatic cells, sheep fibroblasts. The first trials led nowhere. However, inspired by male gametogenesis in other animal models (Martínez-Soler *et al.* 2007), we attempted to directly express protamine 1 in sheep fibroblasts. The results were surprising as, 48 h after transfection, a sizeable

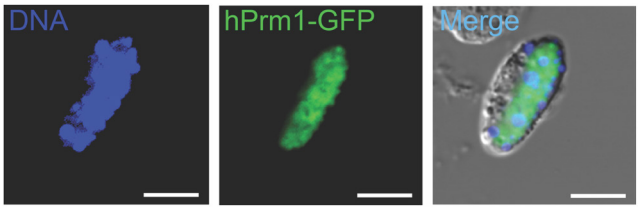

Figure 2 Nuclear remodeling in sheep fibroblasts expressing GFP tagged human Protamine 1 (left, DNA- DAPI; center, hPrm1-GFP; right, merge).

proportion of the interphase nuclei compacted into a shape reminiscent of spermatid nuclei (Fig. 2). Chip Seq analysis showed that the protamine 1 was bound to large nuclear domains, and, most importantly, the genome protaminization was fully reversible after nuclear transfer (Iuso *et al.* 2015). Furthermore, the blastocyst rate in embryos reconstructed with protaminized somatic cells was twice as high as the control, untransfected cells. Although the final proof, the production of cloned offspring, is still missing, we can state with high certainty that protaminization confers improved development up to the blastocyst stage (Czernik *et al.* 2016).

The rationale of this approach is that while the oocyte has no molecular tools to unravel the large-scale genome organization in nucleosomally organized, differentiated cells, the protaminized nucleus brings a unique DNA format similar to that delivered by the fertilizing spermatozoa, which the oocyte can handle. More studies are required to optimize the somatic nuclei protaminization approach. Ideally, the perfect protocol should lead to a protamine-to-nucleosome ratio similar to that typically found in the spermatozoa of the studied species (Yoshida *et al.* 2018).

Maternal specific nuclear reprogramming

Oocyte chromosomes, just like the paternal ones, prepare for totipotency. Indeed, the maternal chromatin must be streamlined in preparation for fertilization.

Very little is known about the reprogramming of oocyte chromosomes during the final maturation stage. There is a great deal of information about meiotic recombination through crossing over (Hughes *et al.* 2018) and epigenetic modifications in preparation for meiosis (De La Fuente 2014) but not about the preparations for totipotency.

A nuclear reprogramming strategy cannot ignore the special features and large-scale nuclear reprogramming occurring in fully grown germinal vesicle (GV) oocytes in preparation for totipotency (Fig. 3). Again, most basic research was performed on mice. When oocytes reach their full size, their chromatin undergoes a marked change and condenses around the nucleolus to become the so-called surrounded nucleus (SN)-type oocytes (Zuccotti *et al.* 1995). Functional studies have shown that this transition is necessary for the oocyte to gain its full developmental competence and is accompanied by a marked decrease in RNA polymerase I and II transcriptional activity (Fair *et al.* 1995, Bouniol-Baly *et al.* 1999, De La Fuente 2006, Inoue *et al.* 2008, Dumdie *et al.* 2018). The details of the process remain elusive; however, the chromatin condensation seems to be linked to histone deacetylase activity since it can be reverted by TSA treatment (De La Fuente *et al.* 2004). Nevertheless, TSA treatment does not influence the transcriptional activity, suggesting the presence of a different mechanism behind the oocyte-specific transcriptional program termination before the transition

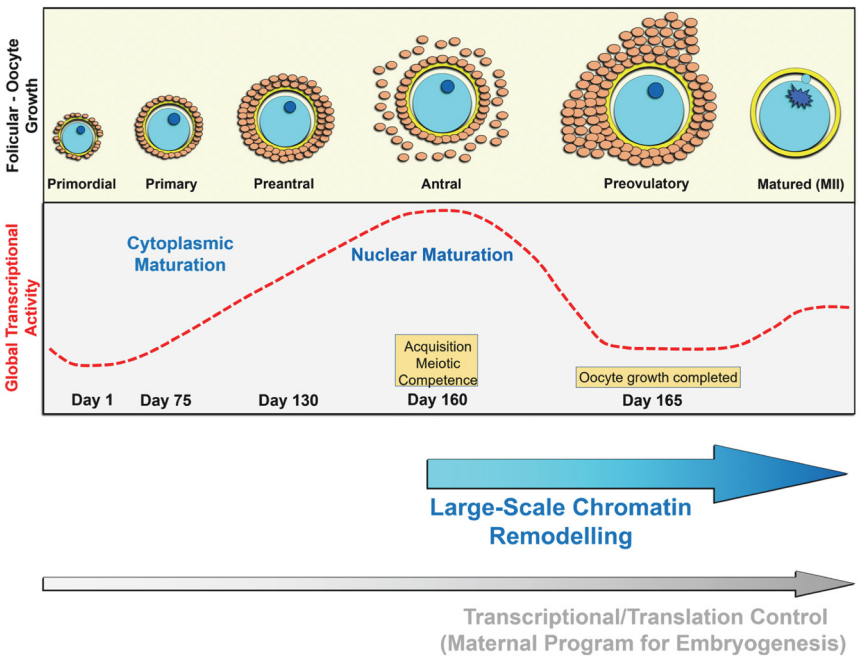

Figure 3 Time-scale schematic representation of oocyte growth and nuclear remodeling.

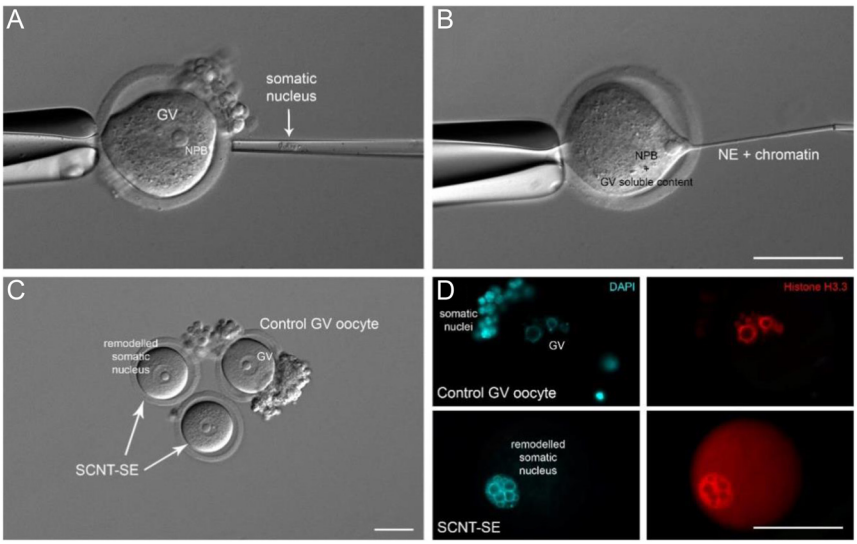

Figure 4 The process of selective enucleation coupled with nuclear transfer. (A) First, a somatic nucleus typically derived from a cumulus cell (arrow) is transferred into a germinal vesicle stage oocyte, which still contains its original nucleus (germinal vesicle, GV) with a prominent nucleolus precursor body (NPB). (B) In a next step, the oocyte is selectively enucleated. The nuclear envelope (NE) and the chromatin in slowly pulled out by a fine pipette. The soluble GV content and the NPBs are expelled into the cytoplasm and available to be incorporated by the somatic nucleus already present in the cytoplast. (C) After 20 h, the remodeled somatic nuclei (SCNT-SE, arrows) are morphologically very similar to control germinal stage oocytes (GV). (D) The uptake of histone H3.3 to the remodeled somatic nucleus. Top image shows the localization of this histone variant in the chromatin of the germinal vesicle (GV). Note that H3.3 levels are very low in cumulus somatic cells, which were used as donors. After an overnight incubation and remodeling, this histone variant is efficiently incorporated into the somatic chromatin, bottom. The samples were extracted upon fixation, thus, only incorporated H3.3 is detected. Scale bar, 50 µm.

to totipotency (De La Fuente *et al.* 2004). This is likely achieved by the dissociation and degradation of RNA polymerase II (Fulka *et al.* 2009, Abe *et al.* 2010).

The published data on mature oocytes showed that they have a highly accessible chromatin (Lu *et al.* 2016, Gu *et al.* 2019), enriched by several histone variants. These include the linker histone variants H1FOO, H3.3, or TH2A/TH2B (Tanaka *et al.* 2001, Akiyama *et al.* 2011, Shinagawa *et al.* 2014). The accumulation of these specialized histones at high levels seems to be a universal feature of oocytes (McGraw *et al.* 2006, Zhang *et al.* 2018) and, at least in mice, was shown to have beneficial effects on reprogramming (Gao *et al.* 2004, Shinagawa *et al.* 2014, Wen *et al.* 2014*a*). However, the simple presence of these histone variants might not be sufficient to induce full totipotency, as neither H3.3 nor TH2A/TH2B is exclusively expressed in oocytes. Therefore, other specialized chromatin factors, such as histone chaperones and remodeling complexes, likely give the oocyte chromatin its unique features (Zhang *et al.* 2016, 2020*a*, Ooga *et al.* 2018).

Our knowledge of oocyte chromatin modifications leading to nuclear totipotency is limited. One possible explanation for this lack of knowledge might be that since the production of Dolly, the oocyte potential to restore totipotency has been studied exclusively with nuclear reprogramming of somatic cells in mind. However, this event occurs in mature metaphase II oocytes after artificial activation, concomitant with DNA replication. Post-activation somatic cell nuclear remodeling/reprogramming works but again drifts significantly from the physiological path, where both oocyte and spermatozoa undergo nuclear reprogramming in a replication-independent fashion. Missing are studies addressing the molecular mechanism leading to totipotency to the resident chromosomes in oocytes.

Insights from germinal vesicles remodeling of somatic cells nuclei

More informative in this respect are studies in which mouse somatic nuclei were transplanted into the GV, the giant nucleus of an immature Xenopus oocyte (Miyamoto *et al.* 2018). Under these conditions, it is possible to detect changes in chromatin accessibility, induced in a replication-independent manner, just like during oocyte-specific reprogramming. Large-scale chromatin modifications were captured using a modified Assay for Transposase-Accessible Chromatin Sequencing (ATAC-seq) (Buenrostro *et al.* 2013). The assay revealed open chromatin regions by exploiting the Tn5 transposons capacity to insert into accessible chromatin domains. The data showed that somatic cell nuclei undergo extensive transcriptional reprogramming toward an oocyte-like state within 2 days, without replication. Expectedly, genes with a pre-existing open structure were easily reactivated, while only a few of the genes that typically become accessible only after nuclear transfer acquired an open chromatin state. Increased nuclear actin polymerization due to overexpression of *Toca1/Fnbp1l* seems to play a primary role in inducing chromatin 3D rearrangement (Miyamoto *et al.* 2011). These findings are enlightening in many ways and more informative than the available post-nuclear transfer data

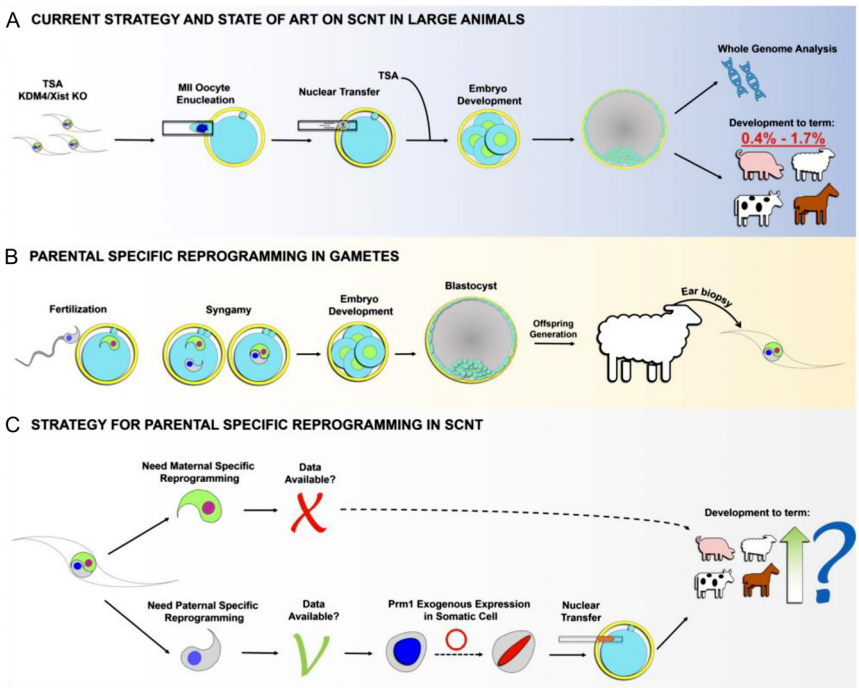

Figure 5 Schematic overview of the current (A) and suggested (B and C) strategies for nuclear reprogramming.

on nuclear reprogramming. Unfortunately, the size and organization of mammalian oocytes markedly differ from those of Xenopus, preventing us from repeating such an important study in mammals. However, a compromise might be found in using Selectively Enucleated GV (SEGV) oocytes. Selective enucleation was first described by Modlinski in 1975 (Modliński 1975) and is used to obtain cytoplasts depleted of chromatin, tDNA, DNA-bound factors, and the nuclear envelope (Gręda *et al.* 2006). This method is somewhat equivalent to oocyte fractionation and allows the analysis of the reprogramming processes that occurs primarily under the influence of the soluble nuclear fraction found in the GV. In some way, injecting somatic nuclei into SEGV oocytes mimics, with a good approximation, the Xenopus GV nuclear transfer experiment (Fulka *et al.* 2019). The somatic nuclei exposure time to the SEGV oocyte cytoplasm can be easily controlled by culturing them in the presence of the meiotic progression inhibitor dbcAMP (Fulka *et al.* 2019). Thus, somatic nuclei can remain in the SEGV for up to 20 h, a time frame similar to the Xenopus experiments (Miyamoto *et al.* 2018). Using this model, long-range replication-independent somatic cell chromatin modifications can also be monitored in other species. Initial results indicated that the somatic nucleus undergoes an incredible nuclear remodeling, gaining a morphology and size similar to control GV-stage oocytes (Fig. 4), anticipated by transcriptional and replication silencing of the transferred nucleus (Fulka *et al.* 2019). The epigenetic modification so far detected indicates enrichment of histone variant H3.3, which is essential for normal development and indicative of an extensive reprogramming in the somatic chromatin (Fulka *et al.* 2019, Fig. 4). However, the soluble GV fraction seems to be rather inefficient in replacing somatic histones H3.1/H3.2, typically found in closed chromosomal domains (Wen *et al.* 2014*a*,*b*). We speculate that this persistence of H3.1/H3.2 is caused by the lack of replication that does not occur in this type of cytoplast. SEGV cytoplasts just started to deliver the initial data, confirming their importance as an unparalleled model to study nuclear reprogramming under physiological conditions. Another sub-nuclear compartment to consider in nuclear remodeling studies are the atypical nucleoli present in oocytes and early embryos. Initial experiments have shown that the oocyte nucleolus is essential for successful development following SCNT (Ogushi *et al.* 2008), likely impacting the 3D genome organization and remodeling of some specific sequences such as the major and minor satellites (Fulka & Langerova 2014).

Concluding remarks

Ideally, an efficient nuclear reprogramming strategy should significantly increase the term development rate, eliminate placental abnormalities, and be applicable to all species. As remarked earlier, the Achilles' heel of current procedures is the difficulties in controlling nuclear reprogramming of somatic genes. The resistance is particularly severe in extra-somatic placental genes. Twenty-five years after Dolly was born is a remarkable turning point. The lesson gained from the over three thousand published SCNT reports is that we need to radically change our approach.

Put simply, we need to reverse our focus, and instead of insisting on epi/genetic modification occurring during post-nuclear transfer genome reprogramming, we need to learn how maternal and paternal alleles are prepared for fertilization, and hence for totipotency. Research on the spermatid epigenetic remodeling during the transition to mature spermatozoa is advanced and comprehensively described in a recent reviews (Meyer *et al.* 2017); lesser knowledge is available on the female gamete. Indeed, profound, large-scale chromatin modification takes place during the last nuclear maturation phase of the follicle-enclosed oocyte (Fig. 4). Luckily, the available technologies provide unique insights into chromatin organization at different levels. The main structural 'units' comprise chromosome territories, compartments A/B, topologically associated domains (TADs), and loops, all help shape the 3D structure, and some are likely cell-type specific. These techniques will help us appreciate the unique chromatin organization in oocytes (Flyamer *et al.* 2017, Ke *et al.* 2017, Chen *et al.* 2020, Zhang *et al.* 2020*b*). The gained information will be extremely useful in providing us with data on the epigenetic format we need to confer on the maternal alleles in somatic cells (Fig. 5).

If we wish to enhance the cloning process in large animals, the key prerequisite is a detailed understanding of oocyte nuclear reprogramming when preparing for fertilization. Unfortunately, one of the negative legacies of Dolly is the ethical aftermath that has instilled a negative perception, especially in Europe, rendering funding for SCNT challenging to obtain. This negative perception is unjustified, for robust reports on the normalcy of cloned animals are in place (Sinclair *et al.* 2016). We believe that 25 years after the birth of Dolly, we have at our disposal unprecedented technological and analytical tools, knowledge, and several proof-of-principal reports that the development of a safe and effective SCNT procedure can be achieved in the medium term.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

All authors have contributed to write this review article.

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