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Nuclear reprogramming: what has been done and potential avenues for improvements

Review Article

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Abstract: A major challenge for reproductive biologists is the development of novel strategies to improve cloning efficiency. Even in species for which cloning is relatively successful, like cattle, the efficiency is still unacceptably low. In this review article we critically analyse all approaches that have been suggested by different laboratories in the field so far. As will be discussed below, so far none of these gives rise to a dramatic increase in cloning efficiency. Possibly, a multi-step approach including a pre-treatment of donor cells to modify their chromatin, along with improved culture system for cloned embryos would be the most promising.

Keywords: *Sheep • Cloning • Nuclear reprogramming*

1. Introduction

The generation of individuals by transfer of a nucleus from a somatic cell into an enucleated egg was achieved first in *Xenopus laevis,* as a demonstration of nuclear equivalence between zygotic and differentiated nuclei [1]. The same experiment was repeated 30 years later using the sheep as a model [2]. However, the initial emphasis that has welcomed the production of Dolly the sheep has been progressively quenched in the light of the multitude of developmental defects described in clones [3]. In particular, an emerging insight is that development and function of extra-embryonic membranes is particularly affected by somatic cell nuclear transfer (SCNT) [4,5]. Such placentation dysfunction has dramatic effects on embryonic development and growth, leading to foetal losses in the most severe cases, and also the occurrence of stillbirth and postnatal mortality [6,7], particularly in sheep [8]. It is possible that the epigenetic mechanisms controlling gene expression in the extraembryonic tissue are less robust hat those acting on the foetus proper and therefore are more easily destabilized by invasive embryo technologies such as cloning. It is now commonly accepted that these abnormalities arise from an incomplete "nuclear reprogramming" of the somatic cell nucleus by the oocyte cytoplasm [9].

The essential prerequisite for nuclear reprogramming of a transplanted somatic cell nucleus is the remodelling of its chromatin, such that it becomes compatible both with the changes in cell cycle dynamics during early cleavage and with the coordinated regulation of the full program of gene expression required for development to term [10]. A complete nuclear reprogramming is therefore achieved only when the repressive epigenetic marks imposed during cell commitment are completely reversed by the oocyte cytoplasm [11]. The efficiency of this reversal determines the developmental success of the nuclear transfer embryo.

Global changes in DNA methylation and modifications of histone proteins are the molecular fingerprint of the physiological programming taking place during normal development, and a great deal of effort is currently being put toward establishing how these global changes are preserved or altered in cloned embryos [3].

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The ideal cloning procedures should induce a full reversal of the cell memory in a differentiated cell, without affecting genes and chromosomal regions that should remain unmodified during embryonic development. In fact, many genes do not normally change the organisation of their chromatin during pre-implantation development, and some chromosomal regions need to be maintained in a repressed state throughout development. Importantly, these regions on the chromosomes should not become aberrantly altered as a consequence of the cloning procedure, particularly those harbouring imprinted genes. Imprinted genes are a developmentally important group of genes which are expressed from the paternal or maternal genomes depending on the parental origin of the allele. Such allele-specific gene expression is programmed by chromatin features established in the germ line which are maintained throughout development. However, despite the progress achieved in our knowledge of nuclear reprogramming, witnessed by 11 mammalian species successfully cloned [3], and the simplification of micromanipulation procedures [12,13], the frequency of offspring production in SCNT remains low, between 1-5% of transferred embryos.

2. Abnormal nuclear reprogramming in clones

The reversal of cellular differentiation through SCNT is an unpredictable event [5]. The main factor responsible for the developmental capacity of clones is the oocyte's failure to restore a totipotent state to the transplanted nucleus, a process, largely unknown, defined as "Nuclear Reprogramming" [10].

Nuclear reprogramming is very limited under the current state of the art, as witnessed by the epigenetic deregulation and abnormal gene expression in pre and post implantation embryos [4,11], in newborn animals and extraembryonic tissue [6,7]. Although abnormalities have been reported in cloned foetuses/offspring [14], often they result from placental fluid imbalances affecting kidney function and therefore general homeostasis [15]. The extra-embryonic tissues are in fact severely affected at all stages of foetal development [16], and even in full term clones [8]. So far, functional and morphological abnormalities of placenta have been studied only in mice, cow and sheep clones, while data from other cloned animals are missing.

In mice [17] and cattle [18], a placental hypertrophy (placentomegaly) has been described, in contrast to findings in sheep [8]. In mice, the main histological abnormalities described are an increased number of glycogen cells and enlarged spongiotrophoblast cells [17]. Enlargement of trophoblast giant cells and disorganization of the labyrinth layer were also seen [17]. The placentae of cloned cattle display fewer placentomes, often larger than normal and irregular in size [19,20]. Histological examination revealed an hypotrophic trophoblastic epithelium and reduced vascularization [21]. The latter two alterations were also found in sheep cloned placenta [22] where ultrastructural studies also revealed features indicative of placental aging, such as thickening of the trophoblast basement membrane [22]. Despite the fact that the sheep was the first animal ever to be cloned, it is also the one which displays the most severe placental abnormalities responsible not only for fetal losses, but also for peri and postnatal demise of cloned lambs. The situation seems to be different in other large animals, where clones surviving the critical postnatal phase grow normally till adulthood, cattle in particular [23].

The phenotype of the first clones and particularly the corresponding placentae clearly indicated that imprinted genes are deregulated in clones. Therefore, studies on imprinted gene expression paralleled the cloning experiments first in mice [9,11], then in large animals [24,25]. The results indicated that imprinted genes, especially those involved in growth regulation, were abnormally expressed in cloned mice and other animals tested. This irregular pattern of gene expression explains the growth abnormalities described in clones, particularly in the placenta. Why the placenta is the organ where imprinted genes are often deregulated in clones is not clear. A plausible explanation might be that extraembryonic tissues have a short functional life, compared to the individual; therefore the epigenetic marks securing developmental genes might be not safely repressed. Furthermore, non canonical aneuploid/polynucleated cells are usually more tolerated in extraembryonic tissue [26] than in the foetus proper. In fact, tetraploid embryos develop a functional placenta which is able to nourish until term a foetus entirely derived from a clump of stem cells [27]. Further evidence of the "light" repression marks in extraembryonic tissue can be seen as early as blastocyst stage, where the trophoectoderm has significantly weaker DNA methylation - and therefore weaker repressive marks - comparing to the Inner Cells Mass (ICM) [28].

The addition of a methyl group to cytosine nucleotides, usually in GpC sequences found in promoter regions or throughout the body of the gene, is indicative of gene repression [29]. DNA methylation has been assessed with an antibody against methylated 5 cytosine (5meC) to measure the pan genomic epigenetic modification in cloned embryos, using the mouse as a reference model

(DNA de/methylation waves in early embryo development are well characterized in this species [30]).

After fertilization, an asymmetric DNA demethylation takes place after pronuclear organization leading to an active demethylation of the paternal pronucleus [31], whereas the maternal one is left unaffected. A progressive demethylation, probably a consequence of the rapid DNA replication cycles in the absence of oocyte-specific *de novo* DNA MethylTransferase (DNMT) 1 [32], follows during early cleavages [30]. By the blastocyst stage, a progressive methylation is observed in concomitance with the beginning of the differentiation process in ICM cells, while trophoblastic cells are hypomethylated [33]. These demethylation/methylation waves are lacking in cloned embryos, where heavy repressive epigenetic marks are present in both ICM and Trophectoderm (TE) lineages [34,35].

There are, however, differences between species in the de/methylation waves in early embryos [33]. Sheep and rabbit zygotes do not demethylate the paternal pronuclei, and also lack the passive demethylation typical of early cleavages [36,37]. Human zygotes are intermediate in this respect, for asymmetric demethylation of the two pronuclei is shown in only half of the processed zygotes, while in both maternal and paternal pronuclei faint methylation has been found in the remaining half [38]. It is difficult to draw global conclusions from these data. In our interspecific Intra Cytoplasmatic Sperm Injection (ICSI) study we demonstrated that a species biologically resistant to male genome demethylation (sheep), show demethylated male pronuclei in mice oocytes; on the other hand, sheep oocytes can partially demethylate the DNA of mice sperm [39]. These data suggest that oocyte cytoplasmic enzymes, as well as paternal chromatin composition/structure are important for reprogramming, as far as DNA methylation is concerned.

Obviously it is difficult to get definitive answers from these studies, but the main message is that the oocyte fails to reprogramme, as far as DNA demethylation is concerned, the genome of a somatic cell. But abnormal expression of imprinted genes is not the only pitfall in SCNT; pluripotency associated genes, essential for early differentiation and ontogenesis, like OCT-4, are also marked by DNA methylation for repression in somatic cells. Accordingly, many SCNT embryos display absent or reduced expression of these genes [40].

The sheer paucity of the DNA contained in the tiny embryos has long hindered the application of high throughput genetic analysis in clones. Encouragingly, the miniaturization of gene expression detection has been finally brought to the point that a reasonable panel of genes can be monitored at single blastocyst level [41]. DNA microarrays are providing precious information on the gene expression profiles of individual SCNT embryos [42]. The results obtained suggest that the SCNT process itself does not greatly affect gene expression [43]. Moreover, major effects on gene expression were exerted by the culture system, rather than the SCNT procedure in a recent study [41].

The general overview impression of the published data suggests that while major alterations are detected in the global epigenetic reorganization of SCNT clones, namely DNA methylation [36], analysis carried out at transcriptome level are indicative of a consistent reprogramming following NT [43].

These data are in strict conflict with the severe phenotype observed in foetuses and extraembryonic tissues in clones. Probably, minor epigenetic chances not detected by the current methodology exert their effect later, during organogenesis; or the few changes detected are amplified later during cellular differentiation [44]. However, gene expression and epigenetic analysis are of precious value for they indicate the molecular alteration that must be targeted to improve nuclear reprogramming [45].

3. Strategies to improve cloning

SCNT has many potential uses in animal breeding [46], the production of transgenic animals [47-50], and as a tool for conservation efforts [51,52]. Therefore, the development of efficient nuclear reprogramming approaches for the production of normal cloned animals is important.

On the basis of the published data we might assume that all mammalian species can be cloned using probably all cell types as nuclear donors, however, the low frequency of normal development remains low. Unfortunately, solutions proposed to maximize nuclear reprogramming success and in turn the frequency of development have had negligible, if any effect. Many attempts undertaken to improve SCNT, owing to the limited knowledge on reprogramming machinery, are prevalently empirical, other are instead suggested by the epigenetic and/or gene expression data available.

3.1. Donor Nucleus Cell Cycle: G0, prolonged Chromosome Condensation (CC)

Dolly's paper [1] and even the precedent report of cloning embryonic cell lines [53] suggested that the induction of nuclear quiescence was the trick to reset the memory of a somatic cell ([1], see Figure 1). The second cloned animals, a mouse, was produced thanks to a prolonged

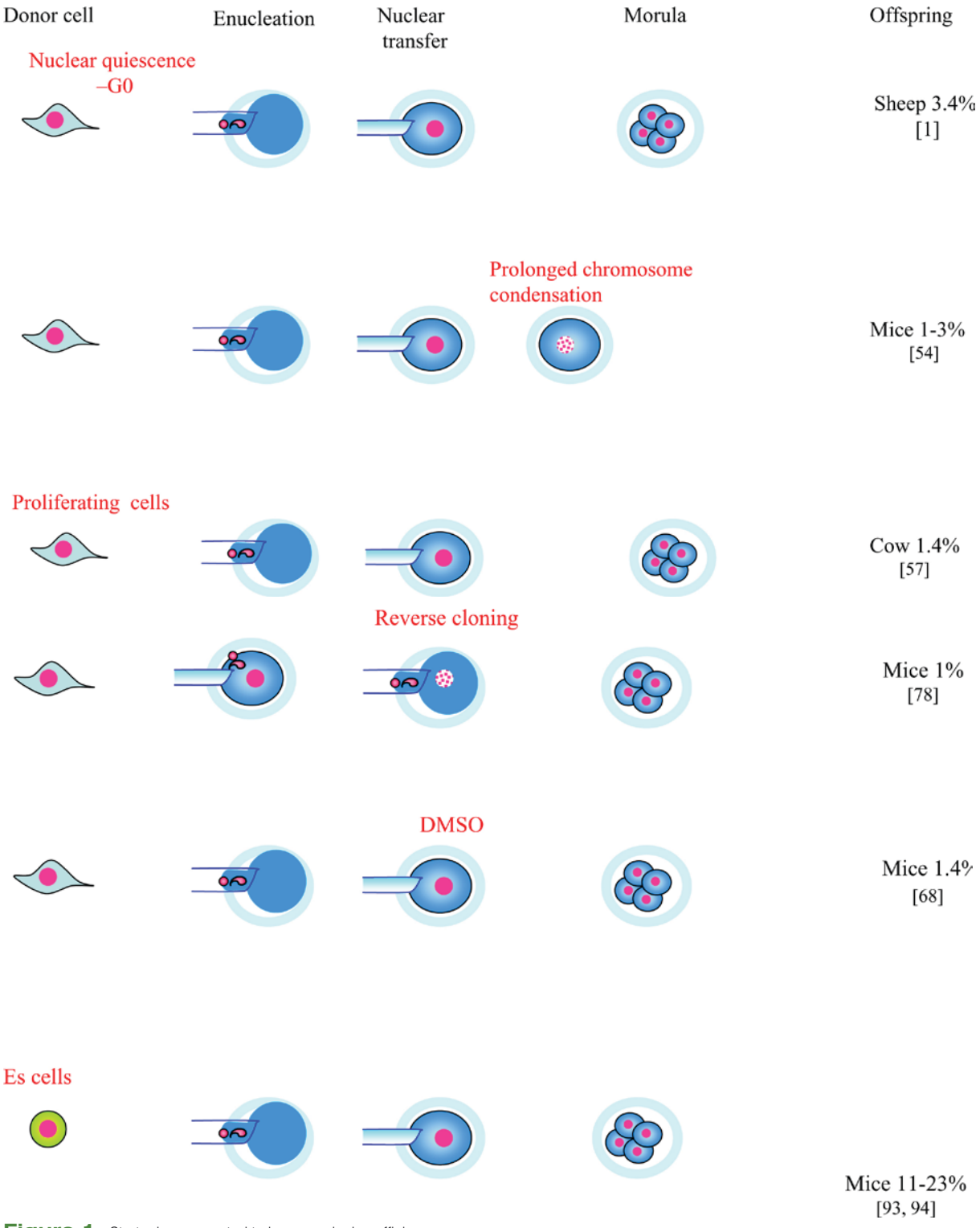
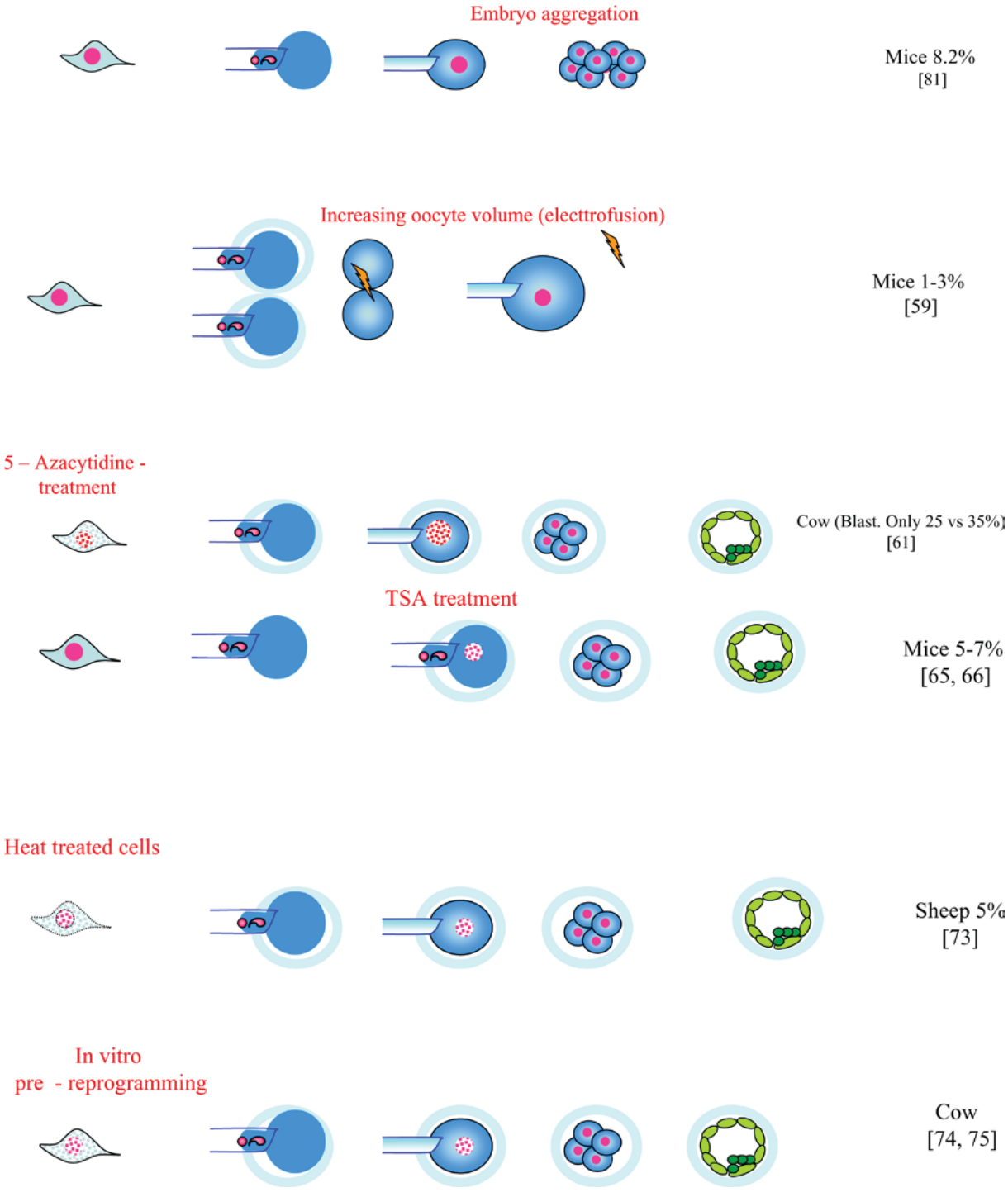


Figure 1. Strategies suggested to improve cloning efficiency.



continued **Figure 1.** Strategies suggested to improve cloning efficiency.

exposure of the transplanted cell nucleus in the oocyte cytoplasm ([54], see Figure 1). Both of the solutions advocated are backed up by scientific basis. During G0, but also following chromosome condensation, cell specific transcription factors are displaced from the genome [55,56] and somehow diluted in the egg cytoplasm, thus facilitating the action of reprogramming factors. These original hypotheses were challenged by other reports that demonstrated that the "reprogrammability" of the nucleus is not strictly dependent on a specific cell cycle stage ([57], see Figure 1), although the compatibility between donor nuclei cell cycle with the metaphase II

oocyte plays an important role indeed [58]. However, nuclear quiescence and delayed activation following NT are widely used in current SCNT protocols [59].

3.2. Oocyte volume

The enucleation involves the removal of a fragment of oocyte encasing the meiotic chromosomes. The size of the oocyte fragment varies according to the operator and with the method of enucleation, usually it is very large in the so called "hand made cloning" [13]. The consequence may be the removal of critical reprogramming factors eventually present around the oocyte chromosomes; alternatively, the reduction in oocyte volume might reduce its potential to develop into a normal embryo. These worries were laid to rest by convincing data which demonstrated that cloning with fully intact, or even bigger recipient cytoplasm (double oocyte produced by fusing two enucleated ones) did not make any change ([59], see Figure 1).

3.3. Demethylating drugs

The cell memory of a differentiated cell is not reset following NT, as indicated by the expression of tissue specific genes in a SCNT embryo [60]. The absence or reduced DNA demethylation of the somatic cells is commonly observed in cloned embryos [9]. Therefore, the use of molecules capable of removing the repressive methyl marks in differentiated cells before NT has been a logical extension of these observations. The drug used to effect this is 5-Azacytidine (5-Aza) . The results were however disappointing. Very few cloned bovine embryos derived from 5-Aza treated cells reached blastocyst stage ([61], see Figure 1). The finding is not surprising. It is known that 5-Aza leads to massive DNA rearrangements and micronuclei [62], thus magnifying the intrinsic drift of SCNT cloned embryos toward aneuploidy [37].

3.4. Hyperacetylating drugs: Trichostatin A (TSA)

Nuclei in cleavage stage embryos are larger than those present in somatic cells. Their chromatin maintains an open structure to allow the intense burst of transcription occurring in large portion of the genome, probably the most intense transcriptional activity the genome can exert. The chromatin histone composition is central to this structure, with histone acetylation playing a major role in keeping such open chromatin conformation. Accordingly, both parental genomes (the paternal first) are loaded with acetylated H4K18 early in the zygote [63,64], probably to facilitate chromatin remodelling. Trichostatin A inhibits histone deacetylases (HDACs), conferring an open chromatin structure through histone hyperacetylation. TSA has been used to treat donor cells before NT [61] or SCNT embryos in the peri-activation period [65] 10 hours post activation. TSA treated cells increased the frequency of development to blastocyst stage [61] in bovine nuclear transfer experiments, but *in vivo* development not assessed. Two studies in mice changed slightly the procedure, using TSA only after NT, I the post-activation phase. Both groups reported an improved frequency of development to term following TSA treatment (5% vs 1% in controls), indicating a positive effect of the drug on nuclear reprogramming ([65-67] see Figure 1). These results are important, representing the most significant breakthrough so far accomplished in SCNT. The results stress the importance of modifying the chromatin of differentiated cells to render it more easily remodelled by the oocyte cytoplasmic molecules.

3.5. Effects of Dimethylsulfoxide (DMSO)

Wakayama accidentally observed that the inclusion of a small amount of DMSO (1%) in the post activation phase of reconstructed embryos resulted in an improved development to blastocyst stage ([68] see Figure 1). It was unclear how the positive effect was exerted, until Iwatani discovered that DMSO affects the global genomic methylation levels [69]. Therefore, a modulation of the epigenetic state of the differentiated cell might be a plausible explanation of the positive effect exerted by DMSO on the cloned embryos. Unfortunately, the effect was limited to the pre-implantation development, for no improvements in living offspring was observed.

3.6. Serial nuclear transfer

Pioneering work carried out in amphibian demonstrated a beneficial effect on nuclear reprogramming by serial nuclear transfer, although the mechanism remained elusive at the time [70]. Serial nuclear transfer was then empirically carried out to improve the developmental competence of frog embryos cloned from differentiated cells [70]. The underlying mechanism was unveiled 25 years later. Using frog erythrocyte nuclei, French scientists demonstrated that the condensation of erythrocyte nuclei in *Xenopus* egg extracts prior to nuclear transfer conferred the capability to undergo the rapid DNA replication cycles typical of early cleavages in this species, resulting in turn in a better cloning efficiency [71]. However, similar studies repeated in mammals lead to opposite results, where a decrease in development was observed following serial nuclear transfer in mice and cattle [59,72]. Probably, mitotic/meiotic conditioning of a somatic nucleus is crucial to reset the chromatin

structure in species like the frog, where the impossibility for a differentiated adult donor nucleus to undergo rapid DNA replication within the egg is the main reason for the compromised development.

3.7. Destabilizing agents: Heat treated cells

The sperm nucleus is rapidly remodelled by the egg in order to release its inherent totipotency. A comparable level of remodelling is unlikely to be sufficient to reprogram a fully differentiated somatic cell. We recently demonstrated that thermal treatment destabilizes high-order structures of chromatin without compromising nuclear reprogramming, as witnessed by the successful embryo development *in vitro* and into viable lambs following embryo transfer ([73], see Figure 1). The importance of our work is that for the first time it has highlighted the need to modify somehow the chromatin of a somatic cell before nuclear transfer, in order to magnify its reprogrammability, as indicated by two recent reports ([74,75], see Figure 1). However, the very high cloning efficiency of the latter paper (41%) has not been confirmed by other groups working on bovine cloning.

3.8. Reverse cloning

Another issue related to the enucleation step is the removal of essential cell cycle – spindle factors in close association with the oocyte chromosomes. This possibility was suggested by cloning experiments carried out in macaque rhesus, which attributed the demise of the clones to the removal of critical spindle factors NuMA and HSET [76]. A modification of the SCNT procedure, "reverse cloning" was developed by the same group to verify the hypothesis [77]. In reverse cloning the donor cell is inserted into the intact oocyte and the enucleation carried out later, in order to allow the "migration" of critical spindle factors NuMA and HSET factors to the incoming set of chromosomes. However, no improvement was obtained in mice [78], suggesting that removal of the spindle does not undermine the development of clones. Moreover, macaque rhesus was successfully cloned using embryonic blastomeres in a previous experiment, thus ruling out any irreversible damage resulting from enucleation [79]. The situation seems to be different in the sheep, where reverse cloning apparently improved nuclear transfer success [80].

3.9. Embryo aggregation

This approach stems for the observation that the expression of a pluripotency associated gene, OCT-4, in cloned embryos is positively correlated with cell number at blastocyst stage [40]. Genetically identical cloned embryos were aggregated at 4 cell stage to increase the cell number ([81], see Figure 1). Embryo aggregation is a very simple and straightforward technique established many years ago. The Zona Pellucida (ZP) is removed by acid or protease digestion, then the naked blastomeres are treated with phytoemoagglutinin to make them sticky, and finally aggregated by pipetting them with a mouth pipette. The rationale of this solution was to "complement" the embryo, practically speaking to increase the chances that the cloned embryo expresses enough OCT4, or to boost OCT4 expression to a level compatible with normal embryogenesis. Clone-clone aggregates did not form more blastocysts, but the majority expressed Oct4 normally and had higher rates of fetal and postnatal development (1% vs 8% in aggregated clones [81]).

3.10. Mitochondrial DNA (mtDNA) composition of the reconstructed embryos

Following natural fertilization, mitochondria brought by the sperm cell are targeted for destruction, leading to a single identical population of maternal mtDNA [82]. In the current NT protocols, the donor nuclei are exposed to a new complement of mtDNA, with the possibility of compromised mtDNA and genomic DNA cross talk limiting the development [83]. Moreover, the presence of mtDNA derived from the somatic cell might create a further complication, defined mtDNA heteroplasmy [84]. It is important to ensure that the somatic nucleus is capable of sufficiently regulating the oocyte mtDNA. The importance of mtDNA composition has been indirectly shown in cloning experiments where autologous SCNT (somatic cells and oocyte derived from the same female donor) developed at higher frequencies, both at blastocyst stage and to term [85], compared to heterologous SCNT (donor cell not related to recipient cytoplasm).

3.11. Activation protocols

In normal development the fertilizing spermatozoa releases the meiotic arrest and triggers the developmental programme of the egg. This fundamental step in skipped in SCNT, therefore the activation stimulus must be artificially applied to the oocyte. There has been a plethora of studies addressing this issue since the era of embryonic blastomere cloning, therefore, the reader is directed to the many reviews published [86].

The activation protocol varies with the species, therefore the cloner must check the reliability of a date activation method, before applying it to SCNT [87]. However, there is evidence in mice that activation is not a major hurdle for SCNT, for oocytes activated in the physiological way, through the sperm, and then

enucleated and reconstructed with a somatic cell, have a comparable development with controls [59]. A report on cattle cloning however challenges these data, providing evidence from an improved development to preimplantation and to term of cloned embryos activated through fertilization [88]. This new insight is compatible with an unexpected role of Ca^{++} in oocyte/embryo development. Classically thought as the intracellular signal dispensable for the start of oocyte meiosis and metabolism following oocyte activation, Ca^{++} released at fertilization has long-term effects on both gene expression and development to term in mice [89].

3.12. Choice of donor nuclei

SCNT has been achieved with a plethora of cell types. The information gained so far is that also in mammals there is an inverse relationship between the differentiated state of a cell and its "reprogrammability"; similar to the amphibian situation [60]. Terminally differentiated cells, like B –T lymphocytes and olphactory neurons do not make offspring following SCNT, unless a "two step nuclear transfer" (where ES cells established from cloned blastocyst are injected into tetraploid blastocyst) is carried out [90,91]. The need for a two step nuclear transfer was skipped in a recent paper which lead to the production of cloned pups from NT of natural B cells [92]. Therefore, we should select for NT less differentiated cells, in theory more easily reprogrammable from the oocyte. This is in fact the case in mice, where ES cells used as nuclei donor for NT resulted in a high frequency of development to term ([93,94], see Figure 1).

Less defined is the "clonability" of somatic stem cells. Studies from independent groups comparing the efficiency of nuclear reprogramming of NT embryos reconstructed with stem cells isolated from adult tissue or with differentiated cells resulted in comparable outcomes [95-97]. However, it is likely that there are differences between the sources of adult stem cells. Taken together, the published data indicate that tissue-specific stem cells exhibited marked variations in the ability to produce cloned offspring, probably as a result of the epigenetic status of the original genomes [98].

Few of the solutions proposed to improve nuclear reprogramming, and in turn the frequency of normal offspring in SCNT, are truly effective. Only two approaches, both tested in mice, resulted in an effective benefit, TSA treatment [59] and the use of ES cells as nuclei donors [93,94]. This finding is however far from being satisfactory. First, the effect of TSA on development to term of clones has been tested only in mice, no data has been published on other species. Similarly, the ES cell cloning applies to mice only, for no embryonic stem cells have been isolated in other "clonable" mammals. Moreover, the advantages resulting from TSA or ES cell cloning are still very limited, if we critically analyze the data. As far as ES cloning is concerned, development into viable pups was higher in ES cloned embryos, but development to blastocyst stage was lower than in control somatic cell clones, therefore, the overall efficiency was basically comparable. Even for TSA, 5% of the TSA treated clones developed into pups, comparing to the 1% in the untreated group. Our view is that cloning still misses the essential breakthrough/s capable of providing the vital leap in clone development, let's say 20-25% development to term, with absent or drastic reduction of adverse phenotypes.

4. Conclusions

SCNT is a complex multi step procedure which includes oocyte maturation, enucleation, cell fusion/injection, oocyte activation and embryo culture, and the efficiency achieved in each one accounts for the final success. Oocyte physiology, activation dynamics and preimplantation embryo metabolism differ markedly between species, therefore, there is not a standardized protocol that can be applied, thus leading to species specific differences in the cloning efficiency. The fact that cloning efficiency is highest in the bovine, the species with the most advanced embryo technologies [23] underlines this. Another critical factor affecting nuclear reprogramming is the timing of zygotic genome activation (ZGA). Species where ZGA is delayed until morula stage may better benefit from the reprogramming machinery. Accordingly, the mouse, with ZGA starting late in the first cell cycle has a much lower efficiency than the bovine, where ZGA takes place during the fourth cell cycle [3].

However, the species specific differences mentioned above have a minor influence on cloning outcomes, if we consider that even in the most suitable species for SCNT, the bovine, the efficiency is still too low. The main factor limiting the full application of SCNT is the abnormal nuclear reprogramming [11], complicated by the high frequency of aneuploidy in SCNT clones [37].

Nuclear reprogramming remains very limited at the moment. The solutions so far tested, like DNA demethylation and histone hyperacetylation [61,65,66], rely on bulk, non-specific effects which might lead to positive as well as negative effects. More realistic instead is a multi-step approach, starting from gamete/oocyte biology, to controlled nuclear reprogramming. A great deal of effort should be dedicated to the optimization of *in vitro* system for the mass production of fully competent recipient oocytes. Robust protocols

are available for the maturation of ovine, bovine and pig oocytes, but other clonable species, particularly wild and rare animals, are unknown from this point of view. Preimplantation development of cloned embryos is often carried *in vitro*, using culture media formulated for normal embryos. There is evidence that SCNT embryos develop better in complex media, suggesting that some of the metabolic pathways of the differentiated cell are still active after nuclear transfer [99]. The development of "compromise" media, between embryos and somatic cells should improve the viability, and in turn nuclear reprogramming in cloned embryos [100,101].

Far more complicated is the nuclear reprogramming issue, even in the light of the recent evidence that other subnuclear organelles, like the nucleolus, play an important role in driving the nuclear remodelling machinery [102]. This work shows that the nucleolus in the embryo originates from the oocyte, even in embryos produced by SCNT, demonstrating that the maternal nucleolus supports successful embryonic development. These results stress further the complexity of genome reprogramming, adding meanwhile further evidence of the "asymmetry" of the parental genomes in normal and cloned embryos, as we recently suggested [103]. An important tool developed is monitoring in living embryos the expression of critical genes through their coupling with fluorescent tags [45]. Such systems are of precious value not only because we can assess objectively the extent of reprogramming, but also because we can evaluate in a short time the effectiveness of a date cloning protocol/variant. These improvements, jointly with the growing understanding on the biochemistry of essential reprogramming steps, like DNA de/methylation [34], are sure indicators for the future improvements waiting for cloning.

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