Published in final edited form as:
Mol Reprod Dev. 2014 February ; 81(2): 183–193. doi:10.1002/mrd.22271.

Reshaping the Transcriptional Frontier: Epigenetics and Somatic Cell Nuclear Transfer

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SUMMARY

Somatic-cell nuclear transfer (SCNT) experiments have paved the way to the field of cellular reprogramming. The demonstrated ability to clone over 20 different species to date has proven that the technology is robust but very inefficient, and is prone to developmental anomalies. Yet, the offspring from cloned animals exhibit none of the abnormalities of their parents, suggesting the low efficiency and high developmental mortality are epigenetic in origin. The epigenetic barriers to reprogramming somatic cells into a totipotent embryo capable of developing into a viable offspring are significant and varied. Despite their intimate relationship, chromatin structure and transcription are often not uniformly reprogramed after nuclear transfer, and many cloned embryos develop gene expression profiles that are hybrids between the donor cell and an embryonic blastomere. Recent advances in cellular reprogramming suggest that alteration of donor-cell chromatin structure towards that found in an normal embryo is actually the rate-limiting step in successful development of SCNT embryos. Here we review the literature relevant to the transformation of a somatic-cell nucleus into an embryo capable of full-term development. Interestingly, while resetting somatic transcription and associated epigenetic marks are absolutely required for development of SCNT embryos, life does not demand perfection.

> "[It] is not difficult to imagine and identify problems with epigenetic reprogramming of somatic cells leading to failed development; it is much more difficult to explain how the process of SCNT actually (sometimes) works."

FORGING A PATH TO THE NUCLEAR LANDSCAPE

The idea of cloning animals by employing nuclear transplantation is historically well established. In fact, Hans Spemann, often referred to as the "father of cloning," described the process when he referred to a "fantastical experiment" in his book, *Embryonic Development and Induction* (Spemann, 1938). The first demonstration that nuclear transplantation could potentially be used to clone adult animals using somatic cells, however, came when Sir John B. Gurdon reported the utilization of nuclear transfer to produce cloned frogs using cells obtained from the gut of feeding tadpoles (Gurdon, 1962). This experiment represents the first reported example of a somatic cell being reprogrammed back to a totipotent state by an enucleated egg and developing into a live, viable offspring. The importance of this work was recently recognized by the world when Gurdon was awarded the 2012 Nobel Prize in Physiology and Medicine.

Initial research with amphibians was carried out in the early and mid-1900s, but it was not until the late 1970s that any significant work to clone mammals using nuclear transfer was

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performed (Illmensee and Hoppe, 1981). Preliminary work was performed on mice with reported success, yet many attempts by different laboratory groups failed to yield live offspring, and in 1984 Jim McGrath and Davor Solter published the results of their research in *Science* with a statement that "cloning mammals by simple nuclear transfer was biologically impossible" (McGrath and Solter, 1984). Scientists at that time thought the problem was related to differentiation, such that as embryonic cells became more differentiated, their genome could not be reprogrammed and therefore could not be used for cloning. This idea seemed to hold true as cells obtained from two-cell mouse embryos could be used successfully for nuclear transfer, resulting in live offspring, but not four-cell or beyond. Work in other species supported this idea. Willadsen (1986) reported the successful cloning of sheep and then cattle, using 8–16 cell embryos as nuclei donors. This early-cleavage stage is analogous to the two-cell mouse embryo as this period marks the maternal zygotic transition, the time point when an embryo starts producing its own mRNA and protein.

The next major step towards the cloning of adult animals was reported by Sims and First in 1994, when cattle were produced from cells of the inner cell mass that were cultured for up to 28 days under conditions that attempted to maintain the potency of the original cells (Sims and First, 1994). This was followed by what Keith Campbell considered to be the defining work that led to the widespread production of cloned offspring from somatic cells: Campbell et al. grew embryo-derived cells for extended passages under standard tissue culture conditions, which led to a clearly differentiated cell type. Using these differentiated cells for somatic-cell nuclear transfer (SCNT), the group was able to produce viable offspring, but only following serum starvation of the cells to induce a quiescent cell-cycle state. From a historical point of view, these were the experiments that predicted that cloning with adult cells would soon follow. As far as major breakthroughs in cloning mammals are concerned, this work utilizing differentiated cells growing in culture to produce cloned animals could just as easily be considered equally relevant as the research that ultimately resulted in Dolly (Campbell et al., 1996).

Dolly of course was the first cloned animal derived from an adult somatic cell. Essentially, her birth involved the continuation of research by Campbell and Ian Wilmut, who were using fetal cells growing in culture for nuclear transfer (Wilmut et al., 1997). In one series of experiments, adult cells derived from mammary epithelial cells were used as nucleus donors, but these embryos were not expected to develop to term. Campbell at least entertained the idea that these cells might work, but others on the team were more skeptical (K. Campbell, personal communication). Regardless of what was actually going through their minds in terms of potential outcome of the experiment and expectations, it is clear that the birth of Dolly brought a completely unexpected surprise. The overwhelming response from the general public and the world as a whole was surprising, given the long history of cloning animals from differentiated cells in amphibians and having previously produced cloned sheep and cattle from cells growing in culture. The major difference, of course, was that Dolly was derived from a cell obtained from an adult animal.

THE SOLID BUT AMORPHOUS BARRIER IN THE PATH TO EFFICIENT SCNT

Today, while more than 20 different animal species have been cloned by employing nuclear transplantation, the process still remains inefficient. Reproductive cloning by nuclear transfer using any donor cell type incurs losses throughout early preimplantation, postimplantation, and pre- and post-natal development. The causes of the observed loss are due to a variety of factors that can be divided into four main categories: micromanipulation trauma, oocyte incompetence, in vitro-culture-induced anomalies, and failed epigenetic

reprogramming of the donated nucleus. There is significant variation in the literature when comparing the efficiency of laboratories in terms of successful production of cloned offspring derived from transplantation of the donor cell nucleus into the cytoplasm of an enucleated oocyte (or sometimes zygote). In reality, the successful production of cloned offspring by SCNT requires that all of these categories be addressed. Although estimates vary, it is not unreasonable to expect only 5–10% of reconstructed embryos transferred into gestational surrogates to develop into a viable offspring. There are a multitude of scientific publications reporting various success rates with cloning animals, and many different factors have been identified that influence the outcome. Regardless of how the data are analyzed and interpreted, cloning animal remains a very inefficient process.

It is also important to point out that the observation and term “large-offspring syndrome” was initially derived from numerous reports of abnormal placentation and extremely high birth weights observed in cloned calves, even when embryonic cells were used as nucleus donors. Similar but less extreme examples were often seen in association with in vitro embryo production in cattle and sheep (Wilson et al., 1993; Young et al., 1998). Yet none of the abnormalities of large offspring syndrome were ever transmitted to the animal’s progeny. These observations led to the model that the inefficiency and abnormal development sometimes observed when using SCNT and other assisted reproduction in animals were a result of failed epigenetic reprogramming.

Epigenetics refers to differential patterns of gene expression conferred through the physical and biochemical properties of chromatin, without a change in DNA sequence. The status of DNA methylation and histone modifications is continuously changing, and subject to a wide variety of different environmental factors. The first few days of preimplantation development represent a critical stage for establishing the embryonic epigenome, which is necessary for directing normal development. Such epigenetic reprogramming represents a crucial event in mammalian development, appears to be conserved across all mammalian species, and is essential for normal mammalian development to proceed (Li et al., 1992; Okano et al., 1999; Dean et al., 2001; for more extensive reviews about establishing and maintaining epigenetic modifications during embryonic development, see also Armstrong et al., 2006; Schwartz and Pirrotta, 2007; Bartolomei, 2009; Mason et al., 2012; Velker et al., 2012; Cantone and Fisher, 2013; Paul and Knott, 2013).

Aberrant gene expression that results from improper epigenetic reprogramming by the oocyte has become a key area of investigation to explain failed and aberrant development following SCNT. Gene expression in embryos, concepti, and even in adult animals produced by SCNT is abnormal (Humpherys et al., 2001, 2002; Rideout et al., 2001; Wrenzycki et al., 2001, 2006; Hall et al., 2005; Ono and Kono, 2006; Aston et al., 2010; Mesquita et al., 2013). These abnormalities have been tied to incomplete epigenetic reprogramming by the oocyte following nuclear transfer and to abnormal patterns of DNA methylation in cloned embryos (Kang et al., 2001; Mann et al., 2003; Santos et al., 2003; Dindot et al., 2004; Kremenskoy et al., 2006; Wrenzycki et al., 2006; Yamazaki et al., 2006; Golding et al., 2011). Further evidence of atypical epigenetic reprogramming in cloned animals comes from a study documenting aberrant patterns of X-chromosome inactivation in calves produced by cloning (Xue et al., 2002). These observations imply that inadequate epigenetic reprogramming, including aberrant DNA methylation, is associated with and may cause anomalous gene expression during a critical stage of development, ultimately resulting in deviant or failed embryo/fetal development (Bourc’his et al., 2001; Beaujean et al., 2004; Niemann et al., 2008; Couldrey and Lee, 2010; Peat and Reik, 2012).

Preimplantation development represents a particularly dynamic period of development during which the epigenome of the egg and sperm are reprogrammed, setting the stage for

subsequent development. This reprogramming occurs in the cytoplasm of the mammalian egg, and is directed by factors contained within it. Although experiments involving nuclear transfer demonstrate they have some capacity to reprogram the epigenome of somatic cells, the ovum did not evolve to carry out this feat. As such, it is not surprising that animal cloning is inefficient, and that mounting evidence supports the idea that problems with epigenetic reprogramming of the somatic nucleus are the primary cause of SCNT embryo mortality (Golding et al., 2011; Mesquita et al., 2013; Salilew-Wondim et al., 2013).

DNA methylation was one of the first and is still the most extensively investigated epigenetic mark in embryo and conceptus development (reviewed by Brandeis et al., 1993; Reik and Dean, 2001; Dean et al., 2005; Latham et al., 2008; Mason et al., 2012; Cantone and Fisher, 2013). DNA methylation within gene promoter regions is associated with a repressive transcriptional state. Somatic-cell nuclei display more extensive patterns of global methylation than embryonic or pluripotent cells, as would be expected of a more transcriptionally restrictive cell fate. Following nuclear transfer, SCNT embryos undergo incomplete global demethylation and aberrant remethylation (Bourc'his et al., 2001; Dean et al., 2003; Santos et al., 2003). Furthermore, nuclei of SCNT embryos typically fail to fully undergo the wave of demethylation observed during normal embryonic development, leading many to conclude that this failure to reprogram the somatic epigeneome is responsible for unsuccessful development of SCNT embryos (Reik et al., 2001; Tollervey and Lunyak, 2012).

There seem to be concomitant mechanisms that set the stage for proper epigenetic regulation via DNA methylation in the early embryo. First, the rapid demethylation in the paternal genome documented in some species (Santos et al., 2002; Fulka et al., 2004; Jeong et al., 2007) is actually a transition from 5-methylcytosine to 5-hydroxymethylcytosine, which is mediated by the TET gene family and modulated by PGC7 (Stella) (Tahiliani et al., 2009; Ito et al., 2010; Gu et al., 2011; Inoue and Zhang, 2011; Iqbal et al., 2011; Koh et al., 2011; Branco et al., 2012; Nakamura et al., 2012; Costa et al., 2013). Yet, this modification is undetectable by antibodies for 5-methylcytosine or by traditional bisulfite sequencing, which also cannot differentiate between methylated and hydroxymethylated cytosine (Huang et al., 2010). Second, the exclusion of DNA methyltransferase 1 (DNMT1) from the nucleus during early murine development clearly associates with passive DNA methylation during DNA replication and cell division (Doherty et al., 2002). Finally, active deamination and base excision repair is a mechanism of DNA demethylation by which levels of DNA methylation are maintained during early development (Barreto et al., 2007; Niehrs and Schäfer, 2012; Seisenberger et al., 2013). Together, these processes drive the early embryo into a state of hypomethylation, as compared to the hypermethylated state of typical somatic-cell nuclei. Interestingly, this demethylation occurs while maintaining the critical chromatic modifications that mark the parent-of-origin genomic imprints necessary for proper development (Bartolomei and Ferguson-Smith, 2011). The need to maintain the epigenetic status of imprinted genes through DNA methylation (and other chromatin modifications) suggest that global hypomethylation of the somatic genome prior to SCNT improves embryonic stem cell derivation, but is unwise if production of offspring is desired (Blelloch et al., 2006; Eilertsen et al., 2007; Giraldo et al., 2009; Peat and Reik, 2012).

Histone modifications are also an integral part of the transcriptional regulation of any cell type (reviewed by Han and Yoon, 2012; Mason et al., 2012; Tollervey and Lunyak, 2012; Velker et al., 2012; Cantone and Fisher, 2013; Ogura et al., 2013; Paul and Knott, 2013). Here, we focus our attention on the importance of histone acetylation, one class of epigenetic modification with an associated group of drugs that have demonstrated effectiveness at improving live births following SCNT. Histone acetylation is typically associated with "active" transcription, although in the context of reprogramming, alterations

in chromatin acetylation cannot be considered solely as activating since sequence-specific transcription factors necessary for the initiation of gene expression must also be available for active transcription. Furthermore, the DNA may not be in the correct spatial configuration necessary to allow for active transcription at the particular stage of development. Thus, it would be more appropriate to consider this histone modification responsible for the generation of a permissive transcriptional process, thereby allowing drugs or other interventions that increase global histone acetylation to make the genome more malleable and amenable to reprogramming.

A MORE EVIDENT STRUCTURE OF THE SCNT BARRIER

During development, progressive morphological and functional changes gradually enable the separation of tasks in order to form an integrated physiological system. This forward-moving process is driven by alterations in gene expression that arise from both the regulative activity of lineage-specifying transcription factors and the progressive alteration of chromatin structure. Epigenetic and structural changes to the chromatin occur shortly after fertilization, and persist throughout development and into adulthood (Ono and Kono, 2006). For an embryo reconstructed through SCNT to develop and thrive, it must successfully use oocyte-derived factors to erase the transcriptional state of the donor cell and alter the transferred chromatin structure to an embryonic state. Thus, the cytoplasm of the oocyte must contain the requisite transcription factors that initiate embryonic patterns of gene expression, and the chromatin structure of the donor cell must be amenable to change. This latter point is viewed as being the determinative step in nuclear reprogramming, and is often cited as being the sole, underlying cause of developmental failure of SCNT embryos (Ng and Gurdon, 2005; Blelloch et al., 2006; Vassena et al., 2007a; Zhou et al., 2009; Aston et al., 2010; Rodriguez-Osorio et al., 2012).

Work during the 1970s demonstrated that genomic DNA is wrapped around a protein octamer of four core histones to create the nucleosome core particle (Kornberg, 1974). Since these initial studies, we have come to understand that the step-wise partitioning of this nucleosome core into distinct, transcriptionally accessible and inaccessible regions of the nucleus has a major influence on gene expression, and is an indispensable component of cellular differentiation. Recent studies examining patterns of DNAse hypersensitivity across multiple different cell types have demonstrated that nucleosome positioning is actually more predictive of cell lineage than the transcriptome (Stergachis et al., 2013), indicating that the chromatin landscape may actually be a stronger determinant of cellular identity than gene expression patterns alone and that the chromatin remodeling step of SCNT is the major determinant of developmental success or failure.

PIONEERING A NEW PATH THROUGH THE SCNT BARRIERS

Studies using a variety of cell-culture models have demonstrated that distinct cell types are initially specified by the activity of a core group of lineage-specifying transcription factors, termed the pioneer transcription factors (Zaret and Carroll, 2011). These key proteins are typically the first to engage target sites in the genome and to induce the genomic structural rearrangements necessary for other ancillary factors to bind. The core mouse embryonic stem cell factors OCT4, NANOG, SOX2, KLF4, and ESRRB are likely the best-characterized pioneer transcription factors, owing to their ability to redirect cultured cells back into a pluripotent fate (Takahashi and Yamanaka, 2006). Once established by lineage-specific pioneer transcription factors, the identity of each developing cell type is maintained and propagated through unique alterations in the way in which the DNA encoding each gene becomes organized within the nucleus.

Advances in sequencing technologies combined with chromatin immunoprecipitation techniques have enabled genome-wide analyses of numerous different families of transcription factors. These studies have revealed that in any given cell type, the vast majority of available DNA-binding sites remains unoccupied, presumably owing to the inaccessibility of the DNA template (Thurman et al., 2012). Interestingly, actively bound sites cluster into an extremely small fraction representing only 0.8% of the genome (Yan et al., 2013). These observations and others have prompted the development of a hierarchical model in which cell-lineage-specific combinations of transcription factors work in a collaborative manner to activate key enhancer regions necessary to direct the control of the gene expression patterns in each developing cell type (Heinz et al., 2010; Gerstein et al., 2012; Hawrylycz et al., 2012). Pioneer transcription factors rearrange the chromatin landscape and reposition select enhancer binding sites within this crucial 0.8% domain, making them available for binding by the general transcriptional machinery (Whyte et al., 2013). Once chromatin structure has been reorganized into a cell-specific conformation, the Cohesin protein complex maintains the unique patterns of chromatin looping that partition the genome into the appropriate accessible and inaccessible regions of the nucleus (Whyte et al., 2013; Yan et al., 2013). Importantly, the Cohesin protein complex remains bound through the S phase of the cell cycle, retaining enhancer sites in a set conformation while seemingly all other transcription factors are evicted during early M phase (Martínez-Balbás et al., 1995; Yan et al., 2013). Thus, enhancer-bound Cohesin and the resulting patterns of chromatin looping represent a core aspect of cellular memory passed from mother to daughter cell—one that must be both erased and subsequently reestablished during SCNT development.

BREAKING THE SCNT REPROGRAMMING BARRIER REQUIRES DRAMATICALLY ALTERING TRANSCRIPTION

Cellular reprogramming is a slow process, whether it is in the context of SCNT or induced reprogramming of somatic cells to a pluripotent state. This process manifests over time, during the mitotic divisions, and requires that chromatin structure be repetitively disassembled and reassembled. Under experimental conditions, most—if not all—somatic cells are considered to be reprogrammable under the appropriate conditions and given enough time (Hanna et al., 2009). The phenomenon of reprogramming can be functionally evaluated by measuring the rate of blastocyst production, the ability to derive embryonic stem cells from SCNT embryos, and the proportion of embryos that survive to produce live offspring (Blelloch et al., 2006); only the latter is a true evaluation of complete reprogramming to the totipotent state.

The oocyte is not a transcriptionally active cell at the time of SCNT, thus all proteins required for cellular function until the maternal-zygotic transition are accumulated during oogenesis and are present to reorganize the newly introduced somatic nucleus. Thus, successful reprogramming of a somatic nucleus into an embryonic nucleus must make use of the transcription factors, nucleosomal components, chromatin modification enzymes already present in the egg, and rests on how well the appropriate transcriptional machinery can access the correct DNA sequences and proceed through transcript initiation and elongation phases in order to produce blastomere-specific mRNAs in the correct temporal fashion. Balanced transcriptional control is also required for the proper response to the changing embryonic microenvironment. Analysis of clones and their offspring from other assisted reproductive technologies revealed that, despite the high variability in gene expression profiles, the survivors have transcriptomes that rarely fall outside the range of “normal” in the context of the entire population because the “abnormal” ones simply do not survive (Humpherys et al., 2001, 2002; Rideout et al., 2001; Turan et al., 2010).

Cell-fate-determination events are progressively imparted throughout development. Despite this gradual process, SCNT embryos very likely become out of balance, their gene expression patterns representing a chimerism of the donor cell type and an embryonic blastomere—and fail at varying points during gestation due to an inability to properly respond to spatial or temporal signals. Latham et al. demonstrated that mouse clones produced from myoblast cells developed poorly in embryo culture media. These SCNT embryos retained some of the donor's transcriptional profile, and with it the preference for the culture conditions from which those somatic nuclei were derived, including a preference for glucose (Gao et al., 2003). These experiments indicated that the donor nuclei were not fully reprogrammed, and although they were capable of carrying out basic cellular functions required by all living cells, the transcriptional profile of the early embryo became a "hybrid" of somatic and embryonic transcripts (Vassena et al., 2007a,b). These data also indicate that certain regions of the somatic-cell genome were more easily reprogrammed by the oocyte than others, and that these differences may be more attributable to the continued, active transcription of select regions rather than the reactivation of somatically repressed loci. Recent studies detailing the transcriptome of mouse SCNT embryos derived from Sertoli cells clearly demonstrate that aberrant transcription starts at the one-cell stage and continues through development (Cao et al., 2013). Thus, the oocyte may frequently fail to restructure the genome or cannot fully block transcription of some active genetic regions. Alternatively, active transcription of somatic genes may tag these loci to be preferentially engaged by the Cohesin protein complex, prolonging the maintenance of transcription from these sites. This would imply that the oocyte presents a somewhat promiscuous environment for gene expression, allowing some actively transcribed genes to continue transcription, which alters blastomere function accordingly.

These observations imply that the chromatin landscape does not quickly remodel upon exposure to the oocyte cytoplasm, and that it very likely takes several cell cycles to reorganize and reposition chromatin domains. Studies examining the generation of induced pluripotent cell lines indicate that the in vitro process of reprogramming cells in culture is a stochastic process that requires many rounds of cell division (Buganim et al., 2012). There is also a positive correlation between higher number of cell divisions before embryonic genome activation and developmental success. For example, cattle undergo three rounds of mitosis before the blastomere nuclei initiate transcription of the somatic-cell genome, whereas mice begin transcription after a single round of DNA synthesis and mitosis. The efficiency of offspring production in cattle cloning is on the order of 5–10 times that of mice. Furthermore, initial success at cloning of mouse lymphocytes was only capable of producing offspring when embryonic stem cells were used as an intermediate step (Hochedlinger and Jaenisch, 2002). This method essentially prolongs the exposure of the somatic mouse nuclei to embryonic conditions and provides a longer time for the proper transcriptional profile to become established. The greater number of cell cycles prior to initiation of embryonic transcription very likely allows oocyte- (or embryonic stem cell-) derived pioneer transcription factors time to move select enhancer regions into and out of the crucial 0.8% space. This could prompt the Cohesin complex to reorganize from the binding pattern found in the donor cell towards one appropriate of an embryonic blastomere. Transcriptome analysis further indicates that the remodeling process in SCNT embryos is hasty, leaving some epigenetic information from the donor cell to persist while other crucial aspects of chromatin structure—like genomic imprints—can be lost (Mann et al., 2003).

The above observations identify two important points about how to evaluate nuclear reprogramming: (1) alterations in gene transcription may not be an accurate indicator of true nuclear reprogramming and (2) examinations of epigenetic alteration of enhancer regions are likely to be more informative of nuclear reprogramming than chromatin modifications/DNA methylation levels within the promoter regions of candidate genes. Work from multiple

laboratories indicate genomic enhancer regions are characterized by the presence of H3K4me1 and/or H3K4me2 modifications, H3.3/H2A.Z variants, p300 binding, and DNase hypersensitive and histone-free regions (Heintzman et al., 2007; Heinz et al., 2010; Rada-Iglesias et al., 2011). As technologies advance and the prospect of conducting chromatin immunoprecipitation/chromatin capture analysis in embryos become technically feasible, it will be exciting to dissect the dynamics of SCNT nuclear remodeling.

ASSISTED REPROGRAMMING OF SOMATIC NUCLEI CAN WEAKEN THE EPIGENETIC BARRIER

Reprogramming of the somatic nucleus by an enucleated oocyte is a consequence of a complex interaction between the somatic chromatin and the proteins in the oocyte cytoplasm. In most cases, the somatic cytoplasm is also incorporated into the reconstituted embryo, therefore a mixture (albeit disproportionate) of both oocyte and somatic proteins is present. The reprogramming factors of the egg must alter the somatic transcription profile in order to establish a zygotic state and to reinitiate totipotency. During the SCNT reprogramming phase, the nuclear and cytoplasmic components responsible for remodeling chromatin and reprogramming transcription in early-cleavage stages are interacting with epigenetic marks, transcriptional complexes, and chromatin structure that are normally foreign to the oocyte cytoplasm. As the epigenetic state of somatic chromatin at the time of exposure to the oocyte cytoplasm is important, alteration of the somatic cell chromatin status prior to recombination with the egg could reasonably be expected to improve the efficiency of the reprogramming process and enhance the developmental capacity of the SCNT embryo. A number of studies have attempted to alter the chromatin architecture in order to improve reprogramming.

Oocyte Extracts Enhance Reprograming Efficiency

Somatic cells are developmentally programmed to maintain the transcriptional activity of their differentiated state. These cells retain their phenotype by maintaining the chromatin structure and epigenetic modifiers that promote the continued expression of proper transcription factors required for their functional characteristics. If, in fact, reprogramming of the somatic nucleus is enhanced by prolonged immersion with oocyte-specific factors, then it stands to reason that improvements can be made in cloning efficiency by repeatedly exposing them to the oocyte cytoplasm.

Consistent with the idea that proper reorganization of the chromatin and reprogramming of transcription requires extended contact with reprogramming factors of the egg, preincubation of permeabilized somatic cells in homologous or heterologous oocyte extracts have been shown to increase cloning efficiency (Bui et al., 2008; Rathbone et al., 2010; Bui et al., 2012). Early experiments in livestock cloning using cell extracts to remodel chromatin prior to SCNT alluded to improved development rates (Sullivan et al., 2004), but have not been confirmed by subsequent studies. Furthermore, this method exhibits transcriptional and placental anomalies similar to standard SCNT procedures, suggesting that additional fine-tuning is required before this approach becomes useful (Mesquita et al., 2013; Zhou et al., 2008). Additional results reported by Rathbone et al. demonstrated a nearly fivefold increase in live sheep following SCNT with extract-treated donor cells. This was offset by a high rate of postnatal death, leading to a modest 1.9-fold increase in efficiency. Whether or not the postnatal loss was a consequence of the somatic nuclei exposure to the Xenopus egg extracts is difficult to determine. None-the-less, the production of offspring was enhanced by heterologous extract treatments and demonstrates Xenopus egg extracts are equally capable of initiating reprogramming, as previously suggested by work in mice examining exposure to homologous germinal vesicle extracts (Bui et al., 2008). The biochemical nature of the

extract effect on somatic nuclei is, no doubt, complex given the number of proteins and RNAs capable of acting on the nuclear structure. Key factors such as the ATP-dependent chromatin remodeling complex BRG1 (SMARCA4) (Hansis et al., 2004) or the ATP-dependent BAF complex from embryonic stem cells can enhance reprogramming of somatic cells by (Singhal et al., 2010). These data reveal that both the BRG1 and BAF remodeling complexes enhance the interaction of the pioneer transcription factors, especially OCT4, in mouse cells, and promote widespread transcription of pluripotency-related genes. This supports the model that spatial remodeling of the genome is necessary to induce totipotent transcription from a somatic nucleus.

Judicious use of Histone Deacetylase Inhibitors can Enhance Reprogramming

A number of SCNT studies that employ histone deacetylase (HDAC) inhibitors to alter the chromatin prior to and after SCNT have been reviewed (Enright et al., 2003; Rybouchkin et al., 2006; Yamanaka et al., 2009; Akagi et al., 2011; Lee et al., 2011; Ogura et al., 2013). Initial studies used only blastocyst development as the measure of efficiency. These studies generally reported varying degrees of success or no effect (Enright et al., 2003; Rybouchkin et al., 2006). These ambiguous results can most likely be explained by the utilization of toxic or ineffective levels of inhibitors. More recent studies in mice have refined efforts of these earlier studies and applied less-toxic compounds. Scriptaid for example, in very strict doses and time-dependent exposures can significantly enhance the production of SCNT offspring (Van Thuan et al., 2009; Zhao et al., 2010b). The most dramatic example comes from the serial recloning of mice, which was made possible by the utilization of HDAC inhibitors to increase the efficiency and thereby allow re-cloning of mice for up to 25 generations (Wakayama et al., 2013). It is important to note, however, that in this dramatic success story, the line of mice used were hybrids that included the 129 strain, which has a genetic predisposition for production of pluripotent stem cells compared to other outbred or inbred strains. Despite this caveate, globally increasing histone acetylation does appear to improve the efficiency of live-offspring production in other mouse strains and also in outbred pig lines (Van Thuan et al., 2009; Zhao et al., 2010a,b). Whether or not this treatment alone can lead to extended serial re-cloning in these populations is yet to be determined.

PERSISTENCE OF THE SCNT BARRIER

Direct perturbation of the epigenome must be undertaken with caution. It is clear that limited exposure of somatic cells and SCNT embryos to inhibitors of histone modifiers can have beneficial effects on the live-birth rate. Yet, there is no evidence that similar disruption of DNA methylation can improve production of live offspring via SCNT. In general, any perturbation of the cells or embryo that can influence transcription of a subset of genes in a positive way can also negatively influence a different subset of genes.

The studies reviewed here and a multitude of others together show that the “fantastical experiment” described by Spemann has been realized, although the dramatic inefficiency in the procedure still hampers application in a host of areas from animal agriculture to xenotransplantation. The initial experiments by Gurdon and then by Campbell and Wilmut opened the door for an entirely new field of induced pluripotent cell technology that has the potential to dramatically alter the field of regenerative medicine. However, this technology will remain unacceptably risky until the epigenomic barriers can be completely understood and manipulated so that directed differentiation can be performed without the risk of adverse outcomes. Similarly, the utilization of cloning technology will not be fully realized until the embryonic and fetal mortality due to failed epigenetic programming is resolved.

When considering the enormous structural rearrangements that must be accomplished to transition from a terminally differentiated epithelial cell into a chromatin structure appropriate for a totipotent embryonic blastomere, it is amazing that SCNT works at all. Frankly, it is not difficult to imagine and identify problems with epigenetic reprogramming of somatic cells leading to failed development; it is much more difficult to explain how the process of SCNT actually (sometimes) works to make a normal animal. SCNT offspring are not the products of random cells with unique epigenomes amenable to reprogramming, even though our best efforts to reprogram somatic nuclei fall short of complete success. It is important to note that reprogramming during the process of SCNT only has to get "close enough" to allow for all of the "required" genes to be expressed in the correct temporal and tissue specific manner. Evolution has provided redundant systems that allow continued development and survival of cells (and organisms) even in the face of genetic and epigenetic perturbations. Thus, although resetting somatic transcription and associated epigenetic marks is absolutely required for development of SCNT embryos, there is no requirement to get it perfect.

Acknowledgments

The authors would like to recognize the life and contributions of Keith H.S. Campbell, whose scientific vision and intellect will be long remembered in the field of cellular reprogramming. Even more than his scientific contributions, his friendship and enduring passion for knowledge are desperately missed.

Grant sponsor: NIH-Office of the Director; Grant number: 5R24OD011188-03; Grant sponsor: NIH-NICHD; Grant number: 1R01HD058969-01A2; Grant sponsor: NIH-NICHD; Grant number: 1R21HD055631-01A2; Grant sponsor: NIH-NICHD; Grant number: 3R21HD055631-02S1; Grant sponsor: NIH-NIAAA; Grant number: RO3AA020129

Abbreviation

SCNT somatic cell nuclear transfer

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