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Focus on Stem Cells

Reprogramming somatic cells into stem cells

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Abstract

Recent scientific achievements in cell and developmental biology have provided unprecedented opportunities for advances in biomedical research. The demonstration that fully differentiated cells can reverse their gene expression profile to that of a pluripotent cell, and the successful derivation and culture of human embryonic stem cells (ESCs) have fuelled hopes for applications in regenerative medicine. These advances have been put to public scrutiny raising legal, moral and ethical issues which have resulted in different levels of acceptance. Ethical issues concerning the use of cloned human embryos for the derivation of stem cells have stimulated the search for alternative methods for reversing differentiated cells into multi/pluripotent cells. In this article, we will review the present state of these reprogramming technologies and discuss their relative success. We also overview reprogramming events after somatic cell nuclear transfer (SCNT), as they may further instruct *ex ovo* strategies for cellular manipulation.

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Introduction

Reprogramming by nuclear transfer

The quest of finding how to turn a somatic cell into a pluripotent cell has puzzled researchers for many years. Adult somatic cells can undergo reprogramming after fusion with a matured oocyte, a process commonly known as somatic cell nuclear transplantation (SNCT). The competence of blastocysts produced by SCNT has been demonstrated first by the production of live animals (Wilmut *et al.* 1997, Wakayama *et al.* 1998) and secondly by the derivation of embryonic stem cells (ESCs) from the inner cell mass (ICM) cells of SCNT embryos (Wakayama *et al.* 2001). These observations provided definitive proof that somatic nuclei can be reprogrammed to a pluripotent state by factors in the oocyte cytoplasm, and that reprogrammed nuclei can direct embryonic development to term. Yet, despite success in the cloning (refers here to SCNT embryos/animals) of animals from many species, the percentage at which cloned embryos survive development to term is much lower than that obtained with *in vitro*-produced or naturally fertilized embryos. Failure to achieve development to term has generally been attributed to a number of reasons, these include: (i) technical limitations in embryo reconstruction, (ii) the use of incompetent or low quality oocytes and (iii) the lack of cell-cycle synchronization between the donor and recipient cells, however, it is generally accepted that a major problem is the incomplete epigenetic remodelling of the somatic nucleus (Campbell & Alberio 2003). The ability of the oocyte to reprogram/remodel the donor nucleus and result in successful development is heavily influenced by the donor cell type (Kato *et al.* 2000). Comparative studies show significant differences in the capacity of different cell lines to direct embryo development, where the nuclei of cells that are relatively less differentiated support better full-term development compared with those of fully differentiated cells. For example, term development of clones from lymphocytes requires serial nuclear transfer, i.e. the transfer of the somatic nucleus through two enucleated oocytes (Hochedlinger & Jaenisch 2002), suggesting that sequential remodelling is needed to reprogramme at least some terminally differentiated cells. Further, the highest success rates for cloned animals are obtained with nuclei from embryonic blastomeres. Efficiency is decreased with more advanced cell types, such as ESCs, fetal fibroblasts and adult somatic cells in the same order (reviewed by Shi *et al.* 2003). While this has been shown systematically in nuclear transfer experiments with mammals, a similar decrease in reprogrammability is observed with somatic cells from amphibians (Gurdon *et al.* 2003), suggesting that this is a generalized feature of vertebrate cells.

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In summary, the evidence suggests that developmentally advanced and more differentiated cell types have undergone modifications of their cellular structures that restrict their reprogrammability by oocytes. These modifications are of epigenetic origin, since the genetic makeup of the cells is not altered. Also this reveals that the epigenetic state of a donor nucleus restricts the ability of oocytes to fully reprogramme the genome after SCNT, and this must be taken into account when new methods for cellular reprogramming are considered. Thus, it is important to determine what events lead to the reprogramming of a somatic nucleus after fusion with an oocyte. This issue has been the subject of intense investigation, and studies have focussed predominantly on gene expression and the remodelling of nuclear structures.

Stepwise reprogramming after SCNT

Gene expression analysis in mouse shows incomplete reactivation of the pluripotency gene *Oct-4* (Boiani *et al.* 2002) and ten *Oct-4*-related genes in more than half of cloned pre-implantation embryos, whereas the somatic genes prostaglandin-endoperoxide synthase 2 (*Ptgs2*), hyaluronan synthase homologue (*Hsh*) and TNF-stimulated gene 6 (*Tnfsg6*) expressed in cumulus cells used as nuclear donors were correctly inactivated (Bortvin *et al.* 2003). Differences in gene expression have been reported for a variety of genes using microarrays, however, the information varies depending on the stages of development analysed and the microarray platform used in different studies. For example, mouse tissue from ESC or somatic cell-derived NT fetuses show differences with their naturally conceived counterparts (Humpherys *et al.* 2002). In contrast, bovine NT blastocysts show more similarities in the expression patterns with naturally fertilized embryos (Smith *et al.* 2005) than with *in vitro*-produced counterparts (Pfister-Genskow *et al.* 2005, Smith *et al.* 2005). This suggests that significant reprogramming of somatic chromatin to a zygotic/embryonic gene expression pattern is accomplished in NT blastocysts, raising the possibility that defects in development of extraembryonic tissues may account for the low development to term in clones. For instance, some of the differentially expressed genes in these studies included *CD81*, which is imprinted in mouse and putatively in the bovine placenta, and *MEIS2*, which is expressed during mouse placentation (Sapin *et al.* 2000). Furthermore, abnormal expressions of cytokeratin 8 and vimentin were also reported in SCNT embryos, both are required for establishment and development of the chorioallantoic placenta in post-hatching embryos (Pfister-Genskow *et al.* 2005). The differences in gene expression reported between studies may relate not only to the different arrays used, but also to the stochastic nature of nuclear reprogramming after NT (Somers *et al.* 2006). Variation in gene-expression profiles between species may be the result of species-specific differences in developmental events, as the case in the development of rodents and ruminants. For instance, zygotic transcriptional activation occurs very early in mouse embryos, with many genes initiating expression at the two-cell stage (Zeng & Schultz 2005). The zygotic genome becomes active later in ruminants, at the 4–16-cell stage (Kanka 2003). Thus, mouse SCNT embryos have a much shorter window of time in which to reprogramme a fused somatic nucleus, and this may result in an incomplete reprogramming in many genomic regions of the donor chromatin, restricting developmental potential. Recognition of the significance of the speed of development may provide a first concept to consider when designing reprogramming strategies. After SCNT, the forward momentum of fast developing species, such as mice, may restrict reprogramming events to a short window of opportunity. This limitation would be exacerbated in procedures using nuclei from cells of a more terminally differentiated character.

A failure to establish new programs of differentiation in later development may also contribute to failure in post-implantation development after SCNT. Studies of cloned mice and cattle show abnormal patterns of gene expression in the tissues of deceased animals and their placentae (Humpherys *et al.* 2002), as well as in live animals (Yang *et al.* 2005). In contrast to the low frequency of development to term, the efficiency of derivation of ESCs lines from cloned mouse embryos is comparable with that obtained from fertilized embryos (Wakayama *et al.* 2005). Furthermore, the transcription profile of ESCs derived from embryos resulting from a normal fertilization or SCNT are identical, and it has been suggested that only nuclei that have undergone appropriate epigenetic reprogramming are capable of generating ESCs from SCNT embryos (Brambrink *et al.* 2006). These studies show promising results, however, the differentiation potential and genomic stability of these cells need to be investigated. These data also suggest that the oocyte environment provides all the factors necessary for turning differentiated nuclei into pluripotent nuclei, although it needs to be stated that the efficiency in the process is low. Full-term development of mammalian embryos also requires an orchestrated spatial organization of cells, and failure in these early events can lead to developmental abnormalities. One of the first spatial events in developing embryos is the formation of the trophectoderm (TE) which gives rise to extraembryonic structures. Therein may reside a major impediment to the successful generation of adult animals from SCNT. Recent work shows that in mouse embryos, the TE lineage is specified as early as the two-cell stage (Deb *et al.* 2006). Indeed, initial establishment of the TE may be governed by maternal *Cdx2* RNA, with zygotic *Cdx2* expression acting to reinforce early specification and direct later TE development. Given the very early specification of the TE lineage, it is easy to envision the difficulty in reprogramming the chromatin of somatic

nuclei to a state competent for very rapid activation of the *Cdx2* gene and also the other as yet unidentified early genes, which would be required for normal development to take place. This may provide a major explanation for the great number of extraembryonic abnormalities in cloned fetuses, although changes in the expression of non-placental genes in embryos can also lead to placental failure (Copp 1995). This hypothesis is supported by studies showing abnormal expression of TE genes (Hall *et al.* 2005), X chromosome (XC) inactivation (Xue *et al.* 2002) and misregulation of imprinted gene expression in placentae of cloned embryos (Farin *et al.*2004) and in adult cloned mice (Senda *et al.* 2004). In this regard, it would be interesting to determine if nuclear localization of *Cdx2* is observed in cloned embryos as it is in embryos derived from fertilization.

Based on these observations, the process of nuclear reprogramming after SCNT may be divided into two major events. The first step is a reversal of the program of somatic differentiation manifest within the donor chromatin. This leads to timely reactivation of a zygotic program, involving the correct reactivation of embryonic genes and repression of somatic genes. This would require proper regulation of genes responsible for the development of the embryonic lineage, i.e. the steps leading up to the production of the ICM, as well as the extraembryonic lineages. Within this sequence, the activation of pluripotency genes is clearly an essential step towards producing pluripotent cells. *Oct-4* is expressed in the mouse ICM and in the ICM and TE of pig and cattle embryos, and is continuously required to sustain pluripotency in mouse ESCs cells (Nichols *et al.* 1998). Thus, *Oct-4* activation has been used as a marker for reprogramming in many approaches that use ES cells or oocytes as a reprogramming environment. However, *Oct-4* is detected at very low levels (1000-fold lower than ESCs) in mesenchymal stem cells (Jiang *et al.* 2002), suggesting that *Oct-4* cannot be used as the sole indicator of multipotency. *Nanog*, a homeobox containing transcription factor, is expressed in ESCs cells and is essential for the maintenance of pluripotency and self-renewal (Chambers *et al.* 2003, Mitsui *et al.* 2003). Yet, the regulation of *Nanog* expression and its functions are not totally elucidated. The transcription factors *Oct-4* and *Sox2* interact at the *Nanog* promoter to sustain its expression in ES cells (Kuroda *et al.* 2005, Rodda *et al.* 2005). During cell differentiation, the upstream region of the *Nanog* gene is remodelled, resulting in transcriptional repression (Deb-Rinker *et al.* 2005). Chromatin immunoprecipitation of promoters bound by *Oct-4*/*Sox2*/*Nanog* suggest that together they regulate a great number of genes (Boyer *et al.* 2005). These transcription factors are part of the molecular signature of ES cells, known as 'stemness' (Ivanova *et al.* 2002, Ramalho-Santos *et al.* 2002) and form a master circuitry for the maintenance of pluripotency (Boyer *et al.* 2005). Reactivation of the master circuitry will be an important step for achieving stable reprogrammed cells after experimental treatments.

The second step in nuclear reprogramming after SCNT is the initiation of the differentiation program that commences when the TE arises as the first lineage in the embryo. Incomplete programming of embryonic and extraembryonic lineages is reflected by the great variety of phenotypic abnormalities and high rates of embryonic loss after SCNT (Wilmut *et al.* 2002, Loi *et al.* 2006). There is evidence indicating that at the epiblast stage the embryo undergoes further reprogramming. This has been demonstrated by studying the mechanism of XC inactivation. In normal embryos, the paternal XC is inactivated by the expression of *Xist* RNA at the four cell stage. The paternal XC remains inactive in the TE, however, random inactivation occurs in the embryo proper, indicating that the inactive paternal X is reactivated in the epiblast (Mak *et al.* 2004, Okamoto *et al.* 2004). In female clones, receiving a nucleus with a randomly inactivated XC, there is inactivation of both XCs after transfer into the egg. This is followed by random reactivation of one XC in the epiblast of mouse embryos, suggesting that the oocyte initiates the epigenetic remodelling of the somatic chromatin and this is continued in the epiblast (Bao *et al.* 2005). The requirement for the epiblast reprogramming stage may be essential for term development of clones, however, it does not seem to be essential for the derivation of ESCs, in view of the high efficiency obtained with cloned embryos (Wakayama *et al.* 2005).

In summary, we can broadly define two major reprogramming events, which take place following SCNT, first, reversal to pluripotency, and second, establishment of new differentiation programs. These processes overlap at certain stages in developing embryos. Based on this concept, the customized development of stem cells for therapeutic applications will require the reprogramming of somatic cells to an undifferentiated state. Presently, the requirement of creating an embryo prior to the derivation of ESCs has limited the overall acceptance of this method for the generation of personalized immunocompatible ECS cell lines. Thus, defective cloned embryos could still supply cells from which specific cells types could be derived. Alternatively, methods of nuclear reprogramming which do not involve the creation of viable embryos are also a possibility (Hurlbut 2005). For example, a recent report demonstrated in mice that ESCs can be derived from *Cdx2*-targeted embryos. *Cdx2* is the earliest known TE-specific gene that interferes with *Oct-4* transcriptional regulation (Niwa *et al.* 2005). By deleting *Cdx2*, TE formation is defective but the ICM forms normally, allowing for the derivation of ESCs without destroying the embryo (Meissner & Jaenisch 2006). This approach clearly demonstrates that the concept of altered nuclear transfer is a viable possibility for the derivation of ESCs,

however, it is still controversial in view of the definitions of the moral status of embryos (Melton *et al.* 2004). A less controversial approach uses present techniques developed for preimplantation diagnosis to derive ESCs from single blastomeres, demonstrating that ESCs lines can be derived without the need of destroying a whole embryo (Chung *et al.* 2006). Alternatively, the possibility of using oocytes from different species has been evaluated as a mean of overcoming the limited availability of oocytes. The viability of such an approach is supported by reports showing the development of blastocysts following transfer of human, sheep, pig and monkey nuclei into bovine oocytes (Dominko *et al.* 1999, Chang *et al.* 2003) and macaque nuclei into rabbit oocytes (Yang *et al.* 2003). Furthermore, there is a single report of the generation of an ESC line following transfer of a human nucleus into a rabbit oocyte (Chen *et al.* 2003). The caveat of this approach is the generation of embryos with the nuclear genome of one species and mtDNA derived from the recipient oocyte (St John *et al.* 2004) and this could have detrimental consequences for cell viability and differentiation.

Altering the epigenetic state of the cell as a reprogramming route

Reprogramming gene expression

On a genome-wide basis nuclear reprogramming requires the prior remodelling of nuclear structures. Indeed, the idea that 'form follows function' during cellular differentiation (Kosak & Groudine 2004) helps to illustrate some of the differential characteristics of stem cells and somatic cells. The significance of structural remodelling is shown, for instance, by the unique localization of the chromosomal loci for *Nanog* and *Oct-4* and centromere organization in human ESCs (hESCs), indicating that these cells have a distinct nuclear architecture (Wiblin *et al.* 2005). Furthermore, a hyperdynamic nuclear state has been described for ESCs, where loosely bound chromatin-associated proteins confer these cells with increased plasticity (Meshorer *et al.* 2006). Interestingly, these features are not maintained in undifferentiated lineage-committed cells, suggesting that chromatin with loosely associated histones and chromatin-associated proteins have the capacity to respond to signals in a more unrestricted way. Furthermore, when the free association of chromatin proteins is prevented in ESCs, the cells loose their ability to respond to external stimuli. It is probable that the hyperdynamic state of the chromatin in ESCs contributes to the high capacity for reprogramming after SCNT observed with these cells (Wakayama *et al.* 2005). Consistent with the requirement of constant dynamic remodelling, reduced differentiation potential is observed in *MBD3*$^{-/-}$ ESCs (Kaji *et al.* 2006). MBD3 is a member of the methyl-binding domain family of proteins, acting as a transcriptional repressor by binding methylated DNA. MBD3 is part of a multiprotein complex, termed NuRD, which has a chromatin remodelling ATPase, a histone deacetylase and other proteins (Wade 2001). *MBD3*$^{-/-}$ ESCs lacking NuRD activity show defects in differentiation and maintain a leukemia inhibitory factor (LIF)-independent self-renewal capability under differentiation conditions. Thus, the requirement of NuRD-mediated downregulation of pluripotent gene expression for lineage commitment is a further example of epigenetic remodelling of the stem cell state required for differentiation.

Remodelling of the chromatin, in particular, the modifications of the DNA molecule and the histones, has been extensively investigated in ESCs and embryos, representing a fundamental regulatory mechanism with consequences for cell-fate determination and development (Jenuwein & Allis 2001, Reik *et al.* 2001). Indeed, several studies have began to uncover the epigenetic signature of pluripotent cells (Azuara *et al.* 2006, Boyer *et al.* 2006, Lee *et al.* 2006). Epigenetic modifications of the genome are heritable and regulate the access of factors to the underlying DNA, thereby modulating transcriptionally active (euchromatin) or silent (heterochromatin) chromatin states. These epigenetic marks consist of differential DNA methylation and post-translational modifications on histone amino termini. DNA methylation at CpG dinucleotides is a key regulatory mechanism involved in the control of gene expression (Jaenisch & Bird 2003). In somatic cells, CpGs are methylated, and is only in areas of high CpG density (CpG islands), where methylation is maintained at a low level (Wade 2001). These areas are of particular interest because they are usually associated with regulatory regions of protein-coding genes, thus gene expression is affected when methylation is established. During mammalian development, active demethylation of the male genome occurs after fertilization, whereas the female genome undergoes passive demethylation during successive cell divisions (Mayer *et al.* 2000). Low DNA methylation levels are maintained for four to six embryonic divisions depending on the species, and normal levels are restored by *de novo* methylation during gastrulation. Importantly, differences between species, such as mice and ungulates (sheep, pigs and cattle), suggest that the degree of DNA demethylation is associated with the time of embryonic genome activation during maternal zygotic transition (MZT). In mice, zygotic transcription initiates two cell divisions earlier than in ungulates, suggesting that the paternal genomes of early MZT species require faster remodelling of the chromatin. Late activating zygotic genomes, such as amphibians, have no active DNA demethylation during early embryogenesis. The DNA is passively demethylated during cell divisions reaching its lowest level prior to zygotic transcription at the mid-blastula transition at 4000-cells stage (Stancheva *et al.* 2002). This developmental stage is

far more delayed as compared with mammalian embryos. In this embryonic context, DNA methylation might provide a control mechanism for the timely expression of embryonic genes, in particular for those genes whose activating transcription factors are present as RNAs and/or proteins in the fertilized egg (Stancheva *et al.* 2002). This further highlights the impact of this epigenetic event in the control of gene expression. For instance, nuclear transplantation in mammals is accompanied by genome-wide demethylation of the donor chromatin. However, precocious remethylation is detected at the four-cell stage in at least half of bovine SCNT embryos, suggesting that the abnormal DNA methylation pattern in these clones results in low viability (Dean *et al.* 2001). Functional evidence that DNA methylation status of the donor cells restricts development of cloned embryos has been demonstrated using hypomethylated donor cells for SCNT (Blelloch *et al.* 2006). Part of the abnormal methylation in clones may be the result of the aberrant expression of somatic *Dnmt1* and the lack of translocation of Dnmt1o at the eight-cell stage (Chung *et al.* 2003), however, this needs to be examined in cloned embryos. In mouse ESCs, levels of DNA methylation are reduced in female cell lines, contributing to the genomic instability of such cells (Zvetkova *et al.* 2005). In conclusion, erasure of the CpG methylation marks in differentiated cells is a target modification required for the establishment of a naïve state in the reprogrammed cells. Hence, reprogramming strategies may need to include assays of genome-wide and gene-specific DNA methylation in order to determine the degree of reprogramming.

Modification at histone tails defines transcriptionally active or repressed states in cells (Fry & Peterson 2001). In ESCs/thymocytes hybrids global chromatin decondensation of the somatic genome is concomitant to histone H3K4 acetylation and independent of gene expression, suggesting that erasure of the somatic-specific histone modifications is needed for complete nuclear reprogramming (Kimura *et al.* 2004). More specifically, when the *Oct-4* promoter was analysed, a specific epigenetic profile was observed in reprogrammed cells similar to ESCs, which was defined by DNA hypomethylation, histone H3K4 hypermethylation, histone H3 hyperacetylation and H3K9 hypomethylation (Kimura *et al.* 2004). This finely tuned regulation of *Oct-4* suggests that establishment of a pluripotent state depends on a series of modifications that ensure the stabilization of the pluripotent state (Fig. 1).

Reprogramming the DNA replication machinery

In addition to the important role that higher order chromatin structure plays in governing the transcription profile of reprogrammed cells, recent evidence from *Xenopus* demonstrates that it is also important in contributing to the rapid replication of DNA that is a feature of the cleavage division cycles of early embryonic cells. Cleaving *Xenopus* blastomeres undergo a distinctive cell division cycle, characterized by an extended S-phase that is accompanied by very short or absent G1 and G2 phases. A similar dynamic is observed in mammalian embryos (Campbell *et al.* 1994). The replication process in *Xenopus* embryos is initiated immediately after fertilization, and cells divide in synchrony every 30 min for about the first 6 h of development. Rapid DNA replication is achieved, in part, by a chromatin structure that includes very short

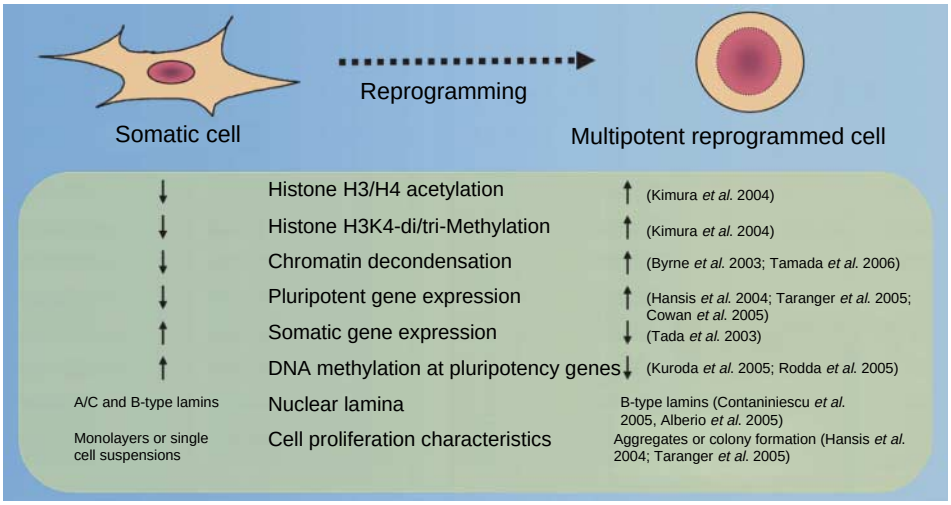

Figure 1 Characterized modifications in reprogrammed cells. Reprogrammed cells undergo epigenetic remodelling of the chromatin demonstrated by the hyperacetylation of the core histones H3 and H4. This is also reflected by demethylation of specific promoter sequences of pluripotency genes, such as *Oct-4*. A characteristic hyper di/tri-methylation of lysine-4 on histone H3 is also observed. Furthermore, DNA methylation is reduced at sequences of activated pluripotency genes, whereas somatic gene expression is low. Remodelling of the nuclear lamina and DNA decondensation have been shown in different reprogramming approaches. This drawing summarizes some of the most relevant modifications that may be required for the acquisition of a naïve state in reprogrammed cells.

loops of DNA that are generated early in the mitotic cycles and that allows sufficient origin replication complex (ORC) proteins to associate with the DNA. When somatic cells are used for NT, abnormal cleavage is observed and the development of all embryos is arrested due to a failure in replication of the somatic DNA (Laskey & Gurdon 1970). However, when somatic cells are exposed to *Xenopus* mitotic (M-phase) extracts, the loops of the somatic cells are remodelled into an embryonic configuration resulting in a significant improvement in DNA replication time (Lemaitre *et al.* 2005). Thus, mitotic conditioning is apparently crucial for remodelling the chromatin loops of differentiated adult cells to an embryonic configuration that supports the rapid DNA replication of embryonic cycles. Cloning experiments in cattle also show that exposure of somatic cells to mitotic extracts can increase the developmental rate of clones (Sullivan *et al.* 2004). It would be interesting to test whether these differences in development are due to a chromatin structure altered to support more rapid DNA replication, or to improved reprogrammability, or both (Sullivan *et al.* 2004). It is important to note that *Xenopus* embryos have an S-phase of 20 min compared with the 8–10 h in mammalian embryos (Schabronath & Gartner 1988, Comizzoli *et al.* 2000). Within this context, it is conceivable that the shortening of the chromatin loops and recruitment of replicon-initiation factors, as occurs in *Xenopus*, is not important in mammals.

Remodelling nuclear structure

A final target for reprogramming strategies is the structure of the nucleus itself. In embryonic blastomeres (Kelly *et al.* 2005) and ESCs (Constantinescu *et al.* 2006), the nuclear periphery delimited by the nuclear envelope is decorated by a thin protein layer composed of B-type lamins. A-type lamins are incorporated into the nuclear lamina in differentiating cells and are found in almost all differentiated cells (Hutchison 2002). This timely expression of A-type lamins suggests that they either trigger differentiation or are the consequence of it. This has not been fully clarified yet, but evidence indicates that A-type lamins limit the plasticity of cells (Hutchison 2002), suggesting that they play a role in maintaining the differentiated phenotype. Furthermore, during embryogenesis, A-type lamins forming the oocyte nucleus are remodelled. At the two to four-cell stage, low levels of A-type lamins are found, but they are not detectable in blastocysts (Kelly *et al.* 2006). The remodelling of the nuclear envelope during early embryo development is a potential reprogramming marker (Sullivan *et al.* 2004). Indeed, NT embryos made with differentiated cells incorporate A-type lamins, however, lamin A is undetectable in blastocysts, indicating that the nuclear lamina of cloned blastocysts is remodelled in a similar manner to fertilized embryos. We have also used the remodelling of the nuclear lamina as a marker for the reprogramming events following the incubation of somatic cells in *Xenopus laevis* egg extracts. We observed energy-dependent remodelling of the lamina, with incorporation of the egg-specific lamin III and removal of somatic lamin A. The removal of lamin A is independent of the incorporation of lamin III, since in oocyte extracts, which lack free lamin III, lamin A is also removed from the somatic cells, leaving these cells with only type B lamins (Alberio *et al.* 2005). Therefore, this egg-free system has the capacity to trigger nuclear lamina remodelling, leading to a nuclear configuration consistent with increased plasticity. However, the differentiation potential of these cells requires further investigation.

Reprogramming strategies

Reprogramming in pluripotent cell/somatic cell hybrids

Heterokaryons, consisting of the stable fusion of two cells that maintain separate nuclei, have been shown to form spontaneously and are able to alter terminally differentiated cells after transplantation (Weimann *et al.* 2003). This ability of cells to fuse and influence each other has been widely used for reprogramming somatic cells. Nuclear reprogramming is triggered by the pluripotent nucleus in heterokaryons. Early studies carried out fusing embryonal carcinoma (EC) cells with somatic cells showed reactivation of the inactive XC, indicating that pluripotent cells have strong reprogramming capacity (Takagi *et al.* 1983). When embryonic germ cells (EG) are fused with thymocytes, demethylation occurs at several DNA loci, including imprinted genes (Tada *et al.* 1997). In contrast, non-imprinted euchromatic genes and heterochromatic centromeric regions are demethylated in mESCs/thymocytes hybrids, without affecting imprinted loci, thus demonstrating an important difference in the reprogramming capacities of EG and ESCs (Tada *et al.* 2001). A recent report using hESCs fused with adult fibroblasts or pelvic bone cells demonstrated a genome-wide reprogramming of gene expression by the stem cells (Cowan *et al.* 2005). Interestingly, when enucleated ESCs are fused to neurosphere cells there is no reactivation of *Oct-4*, however, when nucleated ESCs are used *Oct-4* is reactivated within 48 h (Do & Scholer 2004). If the nucleus of an ESC is needed to reprogramme in fusion experiments, then it would suggest that nuclear activity is required to induce reactivation of the pluripotency gene *Oct-4*. An enucleated cytoplast will, therefore, lack sufficient reprogramming factors to induce a sustained remodelling activity over a long period of time resulting in incomplete reprogramming. Nanog has been identified as an important activator of pluripotent gene expression in mouse neural stem cells (NSC) and also in terminally differentiated mouse thymocytes. Fusion of *Nanog* overexpressing ES cells with NSC results in the activation of *Oct-4* and inhibition of *Olig2* and *Blbp*,

two NSC markers. These changes in gene expression are mediated by remodelling of epigenetic marks of the somatic genomes (Silva *et al.* 2006). Interestingly, overexpression of *Nanog* in NSC does not result in *Oct-4* expression or in the inhibition of somatic genes, indicating that *Nanog* alone cannot confer pluripotency to NS cells. The fusion approach has provided additional evidence on the reprogramming of somatic cells by pluripotent nuclei, however, the use of this strategy for therapeutics is rather limited due to the potential risks associated with the presence of the ESC nuclei in such hybrids (Cowan *et al.* 2005).

Reprogramming by forced expression of defined genes

A recent report shows that transfecting mouse embryonic and tail-tip fibroblasts with *Oct-4*, *Sox-2*, *c-myc* and *Klf4* was sufficient to induce cells to show stem-cell characteristics, including the expression of pluripotency genes, normal karyotype, contribution to all three germ layers in teratomas and chimeric embryos (Takahashi & Yamanaka 2006). *Oct-4* and *Sox-2* are known to cooperate in the activation of *Nanog* which is always activated in the reprogrammed cells. The origin of the reprogrammed cells is still not clear in this study and would be most important to determine whether the low efficiency of the approach is due to the selection of the very few 'less-differentiated' cells in the starting population of embryonic or tail fibroblasts that are, perhaps, more amenable for reprogramming. Erasure of the epigenetic marks and the expression of specific factors, such as perhaps *Oct-4*/*Sox-2*/*c-myc*/*Klf4* and *Nanog*, may provide a powerful combination for more efficient reprogramming of somatic cells.

Reprogramming with amphibian germ cell factors

As discussed previously, reversal of differentiation after SCNT indicates that oocytes possess all the components needed for the establishment of a pluripotent phenotype. However, the limited availability of mammalian eggs has restricted the use of this biological resource, therefore, this has prompted the use of alternative sources of oocytes and *ex ovo* methods for treating large numbers of cells (Table 1). Seminal studies carried out several decades ago show that *Xenopus laevis* oocytes can be injected with somatic cells to study nuclear cytoplasmic interactions as well as protein exchange and RNA synthesis in human cells (Gurdon *et al.* 1979). More recent studies report the reactivation of *Oct-4* gene and inhibition of somatic *Thy-1* gene in human lymphocytes injected into *Xenopus* oocytes demonstrating specific aspects of the reprogramming potential of this heterologous system (Byrne *et al.* 2003). The activation of *Oct-4* requires the demethylation of its promoter and the speed of *Oct-4* reactivation is dependent on the degree of chromatin organization and proteins associated with the DNA (Simonsson & Gurdon 2004). How the *Oct-4* promoter becomes demethylated in the absence of DNA synthesis is not understood and there is no clear evidence of a DNA demethylase enzyme in frog oocytes. In light of this, it is tempting to speculate that DNA demethylation may be the result of a remodelling activity mediated by methyl-binding domain or MBD-proteins and ATP-dependent chromatin remodelling factors.

The main disadvantage of injecting cells into the germinal vesicle (GV) of oocytes is the difficulty in the recovery of viable cells. An alternative, therefore, is to use extracts prepared from *Xenopus* oocytes and eggs (Kikyo *et al.* 2000, Hansis *et al.* 2004, Alberio *et al.* 2005). Previous studies have demonstrated the active remodelling of *Xenopus* somatic cells by the nucleosomal ATPase imitation switch (ISWI) present in egg extracts (Kikyo *et al.* 2000). Recent studies show that nucleoplasmin, a protein present in *Xenopus* eggs, plays an important role in the chromatin decondensation when nuclei from mouse EC cells are incubated in egg extracts. Importantly, the remodelling induced by nucleoplasmin includes centromeric DNA decondensation, reduction of chromatin-associated HP1 protein, loss of histone H3K9 trimethylation and the reactivation of the oocyte-specific genes *c-mos*, *Msy-2* and *H1foo* (Tamada *et al.* 2006). These changes in EC incubated in the presence of nucleoplasmin suggest that the remodelling of the chromatin by this protein may be sufficient to create a configuration amenable for redifferentiation, as determined by gene expression. However, it is important to note that extracts were less effective in remodelling fully differentiated cells.

Hansis *et al.* (2004) incubated human leukocytes in *Xenopus* egg extracts and demonstrated that treated cells can reactivate *Oct-4* and germ cell-specific alkaline phosphatase (GCAP) after 1 week in culture. However, the nature of the remodelling taking place under these conditions is unclear given the brief exposure to extract (30 min at 37 °C) and the 7 days lag time before *Oct-4* expression was detected. However, it was established that the chromatin remodelling factor, Brahma-related group 1 (BRG1), was needed for this gene activation (Hansis *et al.* 2004). It is not known whether demethylation of the *Oct-4* promoter can occur in extracts depleted of BRG1, which could provide a compelling link between chromatin remodelling and the demethylation activity reported in injected oocytes (Simonsson & Gurdon 2004).

We have recently reported differences in cellular activities induced in bovine fetal fibroblasts (BFF) by either *Xenopus* oocyte or egg extracts (Alberio *et al.* 2005). An important difference between these two extracts is their different capacities to sustain transcription and DNA replication in BFFs. Oocyte extracts

Table 1 Summary of reprogramming approaches commonly used.

Reprogramming method	Advantages	Limitations
Injection into *Xenopus* oocytes	Large volume and readily available. Reactivation of the pluripotency gene *Oct-4* gene; Byrne *et al.* (2003)	Cells require permeabilization prior to injection. Injected cells cannot be retrieved easily. Low number of cells can be treated. Labour intensive and low efficiency. Reactivation of oocyte specific genes, suggesting reprogramming to oocyte state rather than ES cells state; Tamada *et al.* (2006). *Nanog* and *Sox-2* expressions, two pluripotency genes, have not been reported yet
Incubation in *Xenopus* oocyte and egg extracts	Large volume and readily available. Large number of cells can be treated and cultured after treatment. Reactivation of *Oct-4* gene after 1 week; Hansis *et al.* (2004). Extensive nuclear remodelling; Alberio *et al.* (2005) and Tamada *et al.* (2006). Carried out at 21 °C where most mammalian cellular activities are shut down, potentially increasing the reprogramming by amphibian factors	Cells require permeabilization prior to treatment. Reactivation of *Nanog* and *Sox-2*, two pluripotency genes, has not been reported yet
ES/somatic cell fusion	Large number of cells can be treated. Reactivation of *Oct-4* gene; Tada *et al.* (2001) and Cowan *et al.* (2005). Inactivation of somatic genes. Reprogrammed cells can contribute various cell lineages when injected into mice; Tada *et al.* (2003)	Low efficiency. Cells maintain the genome of the pluripotent cells, which can lead to genetic instability. Nucleus of the ES cells is required to maintain ability to reprogramme somatic genome; Do & Scholer (2004)
EG/somatic cell fusion	Large number of cells can be treated. Reprogrammed cells can differentiate to various cell lineages; Tada *et al.* (1997)	Erasure of imprints, which may result in genetic instability; Tada *et al.* (1997). Reactivation of inactive X chromosome
EC/somatic cell fusion	Large number of cells can be treated. Inactivation of somatic genes; Takagi *et al.* (1983)	Reactivation of inactive X chromosome
EG cell extracts	Reprogramming of 293T. Reactivation of pluripotency genes and downregulation of somatic genes. Reprogrammed cells can differentiate to mesoderm and ectoderm *in vitro*; Taranger *et al.* (2005)	Needs to be tested with primary cells. No information available on imprinted gene expression
ES cell extracts	Reprogramming of 293T and NIH3T3 cells to express *Oct-4* and downregulation of somatic lamin A; Taranger *et al.* (2005)	Requires the expansion of large number of ES cells to produce small volumes of extracts. Reprogrammed cells propagated for 1 week. Needs to be tested with primary cells
Synthetic molecules	Defined synthetic molecules induce dedifferentiation of C2C12 cells to multipotent progenitor. Large number of cells can be treated	Only demonstrated with C2C12 myoblasts. Dedifferentiation potential requires test with primary cells
Overexpression of specific molecules	*Oct-4*, *Sox2*, *c-myc*, *Klf4* expression can reestablish pluripotency in mouse embryonic and adult somatic cells; Takahashi & Yamanaka (2006)	Needs to be tested with human primary cells. Low efficiency. Expression profiles differ from ESCs

promote transcription but not DNA replication in BFFs, whereas egg extracts, in contrast, promote replication but not transcription. Significantly, we performed our experiments at 21 °C, a temperature at which mammalian cells have impaired functions, but amphibian cells are metabolically active. Our results suggest that at this temperature the amphibian molecules in the extract take control over the mammalian template. It is possible that these conditions may lead to a higher degree of reprogramming. Moreover, it is probable that pluripotency factors present in the amphibian oocytes/eggs are more stable at reduced temperatures, and therefore will produce more consistent effects.

Reprogramming using cell extracts

Cellular reprogramming using extracts from various cell types can drive new gene-expression profiles in somatic cells. This has been shown by the induction of 293T and skin fibroblast cells to express lymphocyte markers (Hakelien *et al.* 2002). This approach has also recently been used for dedifferentiation of 293T cells and NIH3T3 fibroblasts after incubation in extracts derived from pluripotent cells, such as EC cells and ESCs (Taranger *et al.* 2005). The brief treatment of cells with these extracts triggered the formation of colonies with a phenotypical organization of ESCs. Upregulation of a number of pluripotency genes and downregulation of somatic genes, such as lamin A, were subsequently detected up to 4 weeks following treatment. In addition, these cells were able to differentiate to mesoderm and ectoderm lineages (Taranger *et al.* 2005). The phenotypic changes in these cells were the result of epigenetic modifications of the chromatin mediated by chromatin remodelling factors, histone acetylation, expression of specific genes and protein synthesis. It would be interesting to see whether similar reprogramming can be achieved with primary cells of adult individuals, which would represent a major step in the use of such techniques in regenerative medicine.

Reprogramming using synthetic molecules

Using a small molecule library, the compound named 'reversine' was identified. This molecule has the capacity to induce cellular dedifferentiation in C2C12 myoblasts (Chen *et al.* 2004). The resulting dedifferentiated cells acquired osteogenic and adipogenic differentiation capabilities upon differentiation in the respective media. A library of heterocyclic compounds was also used to identify molecules with the capacity to induce neural and cardiomyocyte differentiation in mouse EC cells (Ding & Schultz 2004, Wu *et al.* 2004), and to induce the differentiation of mesenchymal stem cells to osteoblasts (Wu *et al.* 2002). The use of synthetic molecules has a promising potential for driving cells into new phenotypes, although it would be interesting to understand how the action of these substances correlates with physiological events, particularly in view of the sophisticated epigenetic mechanisms that mediate cellular differentiation.

Cytoplasmic reprogramming: the RNA connection

While the major focus of this paper has concentrated on nuclear reprogramming as a means of stably altering gene expression, an additional approach could be to focus on reprogramming the cytoplasm to alter the protein synthetic profile of cells. A definitive role for small RNAs, known as microRNAs (miRNAs), as regulators of translation is now well established (Bartel 2004). These small molecules are non-protein-coding RNAs of 21–22 nucleotides in size with post-transcriptional regulatory activity. miRNAs are expressed in different tissues and have been linked to the establishment of lineage differentiation, although they have also been found in mouse and human stem cells. Several hundred miRNAs have been annotated with predictions that these molecules can regulate up to 30% of protein-coding genes (Wienholds & Plasterk 2005). Translational repression is the most common post-transcriptional mechanism of gene repression mediated by miRNAs in animals, however, they can also mediate transcript-specific cleavage in the cytoplasm by directing the endonuclease activity of the RNA-induced silencing complex or RISC (Wienholds & Plasterk 2005). The expression pattern of miRNA in animals suggests that they are involved in cellular differentiation and in the maintenance of tissue identity, since about 80% of conserved vertebrate miRNAs are tissue specific (Wienholds & Plasterk 2005). Mouse ESCs cells with a targeted deletion in Dicer, the enzyme required to process miRNAs, maintain the undifferentiated state, have reduced differentiation capability and reduced proliferation (Lim *et al.* 2003). Thus, one interpretation is that miRNAs maintain the steady-state physiology of differentiated cells rather than trigger cell differentiation. Single miRNAs can be expressed at very high levels (Lim *et al.* 2003) and regulate hundreds of genes (Lim *et al.* 2005), suggesting that the presence of such molecules stabilize phenotypic identity. This could present a major disadvantage when using highly differentiated cells for reprogramming.

Approaches such as the one recently reported on the use of antagomirs, or chemically modified complementary miRNAs, for *in vivo* silencing of endogenous miRNAs are valuable tools for the study of function of miRNAs. The inhibition of specific miRNAs can result in the up- and downregulation of hundreds mRNAs, depending on the recognition motifs of each miRNAs at the 3′ UTR (Krutzfeldt *et al.* 2005). This indicates that altering the miRNA load of a cell may improve the cellular response to exogenous factors that reprogramme gene expression. For instance, *Oct-4*, *Sox-2*, and *Nanog* bind promoters of 14 miRNA (Boyer *et al.* 2005), suggesting that reprogramming the expression of these transcription factors could have a major effect on the miRNA composition of a cell, making them more compatible with the acquisition of a pluripotent state.

Conclusions

The strategies used in the studies reported here clearly indicate that there may be different ways to induce reprogramming of the somatic genome. Reprogramming cells to a pluripotent state is a consequence of stepwise architectural remodelling of cellular structures, epigenetic modifications of the chromatin and the expression of transcriptional and post-transcriptional regulators. The orchestrated remodelling of target cells will be a condition required to achieve a stable reprogrammed state, which can ultimately be redirected to specific differentiation programs.

There is sufficient evidence demonstrating that cellular identity can be influenced by *in vitro* manipulation and that the fate of reprogrammed cells requires further *in vivo* testing. Therefore, developing cell-based approaches for therapeutics may be a realistic expectation for the near future.

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