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Reprogramming the genome: role of the cell cycle

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In nuclear transfer reconstructed embryos, the co-ordination of donor nuclear and recipient cytoplasmic cell cycle phases is essential to maintain ploidy and prevent DNA damage. However, the stage of the cell cycle at the time of reconstruction and the method of reconstruction may also have a significant impact on the subsequent development of the embryo and fetus through a number of other mechanisms. This paper reviews some of the information currently available and proposes that consideration of the cell cycle may lead to improvement of methods for embryo reconstruction.

Introduction

Differentiated somatic nuclei acquire totipotency after transplantation into oocytes, as shown by the generation of live offspring in a number of species, including sheep (Wilmut *et al.*, 1997a), cattle (Cibelli *et al.*, 1998b), mice (Wakayama *et al.*, 1998a), goats (Baguisi *et al.*, 1999), rabbits (Chesne *et al.*, 2002) and a cat (Shin *et al.*, 2002) and a mule (Woods *et al.*, 2003) (for a review, see Campbell *et al.*, 2001). This reversal of the differentiated state of a somatic nucleus by nuclear transplantation is referred to as nuclear 'reprogramming'. Early studies conducted in amphibians show the capacity of differentiated nuclei to re-direct their gene expression pattern dependent upon nuclear remodelling factors present in the cytoplasm of the oocyte (Di Berardino and Hoffner, 1983). It has been suggested that remodelling of the donor chromatin is essential for proper gene expression in reconstructed embryos. The capacity of the oocyte for nuclear reorganization is demonstrated by the replacement of sperm protamines by oocytic histones after fertilization in mammals (Perreault, 1992). In cloned embryos, the nuclear modifications have been partially studied and some conclusions from these studies indicate that certain conditions have to be taken into account to ensure that development occurs. Initial studies in mammalian nuclear transfer embryos show the importance of cell cycle co-ordination between the donor nucleus and the recipient cytoplast to ensure development (Collas *et al.*, 1992; Campbell *et al.*, 1993, 1994).

It is now accepted that two main types of recipient oocyte are suitable for development to term after single nuclear transfer (NT) (Fig. 1). The difference between these two recipient oocytes is essentially the amount of maturation promoting factor (MPF), a cytoplasmic protein kinase involved in both mitotic and meiotic division (for a review, see Campbell *et al.*, 1996b). All nuclei transferred at the time of activation when MPF contents are high (Fig. 1a) undergo nuclear envelope breakdown, which is followed by premature chromosome condensation (PCC). The nuclear envelope is then reformed and DNA synthesis is observed in all nuclei. In

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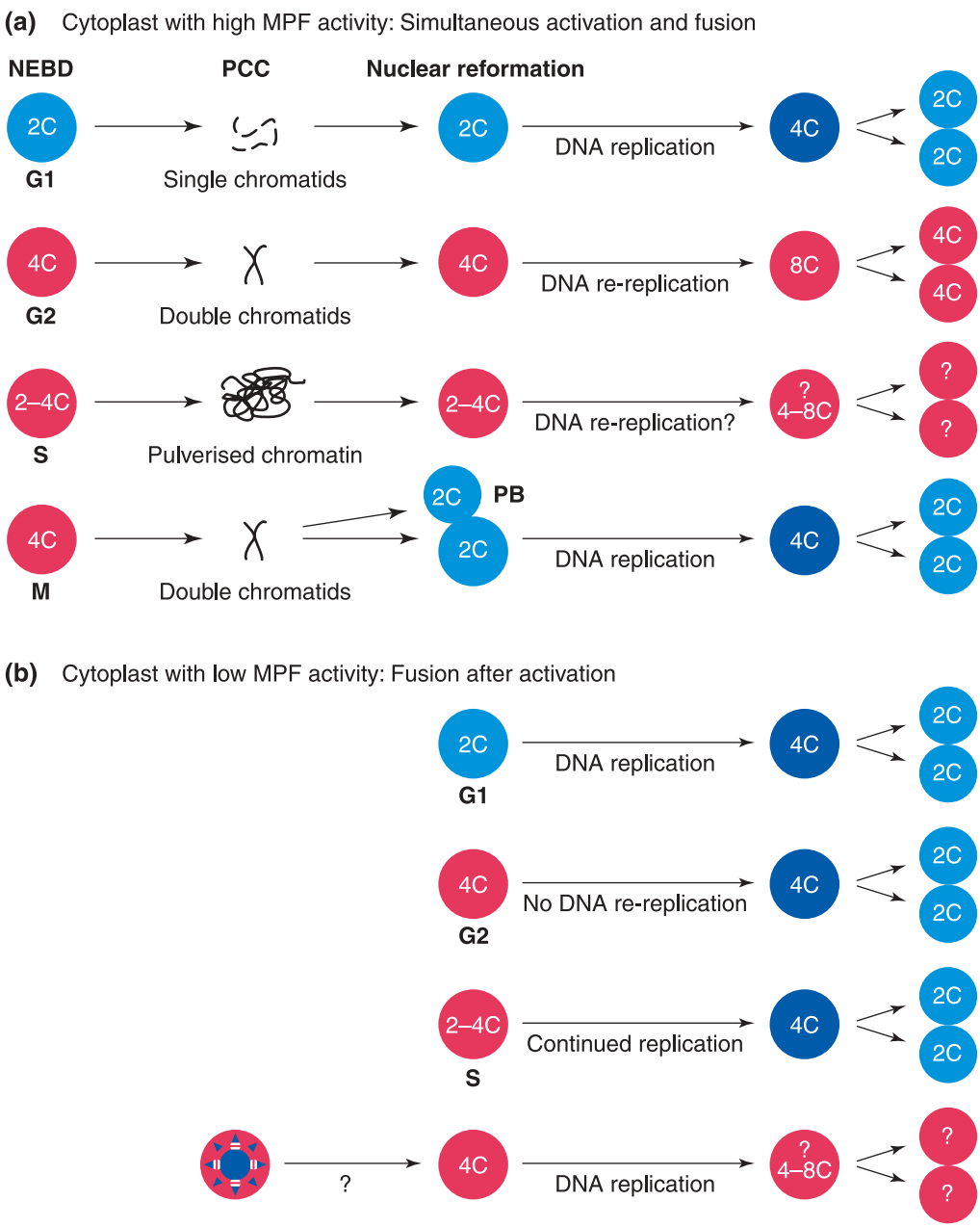

Fig. 1 (a)–(d). For legend see facing page.

this situation it is probable that unless the nucleus is diploid (G1–G0) at the time of transfer, re-replication of previously replicated DNA will occur and that, at the end of the first cell cycle, the DNA content (ploidy) of the nuclei in the daughter cells will be incorrect. The increased amount of DNA present at the end of the first cycle may also adversely affect mitosis resulting in unequal segregation or possible chromosomal abnormalities. Although live offspring have been reported from each of these cell cycle combinations in a range of

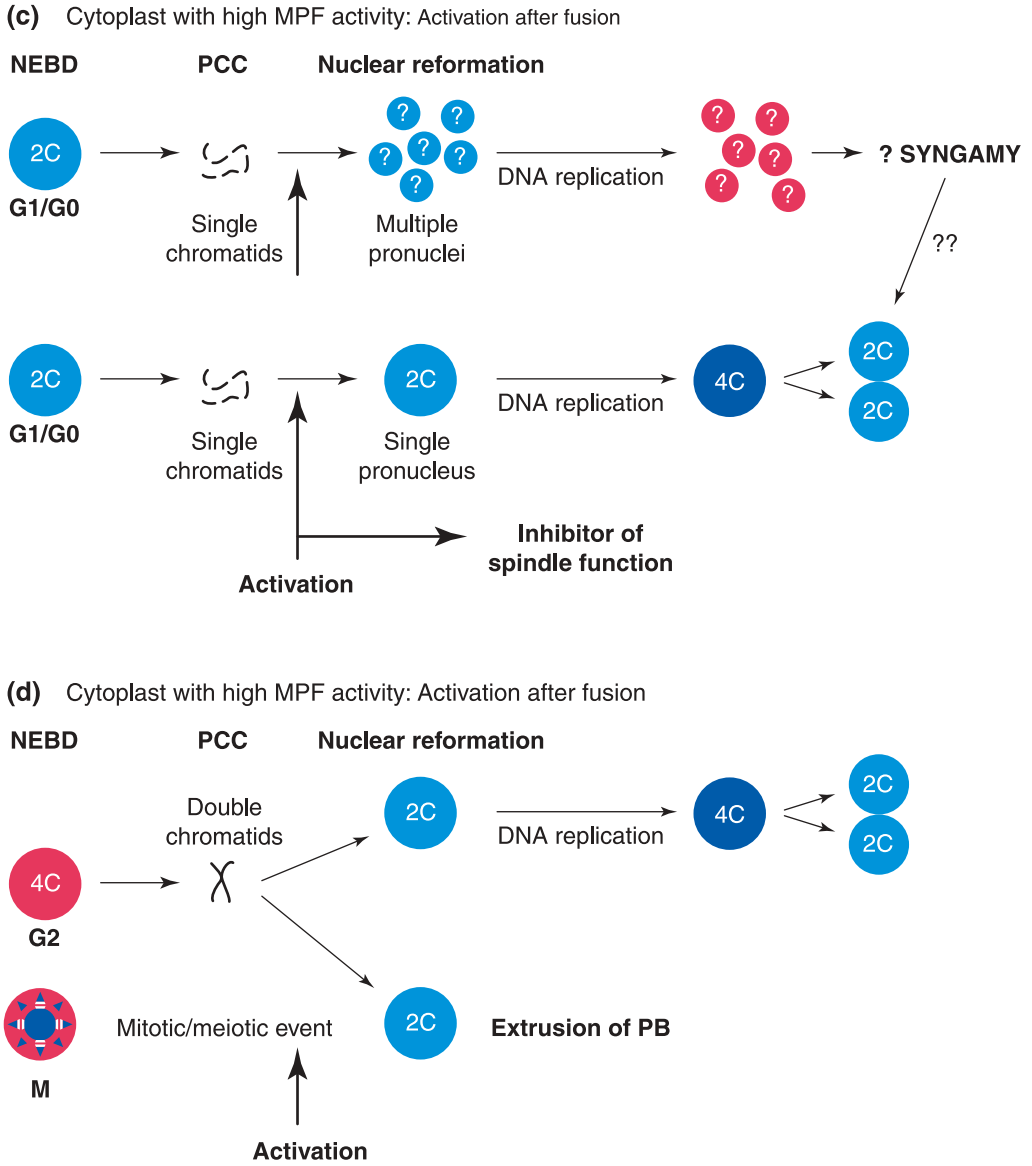

Fig. 1. Effects of nuclear transfer of karyoplasts at defined stages of the cell cycle into cytoplasts at defined stages of the cell cycle on chromatin fate, DNA replication and ploidy of resultant daughter cells. (a–d) In this diagrammatic representation, red circles represent nuclei that are 'out of phase' in terms of DNA content with the stage of the cell cycle of the recipient cytoplast, whereas the blue circles represent nuclei that are 'in phase'. M: mitosis; MPF: maturing promoting factor; NEBD: nuclear envelope breakdown; PB: polar body; PCC: premature condensation.

species (Table 1), the occurrence of the events depicted diagramatically may vary depending upon the species and the age of the recipient cytoplast. In contrast, when nuclei are transferred after the disappearance of MPF activity (Fig. 1b), no nuclear envelope breakdown or PCC are observed. Nuclei that are in G1 or S phase at the time of transfer initiate or continue DNA synthesis, respectively; however, no DNA synthesis is observed in nuclei that are in the

Table 1. Cellular origin, sex and cell cycle co-ordination during NT of live somatic cell clones from different species

Species	Type of donor cell	Sex	Stage of cell cycle	Recipient cell type (cytoplast)					Reference
				MII	MII + Act	TII	Activated	Serial NT	
Sheep	Epithelial-like (E)	Fe	G0	+	+		+		Campbell *et al.*, 1996b
	Epithelial-like (E)	Fe	G0		+				Wilmut *et al.*, 1997
	Fibroblast (F)	M	G0		+				
	Epithelial–mammary (A)	Fe	G0		+				
	Fibroblast (F)	Fe	G0		+				Schnieke *et al.*, 1997
	Epithelial-like (E)	M	G0–G1		+				Wells *et al.*, 1997
Cattle	Fibroblast (F)	Fe	G1	+					Cibelli *et al.*, 1998
	Epithelial–oviduct (A)	Fe	G0–G1		+				Kato *et al.*, 1998
	Cumulus (A)	Fe	G0–G1		+				Kato *et al.*, 2000
	Epithelial–oviduct 1 (A)	Fe	G0–G1		+				
	Epithelial–uterus (A)	Fe	G0–G1		+				
	Fibroblast–skin (A)	Fe, M	G0–G1		+				
	Fibroblast–ear (A)	M	G0–G1		+				
	Fibroblast–skin (N)	Fe	G0–G1		+				
	Fibroblast–ear (N)	M	G0–G1		+				
	Hepatocyte (N)	M	G0–G1		+				
	Fibroblast transgenic (F)	Fe	G0–G1	+					Zakhartchenko *et al.*, 2001
	Germ cells (F)	M	U	+					Zakhartchenko *et al.*, 1999a
	Cumulus (A)	Fe	M	+					Tani *et al.*, 2001
Mice	Cumulus (A)	Fe	G0–G1	+					Wakayama *et al.*, 1998
	Fibroblast (A)	M	G0–G1	+					Wakayama and Yanagimachi, 1999
	Fibroblast transgenic (F)	Fe	M	+				+	Ono *et al.*, 2001
	ES	M	G0–G1	+					Wakayama *et al.*, 1999
		M	G2–M	+					
	ES	Fe	M	+					Zhou *et al.*, 2001
	Sertoli (F)	M	G0–G1	+					Ogura *et al.*, 2000
Goats	Fibroblast transgenic (F)	Fe	G0–G1		+	+			Baguisi *et al.*, 1999
Pigs	Granulosa cells (A)	Fe	G0–G1					+	Polejaeva *et al.*, 2000
	Genital ridge (F)	M	G0–G1		+				Betthauser *et al.*, 2000
	Fibroblast (F)	M	G0–G1		+				
	Fibroblast (F)	Fe	G0–G1		+				Onishi *et al.*, 2000
Cat	Cumulus (A)	Fe	G0–G1	+					Shin *et al.*, 2002
Rabbit	Cumulus (A)	Fe	G0–G1	+					Chesne *et al.*, 2002
Mule	Fibroblast (F)	M	G0–G1	+					Woods *et al.*, 2003

MII: Oocyte activation was carried out at least 1 h after fusion; MII + Act: fusion and activation simultaneous. TII: telophase II enucleation; Activated: activation before fusion; Serial NT: serial nuclear transfer.
A: adult; E: embryonic; ES: embryonic stem cell; F: fetal; Fe: female; M: male; N: newborn.

G2 phase. As cells at different stages of the cell cycle can develop after transfer into pre-activated oocytes, this type of oocyte has been termed 'a universal recipient' (Campbell *et al.*, 1993).

The transfer of M-phase chromatin into pre-activated oocytes has also been reported; however, the fate of the transferred chromatin has not been described (Tani *et al.*, 2001b). It is hypothesized that M-phase chromatin would decondense and form a tetraploid (4C) nucleus that would undergo DNA synthesis; however, this would be dependent upon the cell cycle phase of the recipient (Fig. 1b). If the recipient was at the late stage of the cycle, it is possible that MPF activity may be increasing and M-phase chromatin would enter the first mitotic division and result in daughter cells with diploid pronuclei (Fig. 2).

It has been suggested in both mammals (Czolowska *et al.*, 1984; Szollosi *et al.*, 1988) and amphibians (Hoffner and Di Berardino, 1980; Leonard *et al.*, 1982; Di Berardino and Hoffner, 1983) that optimal reprogramming of the donor nucleus is obtained when the recipient cytoplast remains at MII (unactivated, high MPF activity). In this situation, the fate of the donor chromatin is dependent not only upon the stage of the cell cycle of the donor nucleus but also on the species and the period between fusion and subsequent activation. On short exposure the effects would be as described in Fig. 1a. However, on prolonged exposure a number of effects have been reported. PCC and spindle formation occur, but spindle organization is often disrupted (Czolowska *et al.*, 1984). In some cases, multiple pronuclei are observed (Fig. 1c) (Campbell and Wilmut, 1999); however, this can be avoided by the use of inhibitors or stabilizers of spindle formation, such as Nocodazole (Campbell and Wilmut, 1996) or other agents, such as di-methyl amino purine (Campbell and Eyestone, 1998). In the absence of such treatments it is unknown whether the multiple pronuclei undergo syngamy at the first mitosis and result in diploid daughter nuclei. When a spindle is formed, a diploid pronucleus and a polar body are formed subsequently (Fig. 1d), for example in mice (Kwon and Kono, 1996) and cattle (Alberio *et al.*, 2000).

Although cytoplast recipients can be divided into two main types based on MPF activity, further cytoplast recipients can be defined on the basis of the cell cycle stage at the time of enucleation, the MPF activity and the period of exposure of the donor chromatin to cytoplasmic factors. Four main types of cytoplast recipient can be produced from MII oocytes (Fig. 2).

The cell cycle of the donor cell may also affect development (Campbell *et al.*, 2001) (Table 1). Development to term has been reported in many studies using G0 donor cells from sheep (Campbell *et al.*, 1996b; Wilmut *et al.*, 1997b), cattle (Kato *et al.*, 1998b; Wells *et al.*, 1999), mice (Wakayama *et al.*, 1998a) and cats (Shin *et al.*, 2002). Although it has been suggested that donors in the G0 phase may be more amenable to nuclear reprogramming (Campbell *et al.*, 1996c), live offspring have also been obtained using nuclear donors in the G1 phase, as in cattle (Cibelli *et al.*, 1998b). In addition, M-phase cells have also been used successfully for NT into MII arrested oocytes in mice (Wakayama *et al.*, 1999a), sheep (Liu *et al.*, 1997) and cattle (Alberio *et al.*, 2000; Tani *et al.*, 2001b). Although recent studies in cattle support the previous hypothesis that G0 donors may be beneficial to development when using primary cell populations (Wells *et al.*, 2003), the overall efficiency remains at 1–3% in any species (Gurdon and Colman, 1999).

The reasons for the low efficiency of this technique are little known; however, functional studies have shown aberrant genomic methylation, unstable patterns of imprinting and gene expression in embryos reconstructed by NT. Furthermore, studies investigating structural re-modelling show the formation of multinuclei in zygotes and abnormal nucleolar structure during early development in cloned embryos (Hyttel *et al.*, 2001; Baran *et al.*, 2002; Laurincik *et al.*, 2002). In this review, we aim to discuss the significance of the cell cycle of donor and

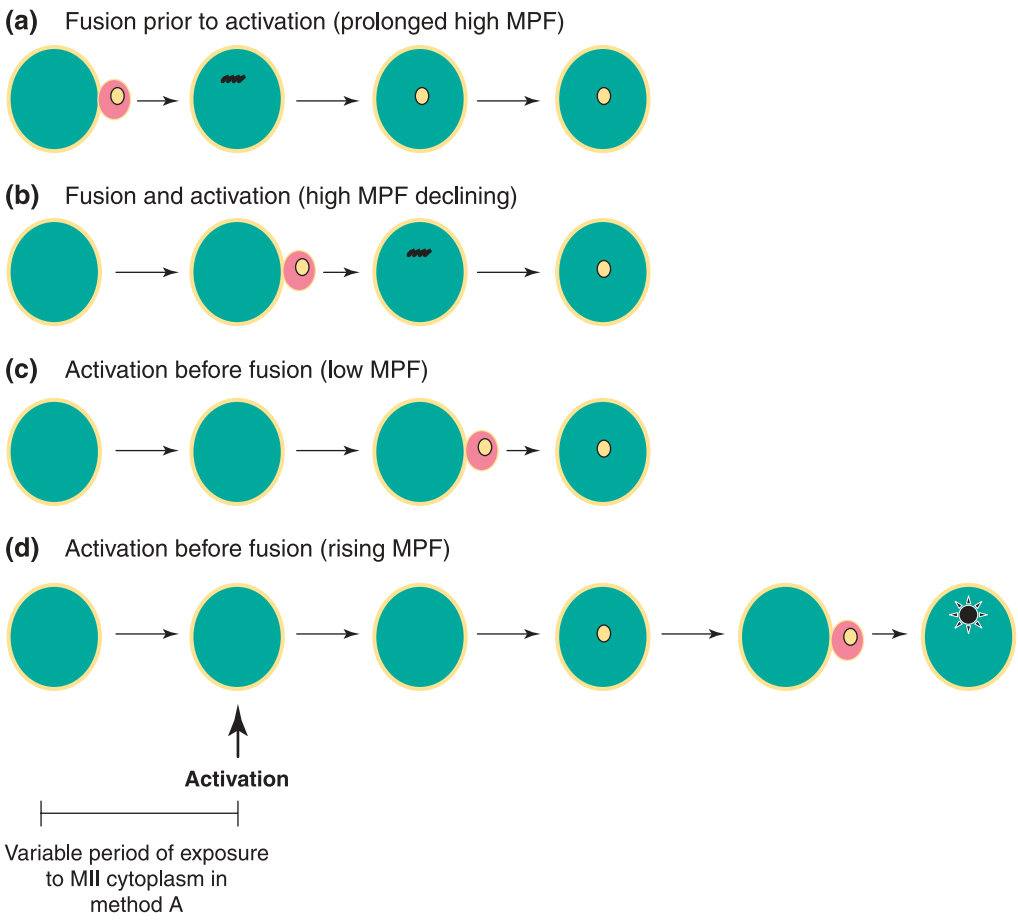

Fig. 2. Diagrammatic representation of major cytoplast recipients prepared from MII oocytes. The cytoplast recipients differ in their maturation promoting factor (MPF) activity and the period of exposure of the donor chromatin to MPF activity. (a) These cytoplasts have high MPF activity. Transfer of the donor cell is carried out before activation, nuclear envelope breakdown and premature condensation would occur. The period of exposure of the condensed chromatin to the recipient cytoplasm can be varied by delaying activation. (b) In these recipients, the oocyte is activated simultaneously with transfer of the donor nucleus; the period of exposure is dependent upon the time required for transfer (that is, injection versus fusion) and the rate of decay of MPF activity (dependent upon species and age of oocyte). (c) In these cytoplasts, MPF activity is minimal at the time of transfer of the donor nucleus. (d) In these cytoplasts, it is possible that donor nuclei could be transferred as MPF activity increases. Thus, G2 or M-phase chromatin would immediately enter a mitotic division; however, there are no reports of this in the literature. Other variations to these cytoplast recipients can be produced dependent upon the timing of enucleation, telophase I or telophase II for instance or enucleation after transfer.

recipient cytoplast on chromatin remodelling and gene expression in cloned embryos and to integrate cell cycle controls with embryonic and fetal development.

Effects of cell cycle on chromatin remodelling

It has been suggested that nuclear envelope breakdown and PCC are two essential structural remodelling events required for correct gene expression in NT embryos (Collas *et al.*, 1992).

Nuclear envelope breakdown induced by high MPF activity of an MII recipient oocyte facilitates the access to the chromatin of factors present in the cytoplasm. The effects of the cell cycle stage of the donor and recipient at the time of reconstruction have been studied using a number of reported markers of chromatin remodelling.

Histone H1

Histone H1 immunoreactivity after nuclear transplantation has been demonstrated in mouse and cattle embryos (Bordignon *et al.*, 2001). The loss of somatic H1 immunoreactivity is more efficient and rapid when either the donor nucleus or the recipient cytoplast is at M phase. Moreover, when the recipient oocyte is not enucleated, no loss of H1 immunoreactivity occurs in the transferred nucleus, indicating that cytoplasmic factors accumulate in the host nucleus and are not available to remove somatic H1 from the transferred nucleus. This observation may explain the significance of the removal of the nuclear component during the enucleation process and its influence on nuclear reprogramming and, in particular, may explain the failure of enucleated zygotes as cytoplast recipients.

Histone H4 acetylation

Histone H4 acetylation is another modification that occurs in normally fertilized embryos. In the mouse, the sperm chromatin decondenses shortly after fertilization and becomes acetylated. This is followed by the acetylation of the maternal chromatin, so that by the time syngamy occurs both genomes show a similar pattern of acetylation (Adenot *et al.*, 1997). In contrast, although the chromatin of the MII arrested oocyte shows very low amounts of acetylation, after parthenogenetic activation the maternal chromatin becomes highly stained. This finding indicates that the process of genome acetylation is dependent upon oocyte activation, either by the spermatozoa or an artificial stimulus, and that the sperm chromatin out-competes maternal chromatin for hyper-acetylated H4 (Adenot *et al.*, 1997). This finding is supported by McLay *et al.* (2002a) who reported that the ability to transfer histones into spermatozoa is acquired during oocyte maturation and is Ca^{2+}-dependent. Histone H4 acetylation activity is linked to transcriptionally active genetic loci (Vettese-Dadey *et al.*, 1996) and is independent of the DNA synthetic activity of the oocyte (Adenot *et al.*, 1997). It now remains to be shown whether NT embryos reconstructed by fusing a donor cell into a pre-activated oocyte will undergo the same level of acetylation as embryos reconstructed with MII oocytes. These studies will provide information about the importance of histone acetylation and transcriptional activity in cloned embryos.

DNA methylation

DNA methylation is another epigenetic modification that the genome undergoes during early embryogenesis (Monk *et al.*, 1987); however, its exact regulation during early development has not been fully elucidated. In *Xenopus laevis*, promoter regions of genes involved in early embryonic development are preferentially demethylated at the mid-blastula transition when genomic transcription occurs (Stancheva *et al.*, 2002). This passive demethylation may be the result of competition between transcription factors present in the form of maternal RNAs and proteins, and decreasing amounts of the maintenance methyltransferase enzyme (*Dnmt1*) in the early embryo (Matsuo *et al.*, 1998). This hypothesis is supported by the fact that early gene activation in *xDnmt1*-depleted embryos is restricted to genes activated at the mid-blastula transition, whereas unmethylated promoter regions of genes that are normally not transcribed at this stage are not activated (Stancheva *et al.*, 2002). In mice, it has been

shown that demethylation of the male genome is accomplished as early as 4 h after fertilization by an active process (Mayer *et al.*, 2000; Oswald *et al.*, 2000). This is followed by further passive demethylation up to the morula stage. By the time of blastocyst formation *de novo* methylation takes place in the inner cell mass, but not in the trophectoderm cells (Santos *et al.*, 2002). The pattern of genomic demethylation in the bovine embryo after IVF is similar to that of the mouse embryo up to the eight-cell stage; however, *de novo* methylation has been reported in later developmental stages, including trophectoderm cells of the blastocyst (Dean *et al.*, 2001). In bovine embryos produced by NT, several studies have shown aberrant methylation patterns during preimplantation development (for reviews, see Han *et al.*, 2003; Reik *et al.*, 2003). Bovine cloned embryos reconstructed with fetal cells showed a reduction in methylation at the one-cell stage, but re-methylation occurred at early stages, resembling the methylation pattern of the donor cell when the embryo reached the morula stage (Dean *et al.*, 2001). In another study carried out with embryos reconstructed using adult skin fibroblasts as nuclear donors (Bourc'his *et al.*, 2001), there was an absence of demethylation during the first three cell divisions and some euchromatin demethylation occurred from the eight-cell stage. From the morula stage, the euchromatin was undermethylated; however, in contrast to embryos produced *in vitro*, centromeric heterochromatin remained methylated in clones. A recent study using bisulphite treatment reported a differential methylation of euchromatic and heterochromatic sequences in NT embryos and also an abnormal hypermethylation of trophectoderm cells in bovine clones (Kang *et al.*, 2002). Demethylation of heterochromatic and euchromatic repeats has also been shown in pig embryos derived both *in vivo* and *in vitro* (Kang *et al.*, 2001b). Moreover, this demethylation pattern was shown to be similar in pig somatic clones (Kang *et al.*, 2001b). This finding contradicts the results obtained in bovine embryos indicating that there may be species differences in the mechanisms of epigenetic reprogramming. Another interesting study reported in bovine tetraploid clones shows an increased demethylation of embryos reconstructed in the presence of the maternal chromosomes, that is without removal of cytoplasmic components present in the vicinity of the metaphase plate of the matured oocyte (Kang *et al.*, 2001c). This finding indicates compartmentalization of factors involved in chromatin remodelling in the oocyte, the distribution of which may also be influenced by the status of the chromatin in the oocyte. Together these studies have shown similarities and differences, this may in part be due to the technical procedure; however, there may also be differences as a result of the biological material. For instance, it has been shown that fetal cells have greater methylation of euchromatin when compared with the pattern in aged fetal cells (Kang *et al.*, 2001a); however, whether these differences are also observed *in vivo* remains to be elucidated. It should also be considered that these groups adopted different methods of embryo reconstruction. Young, matured oocytes were used as recipient cytoplasts in the studies of both Dean *et al.* (2001) and Kang *et al.* (2002); the results indicate that the donor chromatin was exposed to high amounts of MPF for at least 2 h before oocyte activation was conducted. The exposure to high amounts of MPF may be a determinant for epigenetic reprogramming of the genome in cloned embryos. In the study of Bourc'his *et al.* (2001), aged oocytes with lower amounts of MPF were used. Presumably, the chromatin of the transferred nucleus started DNA replication earlier than those transferred into MII arrested oocytes, without undergoing the same epigenetic reprogramming. This hypothesis is supported by the fact that development to blastocysts is significantly improved when young MII oocytes are used in comparison with aged oocytes (Vignon *et al.*, 1998; Zakhartchenko *et al.*, 1999b; Zakhartchenko *et al.*, 2001); however, full-term development related to transferred embryos does not differ between the two approaches. Bovine embryos reconstructed in an environment of low MPF may undergo only minor epigenetic reprogramming and as a consequence they are unlikely to develop beyond the blastocyst stage. No

final conclusions can yet be made about genomic methylation after NT; however, analysing specific sequences of the genome (Stancheva *et al.*, 2002) and considering the methods used for embryo reconstruction may help in the understanding of epigenetic reprogramming.

X chromosome inactivation

An example of epigenetic modification after NT is the inactivation of the X chromosome in reconstructed embryos. In cloned mice, X chromosome inactivation occurs in both trophectodermal and embryonic tissues (Eggan *et al.*, 2000b). In cattle, the paternal X chromosome is inactive in placental tissue of normal clones and bovine fetuses from natural reproduction, whereas both X chromosomes are active in placentae from deceased clones (Xue *et al.*, 2002). Abnormal X chromosome inactivation in the trophectoderm leads to fetal loss in cloned cattle. Fetal abnormalities and low viability have been reported with different NT protocols, indicating that the methods for embryo reconstruction used at present do not facilitate correct reprogramming of the somatic nucleus.

Gene expression in early embryos

Early development is characterized by a switch from maternal transcripts to zygotic transcripts that direct development. Activation of the embryonic genome is species-dependent and related to epigenetic modifications of the chromatin (for a review, see Kanka, 2003). In bovine NT embryos, RNA synthesis is dependent upon the stage of the cell cycle of the recipient oocyte; activated cytoplast recipients (with low MPF activity) are less able to inhibit transcription from the transferred nucleus than MII cytoplasts (high MPF activity). In addition, RNA synthesis is detectable at the four-cell stage in embryos reconstructed with activated cytoplasts but not in embryos reconstructed with MII cytoplasts (Kanka *et al.*, 1999). The effect of the exposure of the donor cell to high MPF activity has also been analysed upon expression of developmentally important genes in somatic clones. A delay of 4 h between fusion and activation leads to an increased number of embryos expressing FGF4 in cattle clones (Daniels *et al.*, 2001). Moreover, the expression of interferon τ is higher in embryos reconstructed by simultaneous fusion and activation compared with embryos in which the fusion activation interval was 3–5 h. Recent studies indicate that early and high expression of interferon τ are indicative of poor quality embryos (Kubisch *et al.*, 1998). In contrast the mRNA contents of the HSP70.1, MASH2 and DNMT are not affected by the time of exposure to MPF, but depend on the stage of the cell cycle of the donor cell (Wrenzycki *et al.*, 2001).

Possible role of cell cycle control in growth and development of NT embryos

During its lifetime, a single cell must duplicate all of its components and give rise to two daughter cells that are identical to each other and identical to the parent cell at birth. The events that occur during a single growth cycle can be divided into the 'nuclear division cycle' and the 'growth cycle'. In an actively proliferating population of cultured cells, the cells maintain a constant macromolecular composition, and a constant size and shape. This requires the co-ordination of the nuclear and growth cycles. Cell growth and division are dependent upon external proliferative signals; however, the mechanisms by which growth and division are co-ordinated are unknown, although a range of mechanisms have been

suggested (for reviews, see Neufeld and Edgar, 1998; Polymenis and Schmidt, 1999; Tapon *et al.*, 2001). The mechanisms that have been suggested include the following:

(1) *Cell division drives cell growth.* This proposal is that growth is a consequence of the nuclear division cycle. However, a number of observations indicate that this is unlikely. Firstly, if the amount of the G1 cyclins is increased, there is no increase in growth rate and a decrease in cell size is observed: that is, Cln3 in yeast Nash *et al.*, 1988), cyclin D1 or E in mammalian cells (Ohtsubo and Roberts, 1993; Quelle *et al.*, 1993; Resnitzky *et al.*, 1994) or in *C. elegans* by inhibiting the proteolysis of cyclins (Kipreos *et al.*, 1996). Secondly, if the cell division cycle is blocked, growth continues resulting in larger cells (Johnston *et al.*, 1977; Neufeld and Edgar, 1998).

(2) *Growth drives cell division*. During the G1 phase of the cell cycle there is a restriction point termed 'Start' or 'R': to pass this point cells must attain a critical size or a certain biosynthetic capacity. However, experiments have shown that activation of G1 cyclin–cdk complexes is sufficient for the G1–S-phase transition. An example of this is given by the yeast *CLN3* gene (yeast G1 cyclin). The product of this gene initiates a START transcriptional programme including transcription of other G1 cyclins (Wittenberg and Reed, 1996). Cln3p is unstable; however, amounts remain more or less constant during the cell cycle possibly regulated by its synthesis. The amount of Cln3p is sensitive to the number of active ribosomes in a cell. As growth decreases there are fewer ribosomes and less Cln3p. As growth increases more ribosomes accumulate and increased amounts of Cln3p are found. When cells are starved, there is a decrease in the number of ribosomes and a decrease in Cln3p, indicating translational control (Gallego *et al.*, 1997; Polymenis and Schmidt, 1997). This type of translational control has also been reported for a number of other cell cycle-related genes, including CDK4 and its partner cyclin D1, $p27^{(Kip1)}$ and MDM2 (reviewed by Neufeld and Edgar, 1998). Therefore, expression may be restricted to conditions favouring maximal growth. Studies carried out in yeast indicate that commitment to cell division is linked to signals that direct ribosome biosynthesis. Thus, the cell is able to adjust the critical cell size threshold before a change in ribosome content and protein synthetic rate occurs (Jorgensen *et al.*, 2002). The interplay between ribosome assembly and cell cycle progression appears to be conserved in higher eukaryotic cell division (Kozma and Thomas, 2002).

(3) *Control of growth and division is co-ordinated*. Both growth and division cycles respond to a common signalling pathway. There are several examples of this type of co-ordination (for reviews, see Neufeld and Edgar, 1998; Polymenis and Schmidt, 1999; Tapon *et al.*, 2001) including:

- *TATA box binding protein associated factors (TAFs)*. In yeast, yTAFs target G1–S-phase cyclins and growth genes (that is, ribosomal proteins) and are responsive to growth state. An increase in growth rate is accompanied by an increase in yTAF, and inactivation of TAF causes G1 arrest.
- *RAS*. RAS activates MAP and PI3-kinase pathways both of which target translation initiation factors and p70 S6 kinase which modulate protein synthesis. This stimulates S-phase progression via stimulation of CDK activating phosphatases, inactivation of $p27^{(kip1)}$ and upregulation of cyclin E expression.
- *Myc*. *Myc* may promote growth via transcriptional activation of genes involved in protein synthesis including ribosomal components and translation initiation factors *Rb*. This tumour suppressor gene has effects on a number of processes including inhibition of transcription via inhibition of RNA polymerases I, II and III.

Table 2. Genotypes and phenotypes of murine cell cycle gene knockouts

Genotype	Phenotype	Reference
RB –/–	Embryonic lethal, neurogenesis and haematopoiesis	Lin *et al.*, 1996
RB +/–	Viable, thyroid and pituitary tumours	Lin *et al.*, 1996
P27 –/–	Gigantism, pituitary hyperplasia, infertility in females	Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996; Fero *et al.*, 1996; Nagahama *et al.*, 2001
P18 –/– P18 –/– p27 –/–	Gigantism	Franklin *et al.*, 1998
P57 –/–	Defects in kidneys, long bones, eye lens, abdominal wall	Zhang *et al.*, 1997; Yan *et al.*, 1997
P27 –/– p57 –/–	Increased embryonic lethality, placental defects	Zhang *et al.*, 1998

- *P53*. *P53* is involved in DNA damage mediated cell cycle arrest. It inhibits RNA pol III, possibly via TFIIIB and may be linked to nucleotide pools.
- *Insulin*. The insulin mediated signalling pathway causes phosphorylation of ribosomal protein S6 which may increase the translational capacity of the cell via production of ribosomal components and translation initiation factors.

(4) *There is independent regulation of growth and division*. There are many exceptions to the co-ordination of growth and division cycles during development. For example in *Drosophila*, embryonic cycles before hatching are growth independent and rely on oocyte stores (Edgar and Lehner, 1996). Similarly, in *Xenopus* during early cycles, the G1 phase of the cell cycle is absent and no growth occurs (Kirschner *et al.*, 1985).

In addition to co-ordinating cell growth with division cycles, these cycles must also be co-ordinated with differentiation, cell death and tissue-specific compartmental controls. It is probable that a range of control mechanisms is involved in co-ordinating these events. In mammalian embryos during early cycles there is little transcription from the zygotic genome and cell division is associated with a reduction in cell size, again indicating an uncoupling of these two events and possibly the involvement of maternally inherited factors in early division cycles. Numerous mechanisms and feedback controls have been described in co-ordinating the nuclear division cycle, that is completion of S-phase before initiation of M-phase (Cerutti and Simanis, 2000; Lew, 2000). The controls operating during mammalian embryogenesis are poorly described; however, species differences are evident. As described above, the cell cycle phase of the recipient and donor cells can have profound effects on the remodelling of donor chromatin. The frequency of successful development to term and survival of embryos reconstructed by NT is extremely low. It has been suggested that only a small percentage of donor cells can be reprogrammed and that those embryos that develop may represent a sub-population of donor cells. An alternative explanation is that only a small percentage of recipient cells are able to 'remodel' and 'reprogramme' the donor nucleus; however, in practice a range of factors is probably involved. The co-ordination of cell growth and division is essential for normal development. The effects of the culture environment upon this co-ordination have been essential in defining some of the controls described above. The culture environment has also been implicated in epigenetic modification of murine ES cells and in the

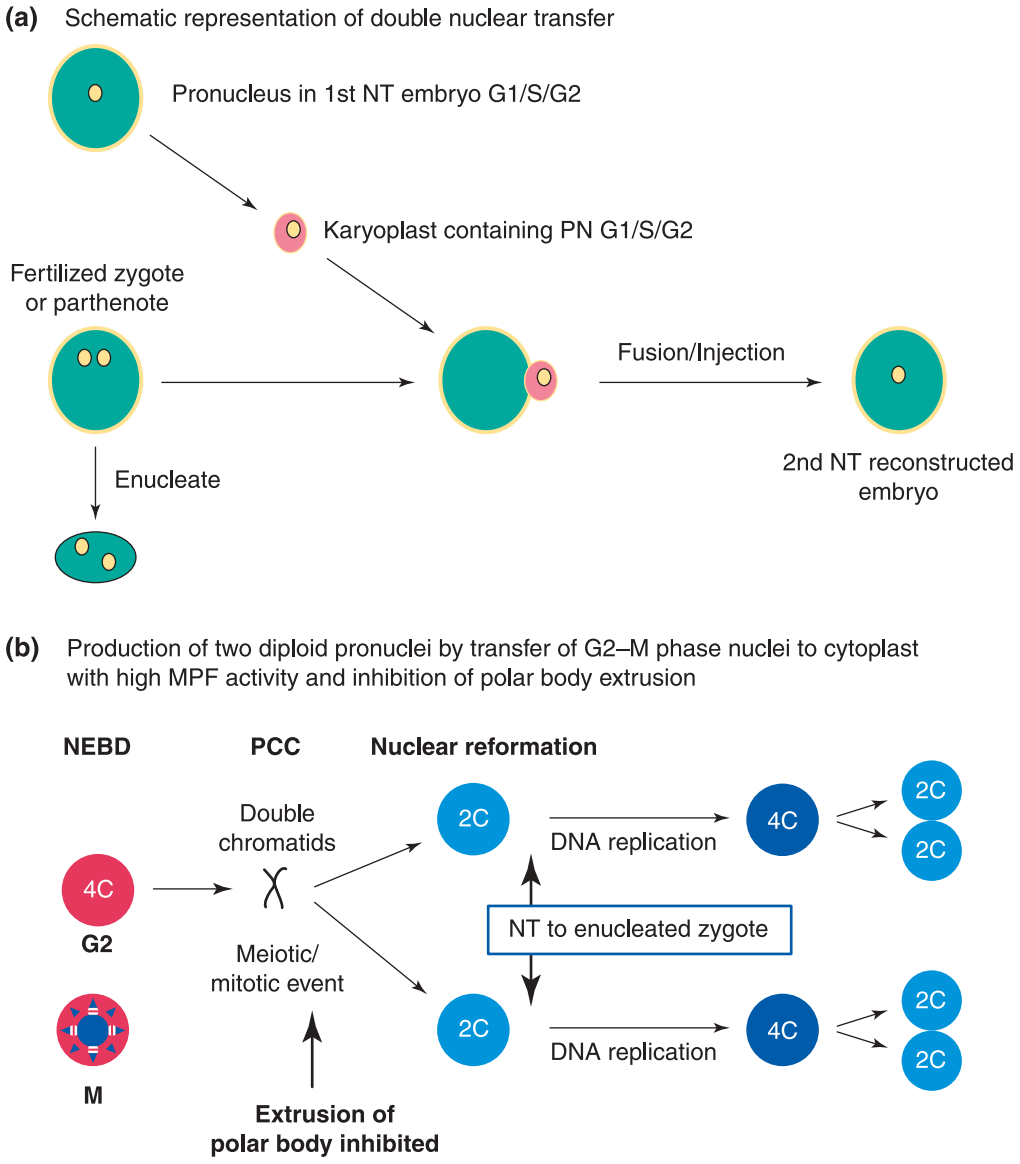

Fig. 3. (a) Diagrammatic representation of the double nuclear transfer (NT) procedure. A diploid pronucleus produced by any of the methods outlined in Figs 1 and 2 may be transferred to an enucleated zygote or parthenote. Both the donor karyoplast and the recipient cytoplast have low maturing promoting factor (MPF) activity by definition, as defined by the presence of an intact pronucleus. In this situation, DNA replication would appear to be co-ordinated between donor and recipient cells. (b) In addition to the methods outlined in Figs 1 and 2, a suitable donor pronucleus may be produced from a tetraploid zygote when polar body extrusion is inhibited (Kwon and Kono, 1996). NEBD: nuclear envelope breakdown; PCC: premature condensation; PN: pronucleus.

development of NT reconstructed embryos, further linking cell cycle control, development and differentiation. In addition, experiments in which cell cycle regulatory genes have been knocked out in mice have resulted in a range of developmental phenotypes (Table 2). Many of these phenotypes resemble the abnormalities observed during development of NT derived

embryos or fetuses, including increased embryonic or fetal lethality, organomeglia, skeletal defects, lung defects and placental defects.

As described above, the presence or absence of the MII spindle in oocytes used as cytoplast recipients has been shown to effect chromatin remodelling. In addition, we hypothesise that removal of the MII spindle (enucleation) may also cause perturbations in cell cycle control, which could have long lasting consequences and contribute to developmental failure of NT derived embryos. Many of the proteins that are involved with cell cycle control are associated with the mitotic or meiotic spindle, including MPF (Czolowska *et al.*, 1986), c-mos (Zhou *et al.*, 1991; Wang *et al.*, 1994) and Cdks (Jiang *et al.*, 1998; John *et al.*, 2001; Menssen *et al.*, 2001; Mollinari *et al.*, 2002; Yoshida *et al.*, 2002). The effects of depleting the oocyte of these proteins on subsequent development are unknown; however, several reports provide indirect evidence of their possible role. Studies on the use of bovine and murine oocytes enucleated at telophase II following activation indicate a greater frequency of development (Bordignon and Smith, 1998; Baguisi and Overstrom, 2000). In humans, a single report on the production of embryonic stem cells from NT reconstructed embryos indicated that NT must be carried out before enucleation for the technique to be successful (Meek, 2001). In both of these situations, it is possible that cell cycle-related proteins have been released from the oocyte chromatin before enucleation and, therefore, may remain in the cytoplast in higher concentrations. Other indications stem from the results of studies using a double nuclear transfer procedure (Fig. 3). In this technique, the first nuclear transfer uses an enucleated metaphase II oocyte as cytoplast recipient. The resultant diploid pronucleus is then transferred into an enucleated, fertilized zygote. This technique has been used successfully in pig cloning (Polejaeva *et al.*, 2000), and studies in mice have indicated that this technique results in fewer abnormalities (Ono *et al.*, 2001). These observations may result from a number of factors, including increased activation due to the use of spermatozoa, or the presence of paternal transcripts or proteins; however, it may also be that by using this procedure the final reconstituted embryo contains a more physiological content of oocyte proteins which contribute to development of the embryo. Other modifications to the techniques used for embryo production also appear to increase the frequency of development and reduce developmental abnormalities, for example the *in vivo* culture of both pig and goat NT embryos (Baguisi *et al.*, 1999; Polejaeva *et al.*, 2000) or the use of embryonic stem cell tetraploids in mice (Eggan *et al.*, 2000a). One explanation of these observations is that the effects of NT and the culture environment on development are interactive and may occur through mechanisms involving cell cycle controls particularly during the early cleavage cycles.

Conclusion and perspectives

Nuclear transfer has numerous roles to play in both research and application; these include animal production and biotechnology, gaining a greater understanding of the mechanisms controlling early development, improving reproductive techniques, the production of autologous stem cells for human therapeutics and genetic conservation (for a review, see Campbell, 2002). At the present time, the efficiencies of the current techniques limit application in several of these areas; however, NT has provided a route for complicated genetic manipulation including double gene knockouts in pigs for research on xenotransplantation (Phelps *et al.*, 2003) or the use of artificial chromosome vectors for the expression of complex proteins, such as human antibodies, in transgenic cattle (Robl, 2003). In the areas of genetic modification the low efficiencies are balanced against the need for the production of only founder animals which cannot be produced by other means at present. For animal production again the benefit of genetic conservation may exceed the low efficiencies; however, as a means for routine

animal production the low efficiencies coupled with the developmental abnormalities reported outweigh the benefits. Improving the frequency of development of embryos produced by NT is a major goal of present research. Studies on the cell cycle proved essential to increasing the frequency of development of embryos created by NT using embryonic blastomeres as nuclear donors and in creating the first mammals by NT from cultured differentiated cell populations. Early studies demonstrated the requirement for cell cycle co-ordination between donor and recipient to prevent DNA damage and maintain ploidy (for a review, see Campbell *et al.*, 1996b). As described above, subsequent studies have shown that the cell cycle of both the donor and recipient cells at the time of NT can have significant effects upon epigenetic modification of the donor chromatin and subsequent development. This review hypothesizes that the cell cycle may have further implications for embryo development via mechanisms that co-ordinate cell growth, cell division and cell differentiation. The method of embryo reconstruction, cell cycle stage at enucleation, presence or absence of the recipient nucleus at the time of nuclear transfer and the activation process may affect epigenetic reprogramming of the donor nucleus. Further perturbations in development may also be associated with the removal of cell cycle regulators during the enucleation process. In summary, a greater understanding of the regulation of cell growth and division during embryo and fetal development with particular reference to epigenetic modification of nuclear and chromatin structure is required. More specifically, identification of the proteins removed during enucleation and their role in normal development may help us to devise a more suitable cytoplast recipient.

This article has concentrated on the potential role of the cell cycle in the development of NT embryos; many factors contribute to successful development and other strategies to improve reprogramming and increase the frequency of development are being pursued. Various studies have implied that the epigenetic status varies among types of donor cell and that specific types of cell may be more amenable to reprogramming. In addition, culture conditions of the donor cell can affect its epigenetic status (for a review, see Jaenisch *et al.*, 2003). Permeabilization of the donor nucleus or modification of chromatin structure by chemical treatments to demethylate or hyperacetylate before transfer have been investigated (for a review, see Vignon *et al.*, 2003). More strikingly, it was demonstrated in sheep that somatic cells denatured by heat treatment were capable of producing live offspring (Loi *et al.*, 2002). In this and other laboratories, cytoplasmic extracts from a range of cell types are being used to alter chromatin structure of somatic cells, not only as a means of improving reprogramming after NT, but also as a method for trans-differentiation of somatic cells for autologous cell therapies (Alberio and Campbell, 2003; Hakelien *et al.*, 2002; Hakelien and Collas, 2003).

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