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Factors Affecting the Development of Embryos Produced by Nuclear Transfer

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ABSTRACT

The development of embryos reconstructed by nuclear transfer is dependent upon numerous factors including the type of recipient cell, method of enucleation, the type of donor cell, method of reconstruction, activation, the cell cycle stage of both the donor nucleus and the recipient cytoplasm and the method of culture of the reconstructed embryos. Many of these points which have been reviewed extensively elsewhere (Sun and Moor, 1995; Colman, 1999; Oback and Wells, 2002; Renard *et al*., 2002; Galli *et al*., 2003b), here we will concentrate on main area, the production of suitable cytoplast and nuclear donor, nuclear-cytoplasmic coordination, oocyte activation, culture of reconstructed embryos, and the effects that this may have on development.

(Key words : nuclear transfer, cytoplast, nuclear donor cell, nuclear-cytoplasmic coordination, oocyte activation)

INTRODUCTION

The concept of nuclear transfer (NT) was first proposed in 1938 by Spemann, who suggested the transfer of a single nucleus into an enucleated oocyte as a method to study cell differentiation and nuclear equivalence. However, due to his death and to inadequate technical skills the first successful NT experiments were not reported until 1952 when Briggs and King demonstrated the production of swimming tadpoles after transplantation of blastula nuclei into enucleated frog eggs. Experiments in amphibians continued and in 1962, John Gurdon reported the production of adult toads (*Xenopus laevis*) after the transfer of nuclei from tadpole intestinal epithelial cells (Gurdon, 1962a; Gurdon, 1962b). This was the first demonstration that the nucleus from a differentiated cell could successfully support development, however, in subsequent experiments using nuclei derived from adult keratinocytes, although swimming tadpoles were produced no adult animals were obtained (Gurdon *et al.,* 1975).

In mammals, due to the size of the oocyte and the requirement for more specialised equipment NT was not reported until 1975 when Bromhall attempted NT using rabbit eggs. In the early 1980s McGrath and Solter reported the first successful NT in mammals, live offspring were obtained after the swapping of pronuclei between fertilised zygotes demonstrating that embryo development could occur after micromanipulation (McGrath and Solter, 1983), however, when nuclei from later developmental stages were transferred into enucleated zygotes no live offspring were obtained (McGrath and Solter, 1984). Using a modification of the technique reported by McGrath and Solter, Willadsen reported in 1986 the birth of live lambs after the production of embryos by NT using early embryonic blastomeres from 8～16 cell embryos as nuclear donors and enucleated Metaphase II (MII) oocytes as cytoplast recipients (Willadsen, 1986). The use of early embryos as nuclear donors and enucleated MII oocytes as recipients continued in other species and both cattle (Robl *et al.,* 1987) and pigs (Prather *et al.,* 1989) were subsequently cloned. The use of early embryos as nuclear donors and the low frequency of development obtained severely limited the application of this technology and efforts were focused on the development of NT techniques from cultured cell populations which would allow the production of large number of genetically identical animals and provide a route for precise genetic modification.

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In 1994, Sims and First reported the production of live calves from inner cell mass cells which had been maintained in culture, however, under the conditions employed these cells grew very slowly, if at all, and were of limited use. In 1996, Campbell and colleagues (Campbell *et al.,* 1996b) reported the birth of live lambs from cultured cells which were established from a blastocyst stage embryo, these cells had in fact differentiated in culture and this report paved the way for the subsequent development of offspring using cells derived from foetal and adult tissues (Wilmut *et al.,* 1997). Since this time offspring have been reported in a range of species including cattle (Cibelli *et al.*, 1998), mice (Wakayama *et al.* 1998), goats (Baguisi *et al.,* 1999), pigs (Polejaeva *et al.,* 2000), cats (Shin *et al.,* 2002), rabbits (Chesne *et al.,* 2002), mules (Woods *et al,*. 2003), rats (Zhou *et al.,* 2003) and horses (Galli *et al.,* 2003a) and from a variety of cell types derived from embryos, foetuses, juvenile and adult animals.

1. The Recipient Cell (Cytoplast)

Studies in mammalian NT have utilised a range of embryonic cells as cytoplast recipients including oocytes, zygotes and early cleavage stage embryos with varying success. Enucleated zygotes of both mouse (McGrath and Solter, 1983; McGrath and Solter, 1984; Kwon and Kono, 1996), cattle (Prather and First, 1990), and pig (Prather *et al.,* 1989) resulted in limited development of the reconstructed embryos. Although studies in the mouse demonstrated that enucleated 2-cell embryos could support development from early blastomere nuclei (Tsunoda *et al.,* 1987), there are no reports of successful development from later stage nuclear donors. The use of matured oocytes (also termed unfertilised eggs) arrested at metaphase of the second meiotic division (MII) has resulted in successful development from a range of cell types in a variety of species and have commonly become the cytoplast of choice. MII oocytes for use as cytoplast recipients can be obtained by a variety of means including; *in vivo* maturation by flushing from the oviduct (Willadsen, 1986), by *in vitro* maturation of oocytes aspirated from antral follicles of living animals or by *in vitro* maturation of oocytes recovered from slaughtered animals. Although the use of *in vivo* matured oocytes may have beneficial effects on early embryo and foetal development (Wells *et al.,* 1997), *in vitro* matured oocytes are commonly used for NT in farm animal species according to cost effectiveness (Farin *et al.,* 2001). MII oocytes have been used as cytoplast recipients for NT utilising a number of protocols, differences in these methods including the method of enucleation, the timing of enucleation, the method and timing of nuclear transfer (fusion or injection) and activation may all effect the development of the reconstructed embryos. Some of these differences will be discussed in more detail with relation to biological differences in the resultant cytoplast and the possible effects on development.

2. The Nuclear Donor Cell

Embryonic blastomeres from early stage embryos were first used as nuclear donors. When it was established that co-ordination of the cell cycle between donor and recipient cells was essential to ensure normal development several laboratories started to investigate the possibility of using differentiated cell types on specific cell cycle stages for somatic cell nuclear transfer (SCNT). After the production of the first mammals from cultured embryonic (Campbell *et al.,* 1996) foetal and adult cell lines (Wilmut *et al.,* 1997) numerous studies provided extensive evidence that somatic cells from different tissues and ages of animals can be used for SCNT (Shiga *et al.,* 1999; Zakhartchenko *et al.,* 1999; Kato *et al.,* 2000). Embryonic stem cells have been used for SCNT and better development was reported in some studies (Zhou *et al.,* 2001; Eggan *et al.,* 2002), although other reports indicate widespread epigenetic instability in ES cloned mice (Humpherys *et al.,* 2001). In another study somatic cells clones showed normal expression of imprinted genes after SCNT (Inoue *et al.,* 2002) although this contradicted a report indicating altered gene expression pattern in clones derived from ES and cumulus cells (Humpherys *et al.,* 2001; Humpherys *et al.,* 2002). The differences between groups could be related to variation in the ES cells used in each study and also may be affected by the manipulation and culture systems used in each study. Unfortunately no conclusion can be made on what is the most appropriate cell type for SCNT. However, what is certain is that cells derived from early embryos, foetuses, adult differentiated and postmitotic cells (Eggan *et al.,* 2004) have successfully been employed for the generation of cloned animals.

3. Nuclear-cytoplasmic Coordination of Donor And Recipient Cells

During a single cell cycle a cell must duplicate all of its components and give rise to two daughter cells which are identical to each other and identical to the cell at birth (Mitchison, 1971). The events occurring during a cell cycle can be divided into those involving cell growth and those involving the nu-

cleus. These two major groups of events are intimately linked, however, for simplicity I will only describe the events occurring in the nuclear division cycle.

The cell cycle of eukaryotic cells is divided into four distinct phases: G1, S, G2 and M. The discrete period of DNA synthesis (S phase) is preceded by a pre-DNA synthesis period (G1), and followed by a post-DNA synthetic period (G2). The replicated genetic material is equally segregated to the two daughter cells during mitosis (M-phase). The nuclear division cycle involves two major events, DNA replication (S-phase) and segregation of the duplicated genetic material (M phase or mitosis). During a single cell cycle all chromosomal DNA must be replicated once. The mechanisms by which a cell coordinates DNA replication and prevents re-replication of previously replicated DNA are unclear however; central to this control is maintenance of an intact nuclear envelope (Blow and Laskey, 1988).

Early studies in NT reconstructed embryos demonstrated the importance of cell cycle coordination between the donor nucleus and the recipient cytoplast in order to prevent DNA damage and maintain ploidy of the reconstructed embryos (for reviews see (Campbell *et al.,* 1996a; Campbell, 2002; Campbell and Alberio, 2003)). It is now accepted that two major types of recipient oocytes (enucleated metaphase II (MII) oocytes and pre-activated enucleated MII oocytes) are suitable for development to term after single nuclear transfer. One of the major differences between these recipient oocytes is the levels of protein kinase activities present in the cytoplasm. MII arrested oocytes contain high levels of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activities, cytoplasmic protein kinases responsible for the changes in nuclear and chromatin structure during both meiotic and mitotic cell cycles cell cycle (Campbell *et al.,* 1996a). The presence or absence of these protein kinases can have beneficial or deleterious effects on the fate of the transferred nucleus in NT embryos.

1) Cytoplast with High MPF

Matured oocytes typically become arrested at metaphase of the second meiotic division (MII) and contain high levels of MPF activity. When an interphase nucleus is transferred into MII oocyte, the presence of high levels of MPF in the cytoplasm induces the transferred nucleus to enter a mitotic division precociously and causes nuclear envelope breakdown (NEBD), premature chromosome condensation (PCC) and dispersion of nucleoli which may be essential for nuclear reprogramming (Collas *et al.,* 1992a). The patterns of NEBD and PCC are dependent upon the stage of the donor nucleus at the time of transfer (Campbell *et al.,* 1996a; Campbell and Alberio, 2003). All nuclei that undergo NEBD, regardless of their cell cycle undergo DNA synthesis following the decline of MPF activity, reformation of the nuclear envelope and nuclear swelling. NEBD and PCC have no apparent deleterious effect on either G1 or G2 nuclei, forming single or double chromatids respectively. However, S-phase chromatin has a typical pulverised appearance thought to be associated with high levels of DNA damage (Collas *et al.,* 1992b) when mitotic chromosomes are transferred the chromosomes remain condensed (Alberio *et al.,* 2000b) (Fig. 1).

2) Cytoplast with Low MPF

On the other hand, if nuclei are transplanted into pre-activated oocytes, in which MPF and MAPK activities have declined following activation or fertilization, no NEBD or PCC are observed. These oocytes are so called 'permissive' to donor nuclei in G1, S or G2-phases of the cell cycle with coordinated DNA replication occurring dependent upon the cell cycle phase of the donor nucleus due to maintenance of an intact nuclear envelope. G1 nuclei undergo a single round of replication, S phase nuclei continue replication and G2 nuclei do not re-replicate (Barnes *et al.,* 1993; Campbell *et al.,* 1993) maintaining correct ploidy in embryos produced under these condi-

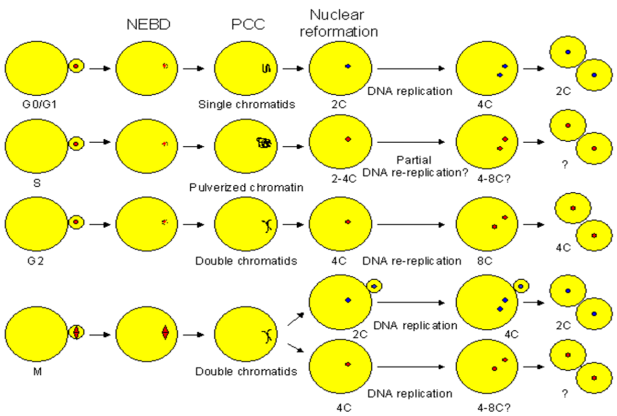

Fig. 1. Effects of nuclear transfer of karyoplasts at defined stages of the cell cycle into cytotplasts with high MPF activity on chromatin fate, DNA replication and ploidy of resultant daughter cells. M: mitosis, MPF: maturation promoting factor, NEBD: nuclear envelop breakdown, PCC: premature condensation, PB: polar body.

tions. Therefore, all of the resultant daughter cells will be diploid (2n). When any of these three cell cycle stage donor cells are transferred into pre-activated oocytes, development to blastocyst can be achieved (Campbell *et al.,* 1994). This type of oocyte has been termed "a universal recipient" (Campbell *et al.,* 1993). When quiescent cells (G0) are used as nuclear donors, an exception to coordinated replication exists. DNA replication requires the presence of chromosome-bound factors that are thought to attach following mitosis and prior to nuclear assembly. In quiescent cells these factors are lost with time and initiation of DNA replication requires permeabilisation of the nuclear membrane (Leno and Munshi, 1994), this would occur in cytoplasts with high MPF activity. The fate of M phase donor nuclei transferred into pre-activated oocytes has not been described. However, an M phase nucleus may undergo chromatin decondensation, pronuclear formation and DNA synthesis, resulting in the production of a tetraploid (4n) daughter cell (Fig. 2).

Although development to term has been obtained with both of these cytoplast recipients, improved development to both the blastocyst stage and to term has been reported when the donor chromatin is exposed to the recipient MII cytoplasm for an extended period (Wells *et al.,* 1999; Wells *et al.,* 2003).

4. Oocyte Activation

Mammalian oocytes are ovulated and arrested at MII until fertilization. During oocyte maturation (progress from the G2/M stages of the first meiotic division to MII) specific reorganization and redistribution of intracellular organelles occurs and the oocytes obtain a full complement of signalling molecules (Miyazaki *et al.,* 1993; Carroll, 2001). The oocytes are released from the meiotic arrest by fertilization and initiate early embryonic development by inducing a series of cellular events within the oocyte. This is referred to as "oocyte activation". The characteristic event of oocyte activation is initiation of intracellular calcium ($[Ca^{2+}]_i$) oscillations, leading to other events including, resumption and completion of meiosis, cortical granule exocytosis, decondensation of the sperm nucleus, recruitment of maternal mRNAs, formation of male and female pronuclei and the initiation of DNA synthesis. In NT transfer reconstructed embryos in addition to the transfer of donor genetic material from the karyoplast to the cytoplast, the cytoplast must be 'activated' in order to initiate development.

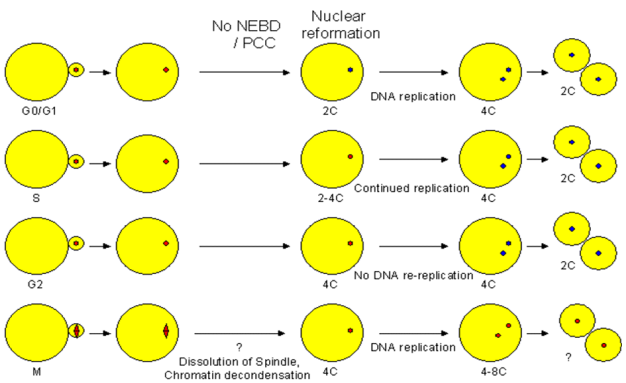

Fig. 2. Effects of nuclear transfer of karyoplasts at defined stages of the cell cycle into cytotplasts with low MPF activity on chromatin fate, DNA replication and ploidy of resultant daughter cells. M: mitosis, MPF: maturation promoting factor, NEBD: nuclear envelop breakdown, PCC: premature condensation, PB: polar body.

3) Events of Oocyte Activation

The fusing of the sperm to the oocyte plasma membrane induces an acute increase in cytosolic free Ca^{2+} concentration (Stricker, 1999) which induces oocyte activation. In all mammalian oocytes, the initial increase in Ca^{2+} is followed by a series of highly repetitive Ca^{2+} transients of high amplitude (Ca^{2+} oscillations). This Ca^{2+} release originates from the point of sperm entry and subsequent Ca^{2+} oscillations arise almost synchronously in the entire oocyte within a second (Miyazaki *et al.,* 1986). These oscillations have been reported in mouse (Cuthbertson and Cobbold, 1985; Kline and Kline, 1992), rat (Ben-Yosef *et al.,* 1993), rabbit (Fissore and Robl, 1992), bovine (Fissore *et al.,* 1992; Sun *et al.,* 1994), porcine (Sun *et al.,* 1992; Machaty *et al.,* 1997) and human (Taylor *et al.,* 1993; Tesarik, 1994). It is accepted that the site of Ca^{2+} release is the endoplasmic reticulum (ER), where ryanodine receptors or inositol 1,4,5-triphosphate (IP3) receptors are present (Kline and Kline, 1992). Although a single Ca^{2+} rise is sufficient for oocyte activation, sustained Ca^{2+} oscillations require a continuous Ca^{2+} influx to refill the endoplasmic reticulum stores (Miyazaki, 1995) for additional development events (Ozil, 1990).

The intracellular Ca^{2+} release induces the cortical reaction (CR). The cortical granules are located within the cortical region of the oocytes and contain specialized enzymes and glycoproteins. The cortical reaction occurs after sperm-oocyte membrane fusion, and is manifested by the release of the contents of the cortical granules into the perivitelline space, thus establishing a block to polyspermy (Raz *et al.,* 1998).

4) Parthenogenetic Activation

Fully matured mammalian oocytes can be induced to undergo activation artificially (parthenogenetic) by a variety of physical and chemical treatments in the absence of the male genome (Kaufman and Gardner, 1974). The activation stimuli are designed to mimic closely the events initiated by the sperm factor released upon fertilization and result in a Ca^{2+} rise in the treated oocyte (Saunders *et al.,* 2002). Such treatments include; Application of an electrical pulse. Short and high voltage DC electrical stimuli cause transmembrane Ca^{2+} influx through the formation of temporary pores in the plasma membranes, allowing an exchange of extracellular and intracellular ions and macromolecules (Zimmermann and Vienken, 1982). Treatment with the Ca^{2+} ionophore (A23187) also induced cortical granule exocytosis, extrusion of the second polar body and pronuclear formation by the contribution of a Ca^{2+} influx to the $[Ca^{2+}]_i$ increase and the release of intracellularly stored Ca^{2+} (Steinhardt *et al.,* 1974; Steinhardt and Epel, 1974; Vincent *et al.,* 1992). It was reported that ionomycin induced a biphasic change in $[Ca^{2+}]_i$ and was used to depleted intracellular Ca^{2+} enhanced stores in mouse oocytes(Jones *et al.*, 1995). Exposure of MII oocytes to 7% ethanol for 5～7 min induces pronuclear formation and successful development to blastocyst by promoting a rapid potentiation of InsP3-methiated Ca^{2+} release through stimulation of InsP3 formation at the plasma membrane (Ilyin and Parker, 1992). In porcine oocytes, intracellular injection of $CaCl_2$ into the cytoplasm induced the exocytosis of cortical granule, decline in the histone H1 kinase activity, changes in the protein synthetic profile, pronuclear formation and subsequent development (Machaty *et al.,* 1996).

Instead of calcium-dependent mechanisms, another method of artificial activation of MII oocytes is to prevent the production of cyclin B thereby attacking a portion of the calcium-signaling pathway downstream of the initial calcium signal. Cyclin B is a component of MPF and is continuously synthesized in order to maintain adequate levels of active MPF. Inhibition of protein synthesis by treatment with puromycin or cycloheximide induced MII oocytes to enter the first interphase in mouse (Siracusa *et al.,* 1978; Moses and Kline, 1995; Moos *et al.,* 1996) and human oocytes (Balakier and Casper, 1993) but not pig oocytes (Nussbaum and Prather, 1995). Greater activation and subsequent development have been obtained when cycloheximide or puromycin treatment is used in addition to a calcium transient inducing stimulus (Presicce and Yang, 1994; Nussbaum and Prather, 1995; Tanaka and Kanagawa, 1997).

5) Oocyte Activation and Oocyte Aging

The ability to artificially activate MII arrested oocytes changes with the age of the oocyte generally determined from the initiation of maturation. Aged oocytes are easier to activate than freshly matured oocytes (Siracusa *et al.,* 1978; Swann and Ozil, 1994; Tanaka and Kanagawa, 1997) because young oocytes continuously synthesize new CSF, which preserves MPF and maintains the meiotic arrest (Fissore and Robl, 1992; Yang *et al.,* 1994). Young oocytes generally require the combination of a calcium stimulus with inhibition of protein synthesis or application of a kinase inhibitor (i.e. 6-dimethylaminopurine (6-DMAP)) (Susko-Parrish *et al.,* 1994), or by inhibition of cdk activity (roscovitine, bohemine (Alberio *et al.,* 2001a)), however, aged oocytes can be activated by a single stimulus which causes a Ca^{2+} increase due to the inactivation of the existing CSF in the cytoplasm of the oocytes and in many cases will activate spontaneously (Plante and King, 1996; Suzuki *et al.,* 1999).

Following artificial activation meiosis resumes and in most mammalian species the resulting parthenotes are haploid. However, if extrusion of the second polar body can be prevented, the embryos become diploid and this improves subsequent development. In bovine oocytes, the combination of ionomycin and cytochalasin B resulted in completion of the second meiotic division but prevented extrusion of the second polar body resulting in diploid embryos (Navara *et al.,* 1994). Similarly an activation stimulus in combination with the protein kinase inhibitor 6-DMAP also prevents extrusion of the second polar body and improved development to the blastocyst stage in bovine parthenogenetic embryos (Susko-Parrish *et al.,* 1994). 6-DMAP induced the second meiotic spindle to disintegrate, inducing oocyte entry directly into interphase with only one diploid pronucleus.

4. Culture of Reconstructed Embryos

Nuclear transfer reconstructed embryos may be cultured *in vitro* to the blastocyst stage after which they will be transferred to a surrogate recipient for development to term. Alternatively, embryos may be cultured *in vivo* in the ligated oviduct of a suitable host animal (in general sheep) until a stage suitable for transfer to a final surrogate mother is reached.

A number of *in* vitro culture media have been developed for individual species, these have included co-culture systems uti-

lizing primary oviductal cell monolayers or established cell lines (Thompson, 2000; Menezo and Herubel, 2002). Traditionally foetal calf serum was used as a media supplement, however more recently defined culture media have been developed i.e. mSOF (synthetic oviduct fluid media) for cattle and sheep (Walker *et al.,* 1992; Matsuyama *et al.,* 1993), NCSU23 (North Carolina State University) for pigs (Machaty *et al.,* 1998) and CZB (Chatot, Ziomek and Bavister) or KSOM for mice (Chatot *et al.,* 1991). The use of low oxygen systems in the absence of co-culture has also been reported to improve development (Watson *et al.,* 1994). The culture conditions consist in either one or two steps culture media, in which the requirements are adjusted for the embryo at different stages of development.

More recently an alternative strategy was reported for the culture of NT reconstructed porcine embryos (Polejaeva *et al.,* 2000). Due to the low birth rates reported following *in vitro* culture of unmanipulated embryos, clones were immediately transferred into the oviduct of a synchronized recipient for development to term. Due to the low frequency of development expected, large numbers of embryos were transferred and gave rise to offspring normal. It would be desirable to develop a culture system that would promote development of embryos with high developmental potential to term. Present culture systems tend to promote development to the blastocyst stage although the viability of those after transfer is severely compromised considering that only 5～20% reach term. Developing non-invasive screening methods for embryo quality, in which the whole embryo can be assessed for developmental potential, are desirable. Some methods consist in the analysis of ploidy or gene expression or single blastomeres, but the information obtained by these procedures is not entirely useful considering the high rate of mosaicism in *in vitro* cultured embryos, as well as differences in gene expression.

5. Development of Cloned Embryos

Overall the frequency of development to term of so called cloned embryos is low, although difficulties arise in comparing the results from different laboratories and in different species estimates of approximately 2～3% of fused couplets have been reported (Gurdon and Colman, 1999). Losses during early pregnancy account for up to 40% in ruminants (cattle, sheep and goats). It has been reported that failure to form a normal placenta is the main cause of abortion at this stage (Hill *et al.,* 2000). Lack of normal placentome development and vascularisation is also accountable for growth deficiencies as well as for the frequent observation of hydrops later in gestation (Hill *et al.,* 1999; Heyman *et al.,* 2002). Similar placental abnormalities have been observed in mouse and sheep, although not in goats and pigs. Postnatal development is characterized with a higher mortality rate in the first week after delivery. This can be the result of dystocia, related to the increased body size of the foetuses, immature lungs, general weakness, predisposition to infections, and weight loss (Zakhartchenko *et al.,* 2001). Despite the high rate of losses normal cloned animals have been reported in the literature (Chavatte-Palmer *et al.,* 2002; Cibelli *et al.,* 2002), although some authors have shown that gene expression of all cloned mice is altered (Humpherys *et al.,* 2002). Due to the stochastic pattern in the occurrence of abnormalities an incomplete or abnormal 'reprogramming' is suggested as the main reason for the altered gene expression and phenotypic aberrations. The consequences of such alterations are unpredictable and may be far reaching.

CONCLUSIONS

The technique of nuclear transfer (NT) is now well established in a variety of species, however, despite considerable research it still remains an inefficient technique. In general across the species examined only 1～2% of reconstructed embryos are able to develop to term and produce live offspring (Gurdon and Colman, 1999). In addition, many of the resultant offspring suffer from a range of abnormalities and many die within the first few months of birth. Although a number of refinements have been introduced, including the use of defined culture media, piezzo assisted injection for transfer of the donor nucleus and improvements in *in vitro* oocye maturation, the methodology of NT has remained essentially unchanged since the production of the 1st live offspring using embryonic blastomeres as nuclear donors (Willadsen, 1986). There are numerous factors affecting development of the reconstructed embryos: the type of recipient cell, method of enucleation, the type of the cell acting as nuclear donor, the cell cycle stages of both the donor nucleus and the recipient cytoplasm and the method of culture of the reconstructed embryos. Oocyte quality is critical in all reproductive technologies. In particular the cell cycle stage and the quality of the oocyte to be used as a cytoplast recipient for embryo reconstruction is central to the development of embryos produced by NT.

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