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Cloning: Eight Years After Dolly

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Contents

It is now 8 years since the birth of Dolly, the first animal produced by nuclear transfer using a donor cell population established from an adult animal. During this time, the technique of nuclear transfer has been successfully applied to a range of mammalian species for the production of offspring using a plethora of donor cell types derived from both foetal and adult tissues. In addition, when coupled with genetic manipulation of the donor cells, transgenic offspring have been produced with a range of genetic modifications including gene knockouts and gene knockings. Despite the apparent successes of the technology, the efficiency of development to live offspring has remained low and developmental abnormalities still occur. The objectives of this paper are to review some of the successes and failures of the nuclear transfer procedure since the production of Dolly. In particular, we will review the major steps in the procedure and discuss studies from our laboratory and others which have modified the procedure in ways which may impact on development.

Introduction

Originally proposed by Spemann (1938) as a method to study cell differentiation (Spemann 1938) the technique of nuclear transfer was first successfully employed in amphibians by Briggs and King (1952) who used embryonic blastomeres as nuclear donors (Briggs and King 1952), subsequently John Gurdon demonstrated the potential to reprogram differentiated cells by producing adult *Xenopus* using epithelial cells from developing tadpole intestine as nuclear donors (Gurdon 1962a,b; Gurdon and Uehlinger 1966). Unfortunately, later studies in *Xenopus* using adult keratinocytes as nuclear donors, although supporting development of swimming tadpoles did not support development to the adult stage (Gurdon et al. 1975). In mammals, the development of this technology occurred more slowly, early unsuccessful experiments in the rabbit by Bromhall (1975) were followed by the production of mice following pronuclear exchange between fertilized zygotes (McGrath and Solter 1983). However, enucleated zygotes proved limited in their capacity as cytoplast recipients to support development when blastomeres from later stage embryos were used as nuclear donors not only in the mouse (McGrath and Solter 1984), but also in other species including pigs (Prather et al. 1989). The use of enucleated metaphase II oocytes as recipient cytoplasts proved more successful and in 1986 resulted in the production of live lambs using blastomeres from 8 to 16-cell stage embryos as nuclear donors (Willadsen 1986). Although this success in sheep was repeated in other species including cattle (Robl et al. 1987) and pigs (Prather et al. 1989), there were major limitations to the utility of the technology; first the frequency of development was very low limiting the number of identical animals which could be produced; secondly this number was further limited by the number of cells in the embryonic stage from which successful development could be obtained. To counteract these problems, the search for a suitable nuclear donor cell type which could be maintained in culture became the aim of many groups. Embryonic stem (ES) cells were proposed as one such cell type (Wilmut et al. 1992) and these have subsequently been used successfully as nuclear donors in the mouse (Wakayama et al. 1999; Humpherys et al. 2001). In 1994, Sims and First (1994) 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. Since this time, although ES like cells have been isolated from a range of species and used as nuclear donors (van Stekelenburg-Hamers et al. 1995), true ES cells have not yet been isolated from farm animal species.

An alternative approach to the use of a specific cell type as a nuclear donor was to improve the nuclear transfer procedure by gaining basic understanding of the nuclear cytoplasmic interactions and use cultured somatic cells as nuclear donors. Although these studies continue to the present time, early studies provided basic information on cell cycle interactions when using embryonic blastomeres as nuclear donors (Campbell et al. 1993; Campbell et al. 1994). These studies led to the use of cultured cell populations as nuclear donors and resulted in the birth of live offspring in July of 1995 (Campbell et al. 1996a). Although the cells used in these early studies were derived from an early embryo, they were not ES cells, as confirmed by the expression of vimentin, cytokeratin and lamin A/C, which are associated with the differentiated phenotype. In subsequent studies, these results were confirmed and extended to the use of foetal and adult cells as nuclear donors resulting in the birth of Dolly in July 1996 (Wilmut et al. 1997).

Milestones in Animal Cloning from Somatic Cells

Research on the production of live offspring by nuclear transfer using cultured cell populations as nuclear donors and culminating in the birth of Dolly provided a wealth of opportunities in both basic and applied research and in areas from pharmaceuticals to agriculture and human cell therapies. Since this time the nuclear transfer technique has been applied across a range of species for reproductive cloning (see Table 1). In addition to reproduction, nuclear transfer has been

Table 1. Major milestones in animal cloning from somatic cell populations

Species	Date	Donor age	Cell type	Reference
Sheep	1995	Embryo	Epithelial like	Campbell et al. (1996a)
	1996	Foetal	Fibroblast	Wilmut et al. (1997)
	1996	Adult	Mammary Epithelial	Wilmut et al. (1997)
Cattle	1998	Foetal	Fibroblast	Cibelli et al. (1998)
	1998	Adult	Oviduct Epithelial	Kato et al. (1998)
Mouse	1998	Adult	Cumulus	Wakayama et al. (1998)
	1999	Embryo	ES	Wakayama et al. (1999)
Goat	1999	Foetal	Fibroblast.	Baguisi et al. (1999)
Pig	2000	Adult	Cumulus	Polejaeva et al. (2000)
Gaur	2000	Adult	Fibroblast	Lanza et al. (2000)
Mouflon	2001	Adult	Granulosa	Loi et al. (2001)
Cat	2002	Adult	Cumulus	Shin et al. (2002a)
Rabbit	2002	Adult	Cumulus	Chesne et al. (2002)
Banteng	2003	Adult	Fibroblast	BBC (2005)
Rat	2003	Foetal	Fibroblast	Zhou et al. (2003)
Mule	2003	Foetal	Fibroblast	Woods et al. (2003)
Horse	2003	Adult	Fibroblast	Galli et al. (2003)
Deer	2003	Adult	Fibroblast	Eurekalert (2005)

used for the production of transgenic offspring by combining with cell culture and molecular biology, not only by random gene addition (Schnieke et al. 1997) but also by gene knocking (McCreath et al. 2000) and gene knockout techniques (McCreath et al. 2000) which were previously restricted to ES cell techniques in the mouse. Such transgenic animals have been developed for a range of applications including; the production of human proteins for the pharmaceutical industry for example human factor IX in sheep milk (Schnieke et al. 1997); the modification of animal products i.e. increased beta and kappa casein content in cows milk (Brophy et al. 2003); the removal of potential antigens in pigs for xenotransplantation i.e. alpha 1–3 galactosyltransferase (single allele) (Lai et al. 2002), double allele (Phelps et al. 2003) and for research into animal diseases i.e. PrP knockout sheep (Denning et al. 2001).

The ability to produce offspring from somatic cells demonstrates that the somatic nucleus can be reprogrammed and produce all of the cell types found in the embryo and the foetus. ES cells are derived from early embryos and in mouse nuclear transfer has been used to produce embryos from which ES cells have been isolated (Munsie et al. 2000). ES cells provide a valuable potential route to a range of human therapies, however, it has been argued that autologous ES cell populations would not only be advantageous but may be necessary for transplantation. More recently human ES cells have been isolated from blastocyst stage embryos produced *in vitro* following nuclear transfer from cumulus cells (Hwang et al. 2004), providing a valuable route not only to the production of stem cells for therapies but to the production of embryos and ES lines from genetically manipulated or deficient cells for research purposes.

Efficiencies and Deficiencies of the Cloning Procedure

Although nuclear transfer has proved to be applicable across a range of species the frequency of development to term remains relatively low with losses because of developmental abnormalities occurring throughout embryo and foetal development and following birth. A true comparison of efficiencies across the published reports is difficult because of differences in experimental protocols, embryo selection and data presentation. In our opinion development to term based on the number of embryos successfully reconstructed is between 0 and 5%, however, following blastocyst selection live birth rates up to 80% have been reported (Kato et al. 1998). The high frequency of gestational losses associated with the cloning procedure provides a major economic barrier to the widespread use of reproductive cloning in animal species requiring large numbers of surrogate recipients. The ability to predict the potential viability of individual blastocysts prior to transfer would greatly increase the efficiency of the cloning process. The majority of studies base blastocyst quality on morphological criteria, however, recent studies in human embryos have suggested that embryo metabolism may provide a route to quality assessment (Donnay et al. 1999). Following embryo transfer, gestational losses have been associated with placental abnormalities (Hill et al. 1999, 2000; De Sousa et al. 2001; Chavatte-Palmer et al. 2002) and aberrant gene expression patterns have been reported in the placenta of cloned mice (Humpherys et al. 2002). Placental proteins such as pregnancy specific protein (PSP60) (Heyman et al. 2002) or PSPb (Hill et al. 2000) have been suggested as markers to monitor foetal development to allow early termination of potentially non-viable foetuses, however, these studies need to be extended. Following birth offspring have been reported with a range of abnormalities including increased size (Large Offspring Syndrome) (Young et al. 1998) and an increased mortality. 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) unfortunately many of these offspring die, however, obtaining accurate figures from the literature to estimate these losses is difficult. Those animals that do survive appear, in the majority of cases, to be physiologically and reproductively normal (Chavatte-Palmer et al. 2002; Cibelli et al. 2002) as in fact are the next generation.

Many recent studies on embryos produced by nuclear transfer have demonstrated that gene expression patterns in the embryo, foetus and placenta are abnormal (Humpherys et al. 2002) and suggested that inefficient or incomplete 'nuclear reprogramming' is the cause of the abnormalities observed. The term 'nuclear reprogramming' has been used to cover all of the modifications that must occur to ensure normal successful development, however, it is generally accepted that in nuclear transfer embryos the reprogramming of gene expression is occurring by epigenetic mechanisms and does not involve modification at the level of DNA sequence. Such epigenetic mechanisms involve modification of chromatin and nuclear structure which can alter the transcriptional status of individual genes. Such modifications include the methylation of DNA at CpG islands, acetylation, methylation, phosphorylation, ubiquitination and Poly ADP ribosylation of core histones, the replacement of somatic histones with embryonic histone isoforms and modification of nuclear lamina. Epigenetic reprogramming occurs at all stages of development and

has been implicated in disease states including cancer (for review see Campbell and Alberio 2005). In nuclear transfer embryos abnormal patterns of DNA methylation (Dean et al. 2001) and nuclear lamin protein expression have been reported (Moreira et al. 2003). The use of such markers of reprogramming may help to alter and improve the nuclear transfer procedure or to select more viable embryos. However, within the scope of this paper epigenetic reprogramming will not be discussed in detail.

The Nuclear Transfer Procedure, Past and Present Approaches

The production of embryos and offspring by nuclear transfer is a multi-step procedure each of which may affect subsequent development. In brief these steps can be summarized as; the production of a suitable cytoplast recipient, selection and culture of a suitable cell, embryo reconstruction, activation and culture. During recent years modifications to the technique have been driven by three major forces. First, the need to improve the frequency of normal development, secondly as a tool to understand the mechanisms involved in developmental control and thirdly to simplify and streamline the procedure. In addition intellectual property ownership and patent law has further influenced these modifications.

The cytoplast recipient

Source

Central to the successful development of nuclear transfer reconstructed embryos is the production of a suitable cytoplast recipient, oocytes and zygotes to be used as cytoplasts can be produced both *in vitro* and *in vivo*. In farm animal species *in vitro* oocyte maturation can provide large numbers of cytoplasts from slaughterhouse material, however, sub-optimal maturation conditions may compromise subsequent development. The production of oocytes and zygotes *in vivo* would be expected to provide cytoplasts of increased quality (Wells et al. 1997), however, to increase yield and reduce costs such procedures are generally accompanied by ovarian stimulation regimes which have been demonstrated to reduce embryo development, retard foetal growth (Van der Auwera and D'Hooghe 2001) and cause aberrant patterns of DNA methylation during early development (Shi and Haaf 2002). Differences in the development of successful maturation protocols, cost and availability limits the choice of method within a particular species. For rare species where oocytes are not available the possibility of using oocytes from other species as cytoplast recipients has been proposed (Dominko et al. 1998, 1999) and when closely related this has proved successful (Lanza et al. 2000; Loi et al. 2001).

Selection and preparation of the recipient cytoplast

Studies in mammalian Nuclear Transfer (NT) have utilized a range of embryonic stages as cytoplast recipients (see Table 2) including oocytes, zygotes and early cleavage stage embryos with varying success. Enucleated zygotes of both mouse (McGrath and Solter 1983, 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

Table 2. Timing of enucleation of recipient cytoplasts for embryo reconstruction

Stage of enucleation	Species	Reference
*Anaphase –telophase first meiosis (AI-TI)	Sheep	Lee and Campbell (2004)
Metaphase of second meiosis (MII)	Sheep	Campbell et al. (1996a), Schnieke et al. (1997), Wilmut et al. (1997)
	Cattle	(Barnes et al. (1993), Delhaise et al. (1995), Du et al. (1995), Cibelli et al. (1998), Wells et al. (1999a,b, 2003, Zakhartchenko et al. (1999b, 2001, Kato et al. (2000), Do et al. (2001)
	Pig	Betthauser et al. (2000), Onishi et al. (2000)
	Cat	Shin et al. (2002b)
	Rabbit	Chesne et al. (2002)
	Mule	Woods et al. (2003)
	Horse	Galli et al. (2003)
	Rat	Zhou et al. (2003)
Telophase of second meiosis (TII)	Cattle	Bordignon and Smith (1998)
	Mouse (chemically induced)	Gasparrini et al. (2003)
	Goat	Baguisi et al. (1999)
Pronuclear zygote (PN)	Mouse	McGrath and Solter (1983); McGrath and Solter (1984), Kwon and Kono (1996)
	Cattle	Prather and First (1990)
	Pig	Prather et al. (1989)
Double nuclear transfer first NT-MII second NT-PN	Mouse	Kwon and Kono (1996); Ono et al. (2001)
	Pig	Polejaeva et al. (2000)
Two cell embryo.	Mouse	Tsunoda et al. (1987)

*Live offspring not yet reported.

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 unfertilized 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 (see Table 2) and have commonly become the cytoplast of choice.

Preparation of the recipient cytoplast requires removal of the genetic material, which is termed enucleation, a range of procedures have been developed to enucleate the recipient cell these will be described and discussed in relation to their possible effects upon development.

Metaphase II enucleation

At MII the maternal DNA is present as highly condensed chromosomes arranged on a metaphase spindle or plate. In most species, the metaphase plate of MII oocytes is not visible by light microscopy due to the presence of cytoplasmic lipid. Enucleation has been achieved by so called 'blind enucleation' using the first polar body (PB1) as a marker for the location of the MII plate, generally the PB1 and a small volume of cytoplasm located below PB1 are removed using a small glass pipette (20–25 μm diameter). The problem with this approach is that in many cases the metaphase plate is not close to PB1. In addition, the removal of cumulus cells prior to oocyte manipulation can further disrupt the relationship between the MII spindle and PB1. These factors can result in a proportion of the oocytes containing residual DNA following enucleation [for review see (Li et al. 2004)]. In fact it has been reported that $< 50\%$ of metaphase plates are located beneath PB1 in bovine (Nour and Takahashi 1999) and rabbit MII oocytes (Mitalipov et al. 1999). A second problem is that up to one-third of recipient cytoplasm just beneath the PB1 is generally aspirated in order to improve enucleation efficiency. The removal of such a large proportion of the oocyte cytoplasm may result in the oocyte having a reduced capacity for epigenetic reprogramming of the transferred nucleus and subsequent development.

Hoechst staining and UV light

Hoechst 33342 (bisbenzimide) is a DNA specific fluorochrome which is commonly used for staining the oocyte to aid or confirm the enucleation procedure. Enucleation is confirmed by the presence of the genetic material derived from the recipient oocytes by exposure to ultraviolet (UV) light under a fluorescent microscope either following aspiration or during the aspiration procedure. Although offspring have been produced following exposure of the oocyte to UV (Kubota et al. 2000; Onishi et al. 2000; Loi et al. 2001; Forsberg et al. 2002) the use of fluorescence and Hoechst 33342 staining may cause damage to the resultant cytoplast in particular the mtDNA (Dominko et al. 2000). This in conjunction with the volume of cytoplasm removed may further reduce the viability of the resultant cytoplast.

Telophase II enucleation

An alternative to the enucleation of MII oocytes is the enucleation of activated oocytes at telophase of the second meiotic division (Telophase II: TII). Mechanical aspiration of the extruding second polar body (PB2) and surrounding cytoplasm following activation is an effective and reliable enucleation method without the need for visualisation of the DNA by exposure to UV light. In addition significantly less of the oocyte cytoplasm is removed when compared with enucleation of MII oocytes which may be beneficial to the developmental competence of the recipient cytoplast (Bordignon and Smith 1998). However, subsequent development may be related to further manipulations for instance when bovine cumulus cells were transferred into TII enucleated oocytes by cell fusion increased development to the blastocyst stage was obtained as compared with MII oocytes, however, when the donor nuclei were transferred by injection reduced development was obtained (Liu et al. 2000a). This may relate to changes in the oocyte membranes, cytoskeleton or reprogramming capacity which may be related to Maturation Promoting Factor (MPF) activity. In fact it has been reported that bovine NT embryos reconstructed using cumulus cells as nuclear donors and pre-activated oocytes as recipient cytoplasts failed to reprogramme the nuclei and did not develop beyond the eight-cell stage, regardless of the cell cycle of the donor cells (Tani et al. 2001).

Chemical enucleation

Mammalian oocytes have been successfully enucleated by a number of chemical treatments including etoposide (Elsheikh et al. 1998), a combination of etoposide and cycloheximide (Fulka and Moor 1993) or ethanol and demecolcine (Ibanez et al. 2003). However, cytoplasts prepared from chemically enucleated oocytes induce poor cleavage rates and do not support embryo development as compared with those enucleated by conventional mechanical methods (Elsheikh et al. 1998; Gasparrini et al. 2003). It is interesting to note that oocytes chemically enucleated by etoposide-cycloheximide treatment do not contain active MPF kinase at the end of the enucleation procedure (Fulka and Moor 1993). In a modification of these methods demecolcine (colcemid) has been used to induce a membrane protrusion containing a condensed chromatin mass in porcine and rabbit oocytes, this was subsequently mechanically removed (Yin et al. 2002a,b).

Other enucleation methods

Treatment of mouse oocyte with 3% sucrose has been used to help visualize the meiotic spindle by causing a more translucent appearance under the light microscope (Wang et al. 2001). However, in bovine oocytes this technique proved less reliable as several projections formed around the oocyte making identification of the chromatin difficult (Liu et al. 2002). More recently the development of the POL-scope has allowed the location of the spindle to be visualized (Liu et al. 2000b), however, this equipment is expensive and its use varies

between species. Centrifugation of MII oocytes combined with CB treatment has been reported to cause enucleation in cattle oocytes resulting in developmentally capable cytoplasts when used as recipients for blastomeres nuclei (Tatham et al. 1995). The metaphase plate has also bee removed by cutting the oocyte without the need for micromanipulators (Vajta et al. 2004, 2005) and the development of Zona free cloning methods allows more rapid enucleation procedures (Booth et al. 2001; Oback et al. 2003; Peura 2003; Gaynor et al. 2005).

Potential effects of enucleation protocol on cytoplast quality

It can be seen from the above that the method of enucleation can have a number of effects on the potential quality of the resultant cytoplast. Physical aspiration of the chromatin at different stages can remove differing volumes of cytoplasm which may have effects on development. Enucleation of oocytes at TII requires oocyte activation which results in a decrease in MPF activity, chemical enucleation can result in the complete loss of MPF. Also the use of chemicals or UV light may cause damage to oocyte proteins or organelles and reduce developmental competence.

Removal of oocyte cytoplasm

Removal of the MII spindle may deplete the oocyte of proteins or other factors which are required for early embryonic development and/or reprogramming of the transferred nuclear genome. Studies in NT reconstructed primate embryos have demonstrated that an organized spindle cannot be completed in enucleated oocytes possibly due to depletion of a spindle-associated protein, Numa. In contrast, when unenucleated oocytes are used as cytoplast recipients, two organised spindles are observed (Simerly et al. 2003). In these experiments, these authors demonstrated the depletion of Numa by immunofluoresence, however, this may not reflect depletion of this protein but rather an inability of the oocyte to localize this protein because of depletion of other proteins or alterations in control mechanisms. This effect on spindle structure has been observed in a range of species with disorganized spindles being reported in mice, cattle and sheep embryos (Wakayama et al. 1998; Shin et al. 2002a); however, the ability of some embryos to develop normally suggests that subsequent mitotic divisions can occur normally.

The effects of enucleation and the possible depletion of oocyte specific 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, suggest a greater frequency of development (Bordignon and Smith 1998; Liu et al. 2000a). In both of these situations it is possible that cell cycle related proteins have been released from the oocytes chromatin prior to 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. In this technique, the first nuclear transfer utilizes an enucleated metaphase II oocyte as cytoplast recipient. The resultant diploid pronucleus is then transferred into an enucleated, fertilized zygote. Successfully used in porcine cloning (Polejaeva et al. 2000), studies in the mouse have suggested that this technique results in fewer abnormalities (Ono et al. 2001). These observations may result from a number of factors including increasing activation because of the use of sperm, or the presence of paternal transcripts or proteins, however, it may also be that by utilizing this procedure the final reconstructed embryo contains a more physiological content of oocytes proteins which contribute to development of the embryo. More recently it has been shown that by increasing cytoplast volume an increase in cell numbers of NT derived bovine blastocysts is obtained but not an increase in the frequency of development (Peura et al. 1998).

Effects of enucleation on MPF and MAPK kinase activities

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 proteins involved with cell cycle control are associated with the mitotic and/or meiotic spindle including MPF (Czolowska et al. 1986), c-mos (Zhou et al. 1991; Wang et al. 1994) and Cdk's (Jiang et al. 1998; John et al. 2001; Mollinari et al. 2002; Yoshida et al. 2002) Experiments in the mouse have demonstrated that MPF kinase activities may be compartmentalized (Fulka et al. 1995) with the majority of activity remaining with the metaphase plate and not the enucleated oocyte to be used as cytoplast recipient for NT. Such a potential reduction in MPF activity due to enucleation, coupled with the reduction associated with oocyte ageing may help to explain the variable response of donor nuclei to MII cytoplasm and the differences in development reported in previous studies (Wakayama et al. 1998). However, a reduction in MPF activity due to enucleation may be species specific as studies in both porcine and ovine oocytes showed that no decline in oocyte MPF activity was associated with the enucleation procedure (Goto et al. 2002; Lee and Campbell 2004). Alterations in the activities of either MPF or MAPK kinase may affect the degree or timing of chromatin condensation and impact on development.

Enucleation at anaphase–telophase of first meiosis

After considering the potential harmful effects of enucleation we have examined the effects of enucleation at anaphase–telophase of the first meiotic division (AI-TI). At this stage the oocyte chromosomes are highly condensed, both sets are closely associated and attached to the spindle. A small protrusion can be observed on the oocyte surface where the polar body will be extruded. Blind enucleation using a glass pipette is more efficient (97.8%) than at MII (78.0%), a significantly smaller karyoplast (15.8 $\pm$ 2.4 μm) is produced than at MII (35.2 $\pm$ 3.1 μm) and consequently significantly less of the oocyte cytoplasm is removed (0.2% vs 2.3%

respectively) (H. Lee and K.H.S. Campbell, unpublished data). Analysis of MPF and MAPK kinase activities showed that AI-TI enucleation did not reduce kinase activities, or prevent the rise in activities associated with maturation to MII. The oocytes matured apparently normally with the chromosomes removed (Lee and Campbell 2004).

MPF kinase activity and development

Coordination of nuclear and cytoplasmic cell cycle stages is essential for development. When an interphase nucleus is transferred into a MII arrested oocyte a series of morphological changes occur including; (1) nuclear envelope breakdown (NEBD), (2) premature chromatin condensation (PCC) and (3) dispersion of nucleoli. Nuclear envelope breakdown 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 pulverized appearance thought to be associated with high levels of DNA damage. Following oocyte activation, both MPF and MAPK activities decrease, the nuclear envelope reassembles and the nucleus swells. After the nuclear envelope reassembles the zygotic cell cycle enters S-phase, if using donor cells in S or G2-phases aneuploid embryos result due to uncoordinated DNA replication. In contrast when G1 or G0 cells are transferred into MII oocytes, coordinated DNA replication occurs and the resultant blastomeres are diploid. Pre-activated oocytes, which have low MPF and MAPK activities, do not induce NEBD or PCC and are permissive to G1, S and G2 donor nuclei with coordinated DNA replication occurring dependent upon the donor cell cycle phase of the donor nucleus. The role of MPF in nuclear reprogramming is poorly understood, however, studies in cattle have shown that prolonged exposure of the donor nucleus to an MII cytoplasmic environment improves development (Wells et al. 1998). In addition the age of the recipient oocyte may also affect the frequency of development, young MII oocytes having greater developmental competence than aged oocytes (Zakhartchenko et al. 2001). In addition to differences in the frequency of development, exposure of the donor chromatin to differing cytoplasmic environments has been found to effect a range of changes to chromatin structure [for example the loss of somatic histone H1 (Bordignon et al. 2001)] and gene expression which may be related to nuclear reprogramming (Kubisch et al. 1998; Daniels et al. 2001; Wrenzycki et al. 2001). The mechanisms underlying reprogramming are unknown, but we hypothesize that oocyte kinase levels, in particular MPF and MAP kinases are intimately involved. In support of this we have carried out studies using recipient cytoplasts with elevated MPF and MAP kinase activities and obtained an increase in the total cell number of blastocyst stage embryos produced (Lee and Campbell 2004). Such manipulations of oocyte kinase activities may prove useful in species where MPF activity declines rapidly following maturation to MII [i.e. in rats (Ito et al. 2005)]. This increase in 'reprogramming' may reflect the physical displacement and exclusion of somatic transcription factors from the condensed chromatin because of the extent and duration of PCC as has been reported to occur during mitosis (Martinez-Balbas et al. 1995), alternatively, it may also reflect an active process by oocyte components which is time dependent prior to the initiation of embryo development. In particular MAP kinase has been implicated in a number of pathways that may regulate the epigenetic state of the donor nucleus. These include; the phosphorylation of histone deacetylases and disruption of corepressor interactions inducing transcriptional repression (Galasinski et al. 2002), the modulation of transcription by phosphorylation of histone H3 and coupling of phosphorylation to acetylation (Clayton and Mahadevan 2003) and changes in DNA methylation patterns which are dependent upon histone acetylation (Gregory et al. 2001, 2002).

Selection and culture of a suitable donor cell

After the production of the first mammals from cultured embryonic (Campbell et al. 1996b) 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. 1999a; 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 cell clones showed normal expression of imprinted genes (Inoue et al. 2002) although this contradicted a report indicating altered gene expression patterns in clones derived from ES and cumulus cells (Humpherys et al. 2001, 2002). The differences between groups may 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 post-mitotic cells (Eggan et al. 2004) have successfully been employed for the generation of cloned animals.

Embryo reconstruction, activation and culture

Embryo reconstruction

The donor nucleus must be transferred into the recipient cytoplasm in order for development to occur. In the majority of species electrofusion of donor and recipient cells is used. This transfers the complete cytoplasm of the donor cell to the recipient, the effects of the cytoplasmic components are on development are unknown. In mouse, where electrofusion is less successful piezo injection has been successfully applies (Wakayama et al. 1998). In our lab we are currently examining the use of a laser (Hamilton Thorne Biosciences, Beverly, MA, USA) in the nuclear transfer process. The laser is used first to cut the zona pellucida, this then allows a blunt pipette to be used for enucleation thus eliminating the need to manufacture sharpened enucleation pipettes. The laser is then used to soften the plasma membrane of the oocyte

allowing cell or nuclear injection with the blunt pipette, the laser is also used to damage the donor cell membrane just prior to injection to ensure lysis. At the present time no offspring have been produced, however in cattle and sheep development to the blastocyst stage is not affected (W. Maalouf, personal observation).

Activation

Mammalian oocytes are ovulated and arrested at MII until fertilization. During oocyte maturation 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 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.

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^{++} 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 and Epel 1974; Steinhardt et al. 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 granules, a 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-signalling 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).

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 cytostatic factor (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) (Susko-Parrish et al. 1994), or by inhibition of cdk activity (roscovitine, bohemine; Alberio et al. 2001), 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).

Embryo culture

The method of culture and the developmental stage at which nuclear transfer reconstructed embryos are transferred to a surrogate recipient is dependent upon species. In rodents embryos can be returned to the oviduct after production. Immediate transfer of large numbers of embryos has also been used successfully in pigs (Polejaeva et al. 2000), however, in the majority of domestic species because of the small litter size, the cost of surrogate recipients is often the limiting factor to this approach and embryos are generally cultured *in vitro* to the blastocyst stage before transfer. This approach allows embryo quality to be assessed. An alternative to *in vitro* culture is to place the embryos into the ligated oviduct of a suitable host animal (in general sheep) until transfer (Willadsen 1979). One problem with this approach is that the embryos are generally encapsulated in agar for protection and this is a time consuming process.

A number of *in vitro* culture media have been developed for individual species, these have included co-culture systems utilizing primary oviductal cell monolayers or established cell lines [for review see (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. 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 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). Novel culture systems consist of multiple culture media, in which the requirements are adjusted for the embryo at different stages of development. In the mouse rather than using embryo culture media, studies have shown that optimal results are obtained when the reconstructed embryo is cultured in the media that the donor cell was cultured in (Gao et al. 2003).

Novel Approaches to Nuclear Transfer

The basic methodologies underlying nuclear transfer have changed relatively little. Recent improvements have streamlined the methodology for example zona pellucida-free cloning, or simplified the equipment required but this has not improved development. Some studies have attempted to alter the epigenetic state of the donor cell prior to reconstruction by either chemical treatments (Enright et al. 2003) or by transferring chromatin rather than nuclear DNA (Sullivan et al. 2004) but these have also not improved development. In our studies we are attempting to produce a more uniform supply of cytoplasts which have a smaller volume of cytoplasm removed during the enucleation process and an increased level of MPF and MAPK kinases. To date such cytoplasts have produced embryos with an increased cell number and trials are underway to establish their developmental competence.

Conclusions and Perspectives

Since the birth of Dolly the nuclear transfer has been successfully applied to a range of species and adapted for the production of gene knockout and gene knocking animals. Studies on embryos and foetuses have provided basic information on the epigenetic mechanisms involved in nuclear reprogramming. However, the frequency of development to healthy offspring remains low. Unmentioned in this review are the potential implications of other cytoplasmic factors which may affect development, for example the transmission of somatic mitochondria [for review see St John et al. (2004)]. Continued research is essential to improve the frequency of development and to provide basic knowledge on the control of cell differentiation and maintenance of the undifferentiated state. Such knowledge will inevitably lead to an increase in development, and provide new routes to stem cell isolation. From a practical point of view nuclear transfer may be applied to the multiplication of endangered species, the multiplication of elite animals and the production of transgenic animals for research, agriculture and biopharmaceuticals.

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