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Nuclear transfer from somatic cells: applications in farm animal species

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The reconstruction of mammalian embryos by transfer of a blastomere nucleus to an enucleated oocyte or zygote allows for the production of genetically identical individuals. This has advantages for research (that is, as biological controls) and commercial applications (that is, multiplication of genetically valuable livestock). However, the number of offspring that can be produced from a single embryo is limited both by the number of blastomeres (embryos at the 32–64-cell stage are the most widely used in farm animal species) and the limited efficiency of the nuclear transfer procedure. The ability to produce live offspring by nuclear transfer from cells that can be propagated and maintained in culture offers many advantages, including the production of many identical offspring over an extended period (since cultured cells can be frozen and stored indefinitely) and the ability to modify genetically or to select populations of cells of specific genotypes or phenotypes before embryo reconstruction. This objective has been achieved with the production of lambs using nuclei from cultured cells established from embryonic, fetal and adult material. In addition, lambs transgenic for human factor IX have been produced from fetal fibroblasts transfected and selected in culture.

Introduction

Embryo reconstruction by nuclear transfer involves the transfer of a single nucleus to an unfertilized oocyte or zygote from which the native genetic material has been removed. In farm animals, unfertilized oocytes arrested at metaphase of the second meiotic division (MII) as recipient cells have become the 'recipient' cell of choice. The nuclear 'donor' cell used for reconstruction depends upon the application of the technology. For embryo multiplication, blastomeres are used as nuclear donors (Prather *et al.*, 1987). More recently, live lambs were produced using cultured cells derived from embryonic, fetal and adult tissues as nuclear donors (Campbell *et al.*, 1995, 1996a; Wilmut *et al.*, 1997). This approach has recently been applied to cattle and viable offspring have been reported by several laboratories, including ours (Christmann, Chen, Polejaeva, Campbell and Eyestone, unpublished), using cultured fetal cells as nuclear donors (Cibelli *et al.*, 1998).

The development of embryos reconstructed by nuclear transfer is dependent on many factors. These include the quality of the recipient oocytes, the cell cycle stage of both the donor cell and the recipient cytoplasm, the method of oocyte culture and activation, and the developmental stage or differentiated state of the donor nucleus. More recently it has been suggested that the state of chromatin in the donor nucleus at the time of transfer may affect development of reconstructed embryos (Campbell and Wilmut, 1997).

Methods of Embryo Reconstruction

Historically, two types of recipient cell have been used for nuclear transfer: oocytes arrested at MII or pronuclear zygotes. In mice, enucleated, two-cell stage blastomeres have been used as recipients

(Tsunoda *et al.*, 1987). In farm species, development does not occur when pronuclear zygotes are used, except when pronuclei are exchanged between zygotes (cattle: Robl *et al.*, 1987; pig: Prather *et al.*, 1989); thus, MII-arrested oocytes have become the recipient of choice, since development to live offspring has been obtained using this type of recipient.

Sources of recipient oocytes

Oocytes for use as recipient cells in embryo reconstruction can be obtained after *in vivo* maturation from mature, unovulated follicles, or by flushing ovulated oocytes from the oviducts (Prather *et al.*, 1987; Prather *et al.*, 1989; Campbell *et al.*, 1994). Alternatively, oocytes recovered at slaughter in cattle (Barnes *et al.*, 1993), sheep (Pugh *et al.*, 1991) and pigs (Hirao *et al.*, 1994) and matured *in vitro* have also been used as recipients. In cattle, immature oocytes may also be obtained by aspirating ovarian follicles *in vivo* in cattle (for review, see Bols *et al.*, 1994).

Enucleation of recipient oocytes

The term enucleation is used to describe the removal of the genetic material from the recipient cell. Oocytes arrested at MII do not contain a nucleus; rather, the chromatin is condensed as chromosomes arranged on the meiotic spindle. In farm animal oocytes, the MII chromosomes, or metaphase plate, are not visible under the light microscope. However, they are generally situated subjacent to the first polar body, which can be used as a convenient landmark for locating the metaphase plate. Enucleation is accomplished by piercing the zona pellucida with a glass pipette (15–20 μm in diameter), and then placing the pipette tip over the polar body (and thus the metaphase plate) and withdrawing a membrane-enclosed portion of cytoplasm containing the meiotic chromosomes by applying gentle suction. In some cases, oocytes may be treated with the microtubule inhibitor cytochalasin B to render the plasma membrane elastic to facilitate enucleation and reduce mechanical stress and damage to the oocyte as a result of the enucleation procedure. Enucleation is confirmed by staining the karyoplast with a DNA-specific fluorochrome (i.e. Hoescht 3332) either following aspiration (Westhusin *et al.*, 1990) or during the aspiration procedure as is used routinely in our laboratory (Campbell *et al.*, 1993a).

Embryo reconstruction

After enucleation, the genetic material from the donor cell (karyoplast) must be introduced into the enucleated oocyte (cytoplast). In general this has been achieved by fusion, although direct injection techniques have been used successfully by some workers (Collas and Barnes, 1994; Ritchie and Campbell, 1995). Fusion is induced by a number of agents including Sendai virus (Graham, 1969), polyethylene glycol (PEG; Kanka *et al.*, 1991) or application of a DC electric current (Willadsen, 1986). In farm animal species, electrofusion is the most commonly used method. Use of Sendai virus is efficient in mice, although its effects are variable in other species, for example sheep (Willadsen, 1986). The use of PEG requires its fast and efficient removal after fusion because of its toxicity.

Activation of the reconstructed embryos

After introducing the donor genetic material, the reconstructed embryo has to initiate embryo development. Normally, development is initiated by an activation event induced by the spermatozoa at fertilization. Fertilization stimulates a series of intracellular calcium peaks that appear to be necessary and sufficient for activation of development. In the absence of fertilization, treatments must be applied that mimic these events to induce development. During recent years many treatments have been reported to cause oocyte activation. Such treatments have included electrical stimulation using either a single DC pulse (pig: Prochazka *et al.*, 1992; cattle: Kono *et al.*,

1989; for review see Robl *et al.*, 1992) or multiple electrical stimuli coinciding with the reported calcium peaks following fertilization (rabbit: Ozil, 1990; cattle: Collas *et al.*, 1993a), various chemical treatments including phorbol ester (mouse: Cuthbertson and Cobbold, 1985), calcium ionophores (i.e. A23187: Ware *et al.*, 1989; Aoyagi, 1992), ionomycin (cattle: Susko Parrish *et al.*, 1994), components of various second messenger systems (inositol Tris-phosphate, mouse: Jones *et al.*, 1995a; cattle: White and Yue, 1996), ethanol (cattle: Nagai, 1987) and strontium chloride (mouse: O'Neill *et al.*, 1991).

The rate and frequency of oocyte activation are dependent upon the age of the oocyte after the onset of maturation. As oocyte age increases, activation can occur spontaneously as a result of changes in temperature or other manipulations. In addition, pronuclear formation occurs more rapidly (cattle: Ware *et al.*, 1989; Presicce and Yang, 1994). In contrast, activation of 'young' oocytes has proved more difficult. In cattle and pigs, treatment of young MII oocytes with an activation stimulus combined with inhibitors of protein synthesis (that is, cycloheximide or puromycin) can overcome this block, for example in cattle (Presicce and Yang, 1994) and pigs (Nussbaum and Prather, 1995). More recently, soluble sperm factors have been extracted that can induce activation after they are injected into mouse oocytes (Parrington *et al.*, 1996; for review see Swann, 1996).

The role of activation in subsequent development is being elucidated slowly. Calcium oscillations continue throughout the first cell cycle in the mouse and are associated with mitotic division (Kono *et al.*, 1996; Jones *et al.*, 1995b). Activation of mouse oocytes with strontium chloride and continued exposure until after the first cleavage division have been shown to increase the size of measurable calcium oscillations at first mitosis to that observed in fertilized zygotes (Kono *et al.*, 1996; Jones *et al.*, 1995a). In addition, the number of cells at the blastocyst stage also increases (Jones *et al.*, 1996) indicating that the efficiency of activation may have far-reaching developmental effects.

Culture of reconstructed embryos

After reconstruction, the embryo must be cultured to a stage at which it can be transferred to a synchronized recipient animal for development to term, generally at the morula or blastocyst stage. Briefly, two options are available: culture *in vitro* or culture *in vivo*. There are many systems for the culture of embryos *in vitro* and research in this area has resulted in an increase in the frequency and the quality of development (for review see Campbell and Wilmut, 1994). Traditionally, reconstructed embryos from cattle and sheep have been cultured in ligated oviducts of temporary recipient ewes (Willadsen, 1986). Owing to the hole made in the zona pellucida during manipulation, this method requires the encapsulation of each embryo in agar. The function of the agar 'chip' is two-fold: first, it holds the embryo in the zona pellucida and second, it prevents attack of the embryo by macrophages within the oviduct.

Nuclear–cytoplasmic interactions in reconstructed embryos

The successful development of reconstituted embryos is dependent upon a large number of factors. In fertilized zygotes early events are controlled by maternally inherited RNAs and proteins, until the transcription is initiated in blastomere nuclei during early cleavage stages. In reconstituted embryos both the cytoplasm and the transferred nucleus must be able to recapitulate these events. Changes in nuclear structure, chromatin structure and gene activity have been reported; these will be discussed in relation to the differentiated state of the donor nucleus. However, for these so called 'reprogramming' events to be successful the chromatin must remain free of damage and the embryo must maintain normal ploidy.

Nuclear events during the first cell cycle

Interactions between cytoplasmic factors within the cytoplast and the cell cycle stage of the donor nucleus at the time of fusion are crucial to the avoidance of DNA damage and the

Table 1. Effects of various cell cycle stage combinations of donor and recipient cells on chromatin and ploidy of reconstructed mammalian embryos during the first cell cycle

Cell cycle stage of recipient	MPF activity	Stage of ploidy (cell cycle stage of donor)	Effect on nucleus	Effect on chromatin	DNA synthesis	Ploidy daughter cells
MII	High	2C[a] (G0/G1)	NEBD[c]	SCs[d]	+	2C
MII	High	4C[b] (G2)	NEBD	DCs[e]	+	4C
MII	High	2–4C (S)	NEBD	PULVERISED	+	? 2–4C[f]
G1/S	Low	2C (G0/G1)	No NEBD		+	2C
G1/S	Low	4C (G2)	No NEBD		+	2C
G1/S	Low	2–4C (S)	No NEBD		–	2C

MPF, maturation/mitosis/meiosis promoting factor; [a]2C = diploid; [b]4C = tetraploid; [c]Nucelar envelope breakdown; [d]single chromatids; [e]double chromatids
[f]?2–4C = unknown ploidy

maintenance of correct ploidy (for review see Campbell *et al.*, 1996b). Early development is characterized by a series of reduction divisions and no net growth occurs. For simplicity the major events of the first cell cycle can be described as those that concern the nucleus relative to DNA replication during which the genetic material is duplicated and this is followed by mitosis and cleavage when the duplicated material is equally segregated to the two daughter cells. The onset of both meiotic and mitotic divisions is controlled by a cytoplasmic activity factor termed MPF (maturation/mitosis/meiosis promoting factor). MII oocytes arrest at metaphase of the second meiotic division and contain high MPF activity. Upon fertilization or activation, MPF activity declines to basal values until the G2 phase of the cycle when increasing MPF activity induces entry to first mitosis. When nuclei are fused to MII oocytes, MPF activity induces the transferred nucleus to enter a mitotic division precociously, characterized by nuclear envelope breakdown (NEBD) and chromatin condensation. The effects of this premature entry to mitosis, or premature chromosome condensation (PCC) as it has been termed, on the transferred nucleus are dependent upon its cell cycle phase. Nuclei in S-phase undergo large amounts of DNA damage; in contrast, nuclei that are before (2C) or after (4C) S-phase form single or double chromatids, respectively, and appear to avoid DNA damage. NEBD also results in DNA synthesis occurring in all nuclei regardless of their cell cycle stage; thus only diploid nuclei will avoid DNA damage and retain correct ploidy. In contrast, if embryos are reconstructed after the decline of MPF activity, NEBD or PCC do not occur, DNA synthesis is controlled by the cell cycle stage of the donor nucleus and correct ploidy is maintained (see Table 1).

The occurrence of NEBD and PCC may not be related solely to the activity of MPF. In cattle oocytes MPF activity, when measured biochemically, declines within 2–3 h after activation (Campbell *et al.*, 1993a,b; Collas *et al.*, 1993b). However, in embryos reconstructed at different times after activation and examined one hour after application of the fusion pulse, NEBD of the transferred nucleus is observed for approximately 9 h after activation (Campbell *et al.*, 1993a). This discrepancy may be the result of further cytoplasmic factor/s, one such activity is MAP kinase. This cytoplasmic kinase which becomes activated early during the first cell cycle of murine zygotes is incompatible with pronuclear formation (for review see Whittaker, 1996).

Cell cycle co-ordination and development

As detailed earlier, inappropriate choice of donor and recipient cell cycle stages can result in chromosomal damage and aneuploidy during the first cell cycle. Two approaches can be used to avoid these problems; first, diploid nuclei can be transferred to MII oocytes and secondly G0/G1, S and G2-phase nuclei can be transferred after the decline of cytoplasmic activities that induce NEBD

(for review see Campbell *et al.*, 1996b). The former of these approaches requires the synchronization or selection of diploid nuclei.

Embryonic blastomeres as nuclear donors

In early embryos, at any one time, most nuclei are in S-phase. Although synchronization of embryonic blastomeres has been successful in mice (Otaegui *et al.*, 1994), similar methods have proved unreliable in farm animal species (authors' unpublished observations). The use of pre-activated oocytes as cytoplast recipients for unsynchronized blastomeres results in a significant increase in the frequency of development to the blastocyst stage in both sheep (Campbell *et al.*, 1994) and cattle (Stice *et al.*, 1994). As an alternative to preactivation, treatment of MI-enucleated cattle oocytes with 6-dimethyl amino purine results in the oocyte arresting in an unactivated state with low MPF (Susko-Parrish *et al.*, 1994). This situation mimics the use of preactivated oocytes as cytoplast recipients in that the transferred nuclei do not undergo NEBD and upon subsequent activation DNA replication is controlled by the cell cycle phase of the karyoplast, thus maintaining ploidy.

The ability to synchronize the blastomeres of early murine embryos has allowed more detailed studies of cytoplast/ karyoplast cell cycle combinations. Recent reports in mice have demonstrated the use of mitotically arrested cells as donors of genetic material (Kwon and Kono, 1996). Briefly, blastomeres from nocodazole-treated embryos arrested in mitosis are transferred to enucleated MII oocytes. Extrusion of a polar body is inhibited by treatment with cytochalasin B, which results in the formation of two (pro) nuclei. One of these nuclei is then transferred to an enucleated, one-cell embryo. Six identical pups have been produced from a single four-cell embryo using this method, and development has also been obtained from 20-cell embryos (Kono, personal communication). In a further study, Otaegui (personal communication) suggests that during the late G2 and early G1 phase of the donor cell cycle, the chromatin can re-direct development more effectively. Together these two studies indicate that during late G2, there is an M and early G1 permissive state which allows nuclear reprogramming. One possible explanation for these observations is that, during these cell cycle phases, certain factors are released from the chromatin, thus allowing access to oocyte-derived factors. This hypothesis is supported by two lines of evidence; first during mitosis transcription factors become displaced from the chromatin (Schermoen and O'Farrel, 1991; Martinez-Balbas *et al.*, 1995) and second, recent evidence indicates that live offspring can be produced from cultured, differentiated cell lines induced to exit the growth cycle and enter a state of quiescence (see below).

Other cell types as nuclear donors

One of the aims of developmental biologists and biotechnologists is to produce offspring from cell populations that can be maintained in culture. For nuclear transfer the availability of a cultured cell line would facilitate cell cycle synchronization of the donor nucleus and allow optimization of cell cycle co-ordination in the reconstituted embryo. In frogs, development to adult was obtained following the transplantation of intestinal epithelial cell nuclei into enucleated oocytes (Gurdon, 1962a,b). In mammals a number of cell types including embryonic stem cells in the mouse and primordial germ cells may be totipotent (for review see Wilmut *et al.*, 1992); however, as yet development to term has not been demonstrated, i.e. cattle PGCs (Moens *et al.*, 1996), murine ES cells (Modlinski *et al.*, 1996). Recently we reported development to term of ovine embryos reconstructed using cell populations derived from embryo, fetal and adult tissues as nuclear donors (Campbell *et al.*, 1995, 1996b; Wilmut *et al.*, 1997). In these experiments the cells were arrested in a G0 or quiescent state by the reduction of serum concentrations in the growth medium. Quiescent cells exit the growth cycle during the G1 phase and arrest with a diploid DNA content. The role of quiescent donor nuclei in the success of these studies may be related to a number of factors. First, a stable population of diploid cells allows the co-ordination of donor and recipient cell cycle; second, when cells enter quiescence a number of changes occur: these include a reduction in transcription, a reduction in translation, and active degradation of mRNA and chromatin condensation (Whitfield

et al., 1985). These changes may render both the cytoplasm and the chromatin more compatible with the cytoplast and facilitate a greater reprogramming of the donor chromatin by maternally derived cytoplasmic factors as discussed in the previous section. The role of quiescence in changing chromatin structure and the ability of donor nuclei to re-control development after nuclear transfer requires further studies; however at PPL this technology has been transferred to cattle and a live calf produced (Chen *et al.*, unpublished).

Techniques for and uses of Genetic Modification in Farm Animal Species

The aims of any scheme for genetic modification or selection are to obtain stable desirable phenotypes that transmit the required traits through the germ line. In farm animal species we were until recently limited to the technique of pronuclear injection; however, nuclear transfer from cultured cell populations provides an alternative route to genetic modification.

Pronuclear injection

The addition of genetic material or production of a transgenic animal can be achieved by the injection of the required gene into the pronucleus of a zygote. Although this technique has been applied successfully in a number of species including mice, rabbits, pigs, sheep, goats and cattle (for review see Wall 1996), there are a number of disadvantages. Integration does not always occur during the first cell cycle resulting in the production of mosaic embryos (Burdon and Wall, 1992). Integration occurs at random within the genome resulting in variable expression of the gene product (see Wall, 1996). At present only simple gene additions may be performed. The selection of transgenic embryos before their transfer is hampered by mosaicism (Rusconi, 1991). The production of the required phenotype coupled to germ line transmission may require the generation of several transgenic lines. Multiplication of the required phenotype or its dissemination into the population is restricted by breeding programmes.

Nuclear transfer

The production of animals from cells that can be maintained in culture offers a number of advantages over the technique of pronuclear injection. First, the cells to be used as nuclear donors can be sexed, genetically modified and selected in culture before their use for nuclear transfer. The resultant animal is produced from a single nucleus of the desired genotype; therefore, mosaics will not be produced and the genetic modification should be transferred to the offspring. As all of the cells in the animal contain the transgene, then dependent upon its site of integration and tissue specific promotor, transgene expression should be obtained in the tissue of interest. The use of cultured cells will allow multiple genetic modifications and also facilitate precise genetic modifications which are presently not possible. For instance, specific genes may be removed (knocked out), replaced (knocked in) or specific chromosomal regions modified. The combination of cell culture and nuclear transfer allows multiple genetic modifications to be carried out by isolating new cell populations to act as nuclear donors from any of the embryos, fetuses or adult animals created. Thus, modifications not possible within the limited lifespan of a primary cell population may be facilitated by its rejuvenation via nuclear transfer.

Genetically modified, selected, clonally derived cells may be stored until expression data are obtained from the resultant animals. An 'instant' flock or herd of animals may then be produced by nuclear transfer, thus reducing the period required by natural breeding for production purposes.

Applications of nuclear transfer

In the short term, the major applications of such technology are most likely to be in the biopharmaceutical industry. The ability to produce animals from cultured cell populations will allow the precise genetic modification of these cells before embryo reconstruction. Such applications

will not only include the addition of genes previously demonstrated using pronuclear injection, but also the targeting of such genes to precise regions of the genome, the removal or replacement of genes by targeting technology (i.e. removal of the PrP gene for scrapie resistance or removal of the bovine serum albumin gene and its replacement with the human homologue for therapeutic use) or the precise modification of endogenous genes (i.e. down- or upregulation of endogenous genes by changes in promoter sequences). The combination of these techniques will have applications in the production of pharmaceutical proteins, production of animal models of human disease and the modification of animals for organ transplantation.

In the longer term the combination of such technologies with the information obtained from genome mapping projects may allow the transfer of large segments of DNA coding for specific animal production traits. These may include genes or chromosomal segments coding for disease resistance, meat quality, growth, reproductive, nutritional or behavioural traits.

Incorporation into traditional breeding regimens

The application of this technology will allow the production of specific selected or modified progenitor animals for the dissemination of required traits into the population as a whole. In addition embryo multiplication by nuclear transfer will allow multiplication of embryos or animals that contain the genetic modification or exhibit the required traits in order to hasten the rate of dissemination into the population.

Other uses of nuclear transfer from somatic cell populations

As with the Amazonian rainforest, unknown genetic resources are also being lost from farm animal species, for example rare breeds of cattle in parts of Africa. At present preservation of these unknown resources is limited to the storage of semen or early embryos. Unfortunately, owing to many factors this approach has proved difficult. The demonstration that cultures of somatic cells isolated from adult animals (Wilmut *et al.*, 1997) can be used successfully in nuclear transfer offers an alternative route to the storage of genetic material.

Conclusion

The use of cultured somatic cells for nuclear transfer holds great potential for the cloning of large numbers of identical animals for agricultural applications. In addition, the relative ease by which cultured somatic cells may be rendered transgenic coupled with use of such cells for nuclear transfer offers a new and more efficient procedure to generate transgenic animals for both agricultural and industrial applications. The possibility of applying gene targeting techniques may allow for the generation of transgenic animals in which specific genes have been silenced, or 'knocked out', and replaced by other genes (knocked in). For example, Yom and Bremmel (1993) suggested that genes coding for the major proteins in cow's milk could be knocked out and replaced by their human counterparts. Cows modified in this fashion would produce milk containing a human milk protein profile. Such milk would presumably be hypoallergenic and more nutritious for human infants and thus be more suitable for infant formula manufacture than normal cow's milk. Gene targeting could also be applied to generate transgenic swine suitable for donating organs to human patients in need of transplant therapy. Use of gene targeting strategies should prevent organ rejection in recipient patients by 'knocking out' expression of proteins that trigger the immune rejection response.

Numerous technical hurdles must be overcome before somatic cell nuclear transfer can be considered routine. The overall efficiency of the process is quite low; many reconstructed embryos have been required so far to generate a few offspring (Wilmut *et al.*, 1997; Schnieke *et al.*, 1997; Cibelli *et al.*, 1998; Christmann, Chen, Polejaeva, Campbell and Eyestone, unpublished). Losses of potential offspring occur throughout the process, from early embryo development, during gestation and even perinatally. Such losses are presumably due to incomplete reprogramming of differentiated nuclei. A

more thorough understanding of the reprogramming mechanism could lead to the development of better methods of inducing reprogramming during nuclear transfer. For transgenic work, especially if it involves gene targeting, cultured cells must be maintained in culture through numerous population doublings to allow for transfection and selection of genetically modified cells and may suffer karyotypic abnormalities or other mutations during extended culture. Improved methods of cell culture and more efficient gene targeting and selection methods could reduce the incidence of mutations induced during culture and thus lead to more robust cell populations for nuclear transfer.

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