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Nuclear Transfer in the Production of Transgenic Farm Animals

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Introduction

The aim with any procedure for genetic modification is to be able to introduce the change which is desired, precisely and with confidence that there will be no other unintentional change. This has, so far, not been achieved in livestock, but recent results suggest that nuclear transfer from donor cells in which genetic change has been made by site-specific recombination will become possible and meet these aims. This chapter will discuss the limitations of the present procedures for genetic change, describe nuclear transfer and the factors which influence development of embryos produced by nuclear transfer, outline the approaches to gene transfer and modification before nuclear transfer, and consider some applications of genetic modification in livestock.

Methods for genetic modification of mammals

Direct injection

At present, the only method for the production of transgenic farm animals is to inject several hundred copies of the gene into a nucleus of an early embryo. While this approach has been useful, and has provided some commercial opportunities (e.g. for the production of therapeutic proteins in the milk of livestock), it has several serious limitations which are well known (Pursel *et al*., 1989; Wilmut and Clark, 1990). It is believed that injection of the fluid causes breaks in the chromosomes, so stimulating

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repair mechanisms which inadvertently include some of the injected DNA into the chromosome. This sequence of events would certainly account for the facts that most of the injected embryos fail to develop to term and that there is a low frequency of transgenesis. The site of integration is apparently random and it is believed that the influence of the neighbouring sequences accounts for the great variability in the level of expression of the same transgene in different lines. In addition, it has been estimated that direct injection causes mutation to an endogenous gene in between 5% and 10% of mouse lines, and there is no reason to expect the frequency to be different in other species. The final, and most profound limitation of this approach is that it can only be used to add a gene and cannot be used to modify an endogenous gene.

Embryonic stem cells

The limitations of direct injection can be contrasted with those observed with the use of embryonic stem (ES) cells in mice (see Hooper, 1992). In the mouse, but so far only in the mouse, culture methods have been found for the isolation of cells from the embryo, such that they divide, but do not differentiate. The cells may be derived from the early embryo or primordial germ cells (Matsui *et al.*, 1992). In some cases, if stem cells are aggregated with another embryo they retain the ability to colonize all of the tissues of the resulting offspring, including the germline. By site-specific recombination in the cells it is possible to introduce genes or to modify existing genes in a predictable manner, with little risk of inadvertent mutation to other genes (Hooper, 1992). As it is possible to confirm precise genetic changes in the cells before aggregation with the second embryo these cells have provided a very efficient means for the introduction of genetic changes in that species. While this approach offers the opportunity for precise modification it has the disadvantage that an intermediate chimeric generation is required before the effect of the modification can be assessed and that this would require up to 2 years in livestock species.

Potential value of nuclear transfer

The development of methods for nuclear transfer from embryonic cells encouraged the thought that it might become possible to devise methods for nuclear transfer from cells after precise genetic changes had been made in the cells. In livestock, nuclear transfer from inner cell mass cells has become routine (Bondioli *et al.*, 1990) and mouse ES cells retain some similarities to inner cell mass cells (Beddington and Robertson, 1989). These two observations suggested that when ES cells from livestock species become available then nuclear transfer from such cells would provide an ideal

means for the introduction of genetic change. Hypothetically, in such a system, it would be possible to exploit the unusually high efficiency of recombination in ES cells before nuclear transfer to produce groups of genetically identical offspring, with the genetic modification. In addition, use of nuclear transfer would avoid the chimeric generation and ensure germline transmission from the offspring. Despite an extensive research effort over a period of 10 years there is still no confirmation that ES cells have been isolated from any species other than the mouse, although cells which resemble mouse ES cells can be seen for a number of passages during culture of pig, sheep and cattle cells (this conference). However, nuclear transfer has proved to be more powerful than was generally anticipated and genetic modification by this route now seems attainable through the use of primary cell cultures.

Nuclear Transfer

Method of nuclear transfer

Nuclear transfer in mammals is achieved by the fusion of a donor cell to an unfertilized egg or early embryo which has been enucleated (shown schematically in Fig. 5.1). Details of the procedures have been described previously (Campbell *et al*., 1993, 1996b). Development after nuclear transfer is influenced by a great many factors which have been discussed elsewhere (Campbell *et al*., 1996b), including a requirement that normal ploidy be maintained in the reconstructed embryo. When a nucleus is transferred from a cell that has begun to differentiate, the pattern of gene expression must be 'reprogrammed' from that of the differentiated phenotype to that required for early development. The experiments in which cell cycle stage has been varied also suggest that the efficiency of this process is influenced by both donor and recipient cell cycle stage. The role of cell cycle in development after nuclear transfer will be reviewed before a consideration of the new opportunities that are becoming available.

Cell cycle in nuclear transfer

Although the cell cycle stage of both donor and recipient cells influence when DNA replication occurs in the reconstructed embryo, the recipient cell may have a dominant role because of the influence of meiosis (maturation or mitosis) promoting factor (MPF) in the cytoplasm (Barnes *et al*., 1993; Campbell *et al*., 1993). During meiosis, MPF activity increases at the time of formation of the meiotic spindles, and at metaphase II remains high until fertilization or parthenogenetic activation. Regardless of the cell cycle stage of the donor nucleus, transfer to a cytoplast with a high level of MPF is followed

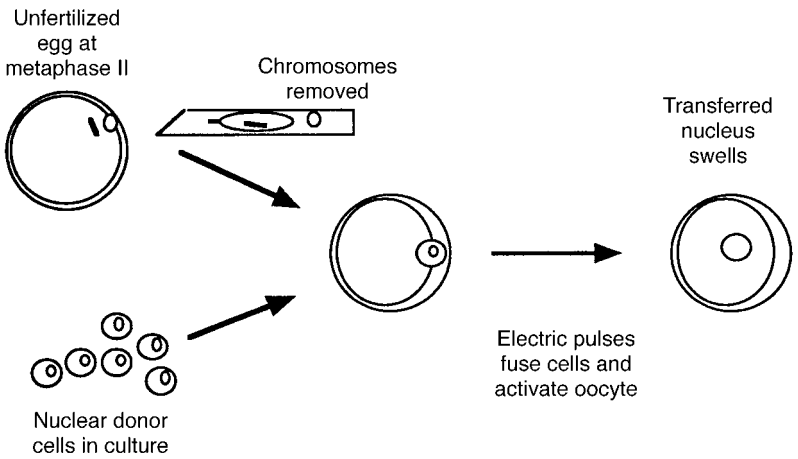

Fig. 5.1. Schematic representation of the method of nuclear transfer used in these studies. There were some variations in the time of oocyte activation in relation to cell fusion which are not shown. These details of the procedures are given in the referenced publications.

by nuclear membrane breakdown, chromosome condensation and, following reformation of the nuclear membrane, DNA replication. By contrast, following transfer to an oocyte with a low level of MPF activity, the nucleus determines whether DNA replication occurs. These observations suggested two different approaches to the maintenance of normal ploidy following nuclear transfer. If the cytoplast has a high level of activity then a diploid nucleus is appropriate, whereas pre-activation of the cytoplast creates a universal recipient able to maintain normal ploidy after transfer of a nucleus at any stage of the cell cycle (Campbell *et al*., 1993, 1994). These different approaches have both been exploited effectively, however, the few comparisons of different recipient oocytes which have been made suggest that there are big differences in embryo development, even between those treatment combinations which were expected to retain normal ploidy (Table 5.1).

By contrast, there are several observations to show that development is influenced by donor nucleus stage, even among those groups in which normal ploidy is expected (Table 5.1). This general conclusion is interpreted to indicate two different effects: (i) a greater opportunity for reprogramming of gene expression during specific phases of the cell cycle; and (ii) a benefit from transfer to similar phases of the cell cycle.

Development after nuclear transfer is more likely to occur if the donor cells are taken from the 'window of opportunity' which includes G2, mitosis G1 and G0 (see Table 5.1). Cells in G0, known as quiescence, have exited the growth cycle and become relatively inactive. Stem cell populations

Table 5.1. Effect of donor and recipient cell cycle stage upon development of mouse and sheep embryos reconstructed by nuclear transfer to the morula or blastocyst stage of development. Cell cycle combinations in which normal ploidy is expected are indicated in bold. (Summary taken from Campbell *et al.*, 1996a.)

		Donor cell cycle stage				
Recipient cell cycle stage	Donor cell type	G0	G1	Early S	Late S/G2	G2
MII	Mouse two-cell		**77.8**	0.0		20.8
MII	Mouse four-cell		**43.0**	0.0	0.0	
G1/S-phase			**60.0**	**14.0**	**0.0**	
Late S/G2			**0.0**	**0.0**	**12.0**	
MII	Mouse eight-cell		**27.0**	0.0		
Post-activated MII	Sheep embryo culture	**12.8**				
MII		**16.3**				
S-phase		**11.7**				

remain in this stage until they receive the stimulus to re-enter the growth cycle. The impression that such a window exists is gained from experiments in several species with different cell types. The response to nuclear transfer varies between species (see below). There is a need for a systematic comparison between these donor cell cycle phases in several different cell types in more than one species.

Comparisons with mouse embryo blastomere donor cells show advantages in using donor cells in G2, mitosis or G1 over other stages of the growth cell cycle. The effect of cell cycle was also studied in sheep. Donor nuclei cells were induced to become quiescent as a means of obtaining a stable, diploid population. Live lambs were born following transfer of nuclei from differentiated cells derived from sheep embryos, fetus and adult mammary gland tissue (Wilmut *et al.*, 1997). The cells were maintained in culture for prolonged periods before being induced to leave the growth cycle and become quiescent.

Normal development of embryos produced by nuclear transfer is assumed to depend upon a pattern of gene expression that closely resembles that observed after fertilization. Immediately after fertilization, development depends upon proteins and RNA produced in the oocyte before ovulation (see Thompson, 1996). At a species-specific stage of development, transcription from the embryonic genome begins and the maternal messages are destroyed. Then there are characteristic changes in gene expression as the cells divide and differentiate. When a nucleus is transferred from a cell that is transcriptionally active, transcription must first

cease before it is initiated at the appropriate stage of development. Abnormal development might reflect the presence of unusual transcripts or the lack of those that are required. It has been suggested that if a donor nucleus is in the 'window', factors which influence chromatin structure and gene expression are removed from the DNA helix. As a result, there is greater access to the DNA for those factors in the oocyte cytoplasm which reprogramme gene expression. Although this hypothesis is compatible with all published information, there have been very few studies of transcription after nuclear transfer or of chromatin structure in donor cells.

Response to nuclear transfer in different species

There is an apparent association between the stage at which transcription from the embryonic genome begins and the efficiency of nuclear transfer. There are few direct comparisons, but it seems that nuclear transfer is relatively ineffective in those species, such as the mouse, in which initiation of transcription from the embryonic genome occurs earliest. By comparison, it has been most effective in amphibians in which the transition occurs very late in development. This observation is interpreted to indicate that further reprogramming of gene expression occurs during each mitosis and that, while there is only one cell division before the transition in the mouse, there are three in sheep and cattle and 12 in amphibians. This association leads to the prediction that the response in rabbits and cattle to the new approach to nuclear transfer will be similar to that seen in sheep, but that there may be differences in pigs and mice, in which the major transition occurs at the four- and two-cell stage respectively (see discussion by Thompson, 1996).

Limitations to the present procedures

There are several limitations to the present methods. Only a small proportion of reconstructed embryos develop to become live offspring, varying between 0.04% with adult cells to 1.7% and 1.0% for fetal- and embryo-derived cells respectively (Wilmut *et al*., 1997). Even when considering only the proportion of embryos that became live lambs after they had developed to morulae or blastocysts in culture before transfer to recipients these same proportions are 3.4%, 7.5% and 4.6%, respectively. If all offspring were to carry a desired genetic change, see below, this would represent a significant gain in efficiency on direct injection, but there is still a major cost in obtaining each lamb.

Secondly, there may be complications at the birth of the lambs. A number of the lambs derived by nuclear transfer died soon after birth. In most cases this has been because of congenital abnormalities in the cardiovascular or urinogenital systems. In addition, some lambs were unusually large, although the extent of the increase cannot be estimated

because of the geneticists habit of transferring embryos between breeds as a means of an instantaneous confirmation that the offspring are indeed derived in the manner described. An increase in birth weight has been associated with several different treatments, including the culture of *in vivo*-produced sheep zygotes for as little as 3 days in medium containing serum (see Walker *et al.*, 1996), nuclear transfer (Willadsen *et al.*, 1991; Wilson *et al.*, 1995), alterations to the relationship between the stage of the developing embryo and maternal endocrine environment (Wilmut and Sales, 1981; Kleeman *et al.*, 1994) and extreme levels of non-protein nitrogen in the diet (McEvoy *et al.*, 1997). As embryos produced by nuclear transfer must also be cultured to a stage at which they can be transferred into the uterus of a recipient female, it is not clear if the increase in size associated with nuclear transfer is due to the nuclear transfer, the culture or to both. Neither the environmental factors causing the increase in size, nor the mechanisms by which the fetus increases in size are known. However, it is clear that there are profound differences in the progress of parturition. Gestation is typically extended by several days and the onset of labour often slow. Despite the prolongation of gestation, lung development in the lambs is often immature.

While it may be acceptable to use nuclear transfer for biotechnology with the present limitations, large-scale agricultural applications must surely depend upon their elimination. In that case the breeder will expect to have a conception rate which approaches that after normal breeding, with little increase in perinatal mortality.

Applications

Several advantages will arise from the opportunity to introduce precise genetic changes in livestock by nuclear transfer. Gene addition will be more efficient and require fewer animals. This offers welfare and economic benefits. The expression of added genes may also be expected to be more predictable once experience has been gained with specific sites of insertion. As the site of the modification will be selected and then confirmed before the offspring are produced there should be no unintended mutation at that site, although the possibility of mutation elsewhere cannot be absolutely excluded. Above all, there will be for the first time the chance to change existing genes. This may be used to modify production of selected proteins or to study the role of the gene product or the regulation of expression of that gene.

The first uses of this new technique will be in biotechnology, rather than agriculture. Although nuclear transfer already offers several advantages over direct injection it is still an expensive procedure and the costs can more readily be justified when the product is a high-value therapeutic protein or the greater understanding of a disease. Furthermore, a greater number of candidate genes has been identified in humans. However, in the longer term it seems probable that as the genome mapping projects identify

interesting new loci so gene targeting through nuclear transfer will offer the means to study either the role of a gene product or the mechanisms which regulate gene expression. In the following sections several different fields of use in biotechnology will be considered in turn, before a brief review of agricultural applications.

Pharmaceuticals

A number of therapeutic proteins are already being produced in the milk of farm animals, including α_1-antitrypsin (Wright *et al.*, 1991; Carver *et al.*, 1993). The transgenic animals were produced by direct injection. Gene targeting will allow a more efficient and precise means for the production of animals with additional genes. This advantage is perhaps greatest in cattle because a female born as a twin to a male calf is almost always an infertile 'freemartin'. In cattle, as in other species, a majority of embryos developing after direct injection die during early development. Whereas it is possible in other species to transfer several such embryos to each recipient, the risk of infertile freemartins limits the value of this strategy in cattle. By contrast, if genes are added before nuclear transfer all of the offspring will be of the same sex, which can be selected as that which is most appropriate for the particular application.

The greatest benefit to arise from use of nuclear transfer is that a specific change may be made. Experience will be required to confirm the most reliable strategy which may be via the insertion of coding sequences into a milk protein gene. This could both direct secretion of the desired protein and stop the production of the milk protein. Alternatively, a fusion gene could be inserted into a site known to allow high levels of expression of such genes. The construct would include both the coding elements for the human protein and the regulatory elements for the milk protein gene being used to direct synthesis to the mammary gland. Experience in this field will assist later applications to modify milk composition.

Xenotransplantation

At present many patients requiring organ transplantation die before a suitable donor becomes available. There is an ever-increasing waiting list for such organs. There is currently considerable interest in the possibility of using pigs as the source of organs for transplantation into humans. One major problem is the hyperacute rejection response, which destroys very rapidly an organ transplanted between species. This rejection is due to the presence of naturally occurring antibodies in human circulation that immediately recognize certain foreign (xeno-) antigens on transplanted tissue. These antibodies initiate a very rapid complement-mediated response that causes the lysis of the

transplanted tissue. Present transgenic strategies include introducing (human) genes whose products suppress the complement-mediated lysis or modify the xeno-antigen (White, 1996). In addition to facilitating such transgenic modification of pigs, gene targeting in conjunction with nuclear transfer would enable quite different strategies to be adopted. Gene targeting could prevent expression of the genes responsible for the xeno-antigen e.g. α_1-3-galactosyl-transferase. Alternatively, strategies using cell-based germline modification could include modifying the pig major histocompatibility complex (MHC) genes so that the longer term MHC-mediated tissue rejection was suppressed, thus reducing/eliminating the requirement for chronic immunosuppression of recipients with drugs. The research necessary for this application will, for the first time, establish routine methods of nuclear transfer in pigs.

Disease models

Medical research benefits from models of human diseases in animals for detailed study of the disease and the development of new therapies. Gene targeting in mice, through the use of ES cells, has been used to provide models of human genetic diseases, such as cystic fibrosis. However, many of the mouse models have significant limitations because of the physiological difference between mice and humans. Furthermore, there are severe practical limitations to routine drug administration and physiological sampling, because of the small size of the animals. The opportunity to target precise genetic changes in livestock could offer more useful animal models.

Sheep have been used extensively for the study of many human respiratory diseases because there are considerable similarities between human and sheep lungs. Sheep may thus be an ideal candidate in which to generate a model for human cystic fibrosis by targeting mutations to the cystic fibrosis gene. Such animals could be used to study the physiological changes in detail, to evaluate new small-molecule-based therapies and for the development of gene therapy strategies.

Disease models in farm animals will be important in understanding genetic diseases in these species. Studies of scrapie in sheep and bovine spongiform encephalopathies in cattle will provide much needed understanding of these spongiform encephalopathies in these species, and indirectly of Creutzfeldt–Jakob disease in humans.

Susceptibility models

Similar benefits may flow from the use of animals to investigate genetic differences in susceptibility to disease. There is a genetic component to most of the major causes of adult death, including cancer, heart attack and stroke. As the human genome projects identify regions associated with susceptibility

to specific diseases then it will be possible to use gene targeting to study the role of candidate genes in the aetiology of the disease. In some cases this may stimulate the development of drugs able to overcome the susceptibility.

Physiological studies

There is every reason to expect that the opportunity to target precise genetic changes in livestock will offer advantages to all of the existing transgenic projects described in this book. Gene targeting has the potential to change any aspect of gene function and to modify the gene product or prevent its production completely. By changes to the regulatory region, the site and level of expression may be changed. Alternatively, the mechanisms controlling gene expression may be analysed by modifications to the regulatory region, leading in turn to the opportunity to modify expression either in livestock or human patients. In just the same way that studies of undesirable gene expression may help in the treatment of disease, similar studies of gene expression in health or exceptional agricultural productivity will confer different benefits. An understanding of the role of specific genes in agricultural production will assist accurate genetic selection or the introduction of targeted changes in livestock.

Conclusions

A new era in biotechnology, biomedicine, farm animal breeding and research is being heralded by the development of methods for precise genetic change. Specifically, nuclear transfer from cultured cells after the introduction of genetic changes will allow entirely new approaches to the study of genes in disease and health. In the past, physiological studies have identified the role of secretions from each organ. Classically, the role of an organ was defined by the removal of that organ, the purification of a product from the tissue and confirmation that normal health was restored by administration of the product. As the gene mapping projects identify new genes in humans and livestock an analogous series of studies can be expected to define the role of specific gene products. In the past such studies were limited to mice, but the new nuclear transfer techniques will provide the same powerful tool in other species. The value of this new opportunity cannot yet be estimated, but it will certainly be large.

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