MODIFICATION OF MAMMALS BY GENE TRANSFER

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ABSTRACT

The first transgenic animals were produced back in the mid-1980’s. This involved the introduction of foreign DNA into fertilised mouse eggs producing transgenic mice for studies of the biological systems regulating growth and development. Since that time, transgenic mice, rats, rabbits, sheep, pigs, goats and cattle have all been produced using the approach known as microinjection. More recently, the biological revelation that a differentiated cell can be developmentally-reprogrammed and following transfer to an enucleated egg produce a copy of the original donor animal, came a new approach to the production of transgenic animals. This procedure commonly referred to as nuclear transfer or ‘cloning’ now provides a new exciting and more sophisticated approach to the production of superior or transgenic animals. The potential applications of these technologies include scientific, agriculture and biomedicine, and it is the production of biologically important molecules or therapeutics that has progressed most rapidly and is nearing commercialisation. These technologies and the livestock produced are likely to have a significant impact on agriculture, livestock production, medicine and will raise new issues including public concerns.

Keywords. transgenics, genetic modification, nuclear transfer, microinjection, pharmaceuticals.

INTRODUCTION

Transgenic technology involves the transfer of a segment of DNA into the genetic material of another species. One of the most exciting uses of this technology is its application to the modification of animals (and plants) to alter their physiology in order to examine biological processes such as development, growth, and reproduction. The vast majority of transgenic livestock have been produced using the procedure known as microinjection. Many of the scientific breakthroughs that enabled the production of these genetically modified animals have occurred over the past two-decade. The production of transgenic animals, in the form of mice, was first described in 1980-81 (Gordon et al. 1980). Since this time, the production of transgenic mice by microinjection of DNA into the pronucleus of zygotes has been the most productive and widely used technique. Likewise during the past 15-20 years, the technique of microinjecting DNA into a fertilised zygote has been extended to a variety of species beyond the mouse, including rats, rabbits, swine, sheep, cattle, goats, and some species of fish and poultry (Pinkert and Murray 1999).

The technology has been applied most rapidly by some biotechnology companies who aim to take advantage of the natural (and selected) ability of livestock such as cattle, sheep and goats to produce large quantities of protein in their milk. By combining this ability of mammals with gene transfer approaches, new animals are being developed that produce large quantities of highly valuable proteins for the treatment of human diseases.
This review will discuss methods available for the production of genetically modified mammals including recent progress and challenges associated with the production of transgenic animals via somatic cell gene manipulation and nuclear transfer or cloning. Potential applications of this technology in biological research, agriculture and biomedicine are also discussed.

DEVELOPMENT AND PRODUCTION OF TRANSGENIC MAMMALS.

Pronuclear micro-injection. The advantages of this technique are mainly that the procedures are now relatively standard and well characterised for most livestock species. Further, although the efficiency of production of transgenic livestock is considerably lower than in mice, many animals are now ‘on the ground’ around the world. The major drawbacks of the approach, apart from cost, are that only the addition of a gene(s) can be achieved, and the insertion site within the genome is semi-random resulting often in unpredictable effect on expression of the transgene.

The process of integration of the transgene (the injected DNA or gene construct) into the genome is still not well understood (Bishop and Smith 1989, Hamada et al. 1993). However, it is clear that one to several hundred copies of the transgene are usually integrated at a unique and apparently random site. Although these injections are performed in the pronuclei of fertilised eggs, it appears that the majority of founder animals are mosaic, at least in mice. This means that not all cells in the body of the founder transgenic animal contain the foreign injected DNA, thus suggesting that the DNA integrates after the first cell division (Whitelaw et al. 1993). The practical consequence of mosaicism is that the founder animals must be bred before expression of the transgene can be truly evaluated as not all cells carry the transgene. With livestock, this adds an additional 2-3 years on to the time frame. The percentage of livestock born that are identified as transgenic varies widely between experiments and is slightly lower than that observed in mice (1-15% vs. 20-30%, respectively).

Traditionally, the cost of production of transgenic livestock has been very high. This cost, when compared with mice does not appear to reflect a difference in the efficiency of the microinjection technology applied to these species. It is rather related to the number of fertilised ova obtainable per female, the availability and the number of recipients needed for the microinjected eggs, the length of the gestation time, the generation interval, and the maintenance costs of these animals. In addition, for physiology reasons, oestrus synchronisation seems more difficult to achieve in farm animals as compared with mice. Despite this, the technology has already been applied successfully to produce transgenic farm animals (Krimpenfort et al. 1991; Hyttinen et al. 1994).

The cost of producing transgenic livestock could be lowered if developmentally-competent and/or transgenic embryos only were transferred to recipient females. This would reduce the number of synchronised recipients required, and therefore in the case of ruminants, lower the cost considerably. One approach for selection of viable eggs is to mature embryos from the one-two cell stage to morulae or blastocysts either in rabbit oviducts or in in vitro culture systems (Krimpenfort et al. 1991; Hyttinen et al. 1994). This approach would enable the taking of biopsies for sexing and transgene analysis by polymerase chain reaction (PCR) (Fajfar-Whetstone et al. 1993; Bowen et al. 1994; Hyttinen et al. 1994).
The level of expression of a transgene usually varies widely between lines of animals, and is largely unpredictable. This usually reflects the influence of the site of integration of the transgene on the level of its expression. The predictability of expression of a transgene can be improved by including dominant control elements in the gene construct or by using very large fragments of DNA that include all the regulatory regions. A considerable amount of research throughout the world has focussed on achieving correctly regulated expression of transgenes.

**Embryonic stem cell manipulation.** Embryonic stem cells (ES cells) are totipotent cells derived from the embryonic inner cell mass of pre-implantation embryos. They can be propagated and manipulated in vitro while retaining their ability to fully participate in embryonic development and thus produce live viable animals. ES cells have been used mostly for deletion of target genes by homologous recombination (see Capecchi 1989) in mice for studies to characterise the function of specific genes. ES cells have also been used to introduce subtle genetic mutations (Valancius and Smithies 1991; Askew et al. 1993; Stacey et al. 1994; Wu et al. 1994). However, this very powerful technology still appears to be limited to mice as true ES cells have not yet been established for other livestock species (see Anderson 1992 for review). In hamsters, (Doetschman et al. 1988) and pigs (Notarianni et al. 1990), ES-like cells that can be maintained in culture and undergo differentiation have been described. Similarly in rats, ES-like cells have been shown to produce chimeras (Iannaccone et al. 1994), but their contribution to the germ-line remains to be demonstrated. Short-term in vitro culture of cells derived from the inner cell mass of blastocysts from cattle and the subsequent production of calves by transfer of nuclei from these cells into enucleated oocytes has already recently been reported (Sims and First 1994). Thus to date, true ES cells have only been described and used in mice. This has severely limited the use of this very powerful approach to delete or site-specifically insert genes to mice. However with the development of nuclear transfer procedures, gene deletion and site-specific insertion has become possible in livestock species.

**Alternative strategies.** Germ-line transformation of animals can also be achieved using engineered retroviruses. In mammals, the use of such vectors has until recently been mainly restricted to mice. However, Chan et al. (1998) have recently used a retrovirus approach that resulted in impressive and promising advancement in the efficiency of production of transgenic cattle. Transfer of a retroviral replication-defective RNA vector into oocytes in MII arrest of meiosis by pronuclear injection, resulted in the production of cattle offspring whereby the majority were transgenic.

The possibility of obtaining transgenic animals using spermatozoa to carry the foreign DNA into the egg has been achieved in a limited number of laboratories (Lavitrano et al. 1989; Gagné et al. 1991), and for a range of species (Rottmann et al. 1991; Schellander et al. 1995; Sperandio et al. 1996). More recently, coinjection of unfertilised mouse oocytes with sperm heads (or membrane disrupted sperm) and exogenous DNA encoding either a green fluorescent protein or Lac Z reporter gene into oocytes has resulted in about 20% of offspring expressing the integrated transgene (Perry et al. 1999). These data suggest that exogenous DNA can reproducibly be delivered into an oocyte by microinjected spermatozoa and provide a new and potentially inexpensive method for the production of transgenic animals. One of the problems of the ‘sperm-mediated’ approach for the production of transgenic animals is that the procedure results in a medium-high rate of mosaicism in the resulting
founder transgenic animals (Chan 1999). As for microinjection, this necessitates the breeding of F1 animals to evaluate the expression of the transgene.

**Production of transgenic animals by nuclear transfer.** The relatively recent demonstration that livestock can be cloned using a procedure known as nuclear transfer is likely to revolutionise the breeding of superior or high value animals, and the production of transgenic animals. Further, this technology will, and has, raised many exciting scientific questions and observations. Perhaps the most far reaching to date is the observation that a fully differentiated cell from an adult animal can be 're-programmed' to generate a new viable animal.

Nuclear transfer in livestock is achieved by the fusion of a donor cell to an unfertilised egg or early embryo, which has been enucleated (Wilmut et al. 1999; Wells et al. 1998; Wells et al. 1999). This procedure involves firstly deriving a cell line from an animal of interest. Such an animal may be a high producing or rare adult cow, sheep, or goat. Similarly, the donor cell line can be derived from a foetus or embryonic source. This cell line needs to be totipotent for nuclear transfer (TNT). This means that it retains the ability to differentiate into all the different cell types (and organs) that make up the adult organism. Oocytes are then recovered, generally from ovarian samples collected from the slaughterhouse, and the chromosomal material is carefully removed. A totipotent cell is then injected into the enucleated oocyte and wedged between the zona pellucida and the cytoplasm membrane (Wells et al. 1997). A small pulse of electricity is then applied to the reconstructed oocyte to fuse the membranes. The embryo is activated into development by chemical means, and is then cultured *in vitro* until the morula/blastocyst stage. Viable embryos are transferred to synchronised recipient animals to develop to term.

Once permanent cell lines with totipotent nuclei are or have been established, there is in principle no limit to the genetic alterations that can be made. Transgenic sheep and cattle have been quickly produced by nuclear transfer of transformed fetal fibroblasts, demonstrating that this is a promising technique for the production of transgenic animals (Schnieke et al. 1997; Cibelli et al. 1998). More recently, the generation of the first gene targeted sheep by nuclear transfer has been reported (McCreath et al. 2000). Indeed, nuclear transfer is rapidly becoming the method of choice for the production of transgenic livestock. Using nuclear transfer, transgenic animals are developed by firstly transfecting the totipotent somatic cells with a gene construct of interest. Transgenic cells are then selected, isolated and used as the donor nuclei source for nuclear transfer (see Figure 1).

**APPLICATION OF TRANSGENIC ANIMALS**

**Traits affecting domestic animal productivity.** One of the first transgenic experiments performed involved the insertion of the growth hormone gene in mice. The body size and growth rates of the transgenic mice were dramatically increased in the mice expressing the growth hormone gene under the control of a metallothionein enhancer/promoter (Palmiter et al. 1982). These experiments stimulated interest in modifying traits that determine productivity of domestic animals. Similar attempts to modify growth in sheep and pigs resulted in a range of phenotypes that, although they included altered lean:fat body composition and feed conversion efficiency, also lead to undesirable side-effects such as skeletal abnormalities and infertility (Pursel et al. 1989). More recent attempts to manipulate growth of domestic animals have involved the insertion of genes such as *c-ski*, growth
hormone releasing factor and insulin-like growth factor I (IGF-I). These studies, whilst demonstrating that progress is being made towards producing animals with desirable growth and body compositional phenotypes, have further illustrated the need to develop a better understanding of the biology of muscle growth and development. Currently, a number of groups around the world (including one in New Zealand) are attempting to delete, or modify the expression of the myostatin gene in sheep and pigs. This gene was recently found to be responsible for the double muscling phenotype in Belgian blue cattle (Kambadur et al. 1997). In sheep and pigs, the deletion may lead to advantageous carcass compositional changes.

![Diagram](image)

**Figure 1. Schematic diagram of steps involved in production of transgenic cattle using a nuclear transfer or cloning procedure.**

Other productivity traits that are major targets for modification include characteristics of the milk of goats and cattle. Manipulation of the composition of endogenous milk proteins is likely to confer advantageous nutritional and/or processing properties on the milk. Some modifications that may be beneficial include: (i) overexpression of milk protein genes such as κ-casein to reduce the size of casein micelles and thus improve the yield of cheese; (ii) expression of modified forms of milk protein genes such as β-casein without phosphate groups to yield a higher moisture and thus softer cheese; (iii) reduction or even inhibition of gene expression, such as α-lactalbumin to reduce milk lactose content. For a more thorough review of possible beneficial changes that could be made to milk, refer to Murray and Maga (1999).
Infection of the mammary gland, in addition to causing animal distress, is a major economic burden of the dairy industry. *Staphylococcus aureus* is the major contagious mastitis pathogen, accounting for approximately 15-30% of infections, and has proved difficult to control using standard management practices. Recently, as a first step toward enhancing mastitis resistance of dairy animals, Kerr *et al.* (2001) reported the generation of transgenic mice that secrete a potent anti-staphylococcal protein into milk. The protein, lysostaphin, is a peptidoglycan hydrolase normally produced by *Staphylococcus simulans*. Three lines of transgenic mice, in which the 5'-flanking region of the ovine β-lactoglobulin gene directed the secretion of a modified form of lysostaphin into milk, exhibit substantial resistance to an intramammary challenge of 10⁴ colony-forming units (c.f.u.) of *S. aureus*, with the highest expressing line being completely resistant. Milk protein content and profiles of transgenic and non-transgenic mice were similar. These results clearly demonstrate the potential of gene transfer approaches to combat the most prevalent disease of dairy cattle.

**Phenotypic analysis of farm animals.** Gene transfer technologies in mammals, and particularly livestock have developed in parallel with farm animal genomics (the identification of trait genes). Early stages of farm animal genomics involved the construction of maps of highly informative markers and genes, followed by the use of these maps to scan broadly across genomes of flocks and herds or resource populations, segregating for commercially important traits, to locate quantitative trait loci (QTL) into 20-40 cM chromosomal regions. More latterly the focus has shifted to identification of the trait genes themselves. Having identified such a gene, the next stage will involve verification that the gene or a mutation in the gene does give rise to the observed phenotype. Gene transfer techniques applied to livestock, particularly those that enable us to precisely target a locus or individual gene will be powerful tools to bridge this phenotype gap. Such approaches have clearly been available for more than a decade in the mouse through ES cells and homologous recombination, and gave rise to notable successes such as identification of the function of the GDF8 gene (myostatin). Through comparative genomic approaches several research groups subsequently identified that double-muscled cattle have a mutation in this gene. The combination of powerful genomics approaches to identify genes important in economically-value traits, and precise gene manipulation and transfer techniques will provide us with the tools to both unravel biological processes associated with meat and milk production and to ultimate commercial exploitation through superior phenotypes.

**Pharmaceuticals.** The concept of using livestock as ‘bioreactors’ for the production of high-value molecules has been around for at least a decade. This approach is formulated on the biological ability of livestock (and in some cases rabbits) to produce large quantities of protein in their milk. A foreign gene under the control of regulatory elements or a promoter that directs expression of the gene to the lactating mammary gland is inserted into the animal. Milk is harvested from the lactating transgenic animal and the high-value protein is purified from the milk. Table 1 shows some examples of current projects being undertaken in this area and their stage of development. In general, many projects aimed at delivering pharmaceuticals from milk are considerably further advanced than those aiming towards production trait modifications. Technology developments are occurring at a much faster rate in the pharmaceuticals area, and many of the first products to be commercialised will also be from this area.
TRANSGENIC LIVESTOCK PRODUCTION IN NEW ZEALAND
Two organisations are working on the development of transgenic livestock in New Zealand. The first of these is the Scotland-based company PPL Therapeutics who have established a sheep farm in New Zealand. This farm or containment/quarantine facility is for the housing of their sheep that are transgenic for α₁-antitrypsin. Recent approval for this work, granted by ERMA New Zealand, will allow PPL to scale the ‘production’ flock up to 4,000 animals. The second organisation in New Zealand working on the development of transgenic livestock is AgResearch, where development of genetically modified cattle is currently being undertaken (see Table 1). In this case the target applications are both therapeutics and potentially functional foods.

Table 1. Some current projects involving the use of transgenic livestock for agricultural, nutraceutical or pharmaceutical applications.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Target</th>
<th>Species</th>
<th>Stage of development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin III</td>
<td>Congential ATIII deficiency</td>
<td>Goats</td>
<td>Phase III clinical</td>
</tr>
<tr>
<td>Anti-cancer Mab</td>
<td>Cancer (?)</td>
<td>Goat</td>
<td>Animal</td>
</tr>
<tr>
<td>Human Serum Albumin</td>
<td>Blood protein</td>
<td>Mouse</td>
<td>Animal</td>
</tr>
<tr>
<td>α₁-antitrypsin</td>
<td>Cystic fibrosis</td>
<td>Sheep</td>
<td>Phase II clinical</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Blood clotting factor</td>
<td>Sheep</td>
<td>Animal</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>Blood clotting factor</td>
<td>Sheep/Goat</td>
<td>Phase I clinical</td>
</tr>
<tr>
<td>Human α-lactalbumin</td>
<td>Neutraceutical</td>
<td>Cow</td>
<td>Animal</td>
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<tr>
<td></td>
<td>(phenylketonuria)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human lactoferrin</td>
<td>Neutraceutical</td>
<td>Cow</td>
<td>Animal</td>
</tr>
<tr>
<td>Human α-glucsidase</td>
<td>Pompe's disease</td>
<td>Rabbits</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Bovine casein</td>
<td>Neutraceutical</td>
<td>Cow</td>
<td>Animal</td>
</tr>
<tr>
<td>Human myelin basic protein</td>
<td>Multiple sclerosis</td>
<td>Cow</td>
<td>Animal</td>
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Production of transgenic cattle with enhanced casein. Casein is the major protein component of the milk of most mammals, making up 80% of the total protein in cow’s milk. The casein fraction of bovine milk is composed of four component caseins, which come together to form a globular micelle. The casein micelle transports insoluble CaP to the suckling young, and also determines many of the properties of milk protein in cheese and other product manufacture.

Genetic modification of casein levels in dairy cattle will provide us with a valuable biological model. Increasing the casein content against a genetic background of intensive selection for milk production will provide us with the opportunity to consider questions relating to the biological ceiling in output of the mammary gland. Further, it will enable examination of the physio-chemical properties of bovine milks of widely differing compositions. Finally, enhancement of the casein to total solids content of milk by the combination of β- and κ-casein may not only augment casein substantially, but
also decrease the size of the casein micelles, and thus additionally improve the functionality of milk protein in product manufacture.

Towards this end at AgResearch Ruakura, bovine fetal cell lines have been derived from Friesian dairy cattle of high genetic merit for milk production, and of desired milk protein variant genotype. These cell lines have been verified as totipotent for nuclear transfer in experiments that produced cloned calves. Genetic modification of the cell line has involved the co-introduction of two casein gene constructs. The first is a $\beta$-casein expression construct spanning an 18-kb genomic region of DNA isolated from a bovine library. The second construct is a $\kappa$-casein expression construct made up of three distinct regions; a genomic region of the $\beta$-casein gene spanning the promoter, transcription unit and 3’ flanking DNA, flanked by the $\kappa$-casein gene and thirdly a puromycin resistance gene for selection. Prior to their introduction into cattle cell lines, both gene constructs have been verified by micro-injection into mouse zygotes and generation of transgenic mice. Bovine $\beta$-casein and $\kappa$-casein protein was detected in the milk of mid-lactation transgenic mice by Western analysis.

Casein gene constructs have been transfected into bovine cell lines and stable clonal lines derived by conventional methods. We have found that stable cell lines can be produced at an efficiency of $1-3 \times 10^{-3}$. Clonal lines have been verified by Southern analysis for the presence and copy number of transgenes, and karyotyped to eliminate lines with chromosomal abnormalities.

A series of nuclear transfer experiments have been performed using casein cell lines. This procedure involves recovery of oocytes from cow ovaries collected from the slaughterhouse, and removal of the chromosomal material. Next, a cell from a clonal cell line is injected into the enucleated oocyte and wedged between the zona pellucida and the cytoplasm membrane. A small pulse of electricity is then applied to the reconstructed oocyte to fuse the membranes. The embryo is activated into development by chemical means, and is then cultured for 7 days \textit{in vitro} until the blastocyst stage. Viable embryos are transferred to the uteri of synchronised recipient animals for development to term. In these studies with casein cell lines, the rate of fusion of the donor cell with the bovine oocyte has been found to range between 70-80%. Of fused reconstructed embryos, approximately 50% develop to the blastocyst stage in our \textit{in vitro} culture system. High quality embryos have been transferred to synchronised recipient cattle within the Ruakura double-fenced containment facility. To date, ten cloned-transgenic calves from three distinct somatic cell lines have been born, and are being raised. In time, the expression of the casein transgenes and changes to the composition of milk will be examined.

**CONCLUSIONS**

The development of transgenic livestock is one of the most exciting areas of biotechnology. Already transgenesis has been applied to a wide range of species, and involves a large number of different genes. The ultimate product applications of the technology can be found in the agricultural, food and pharmaceutical industries. Technological changes are occurring rapidly; the recent development of cloning is likely to revolutionise the development and precision by which transgenic livestock can be developed.
In New Zealand at the present time two organisations are developing or working with transgenic livestock. Both organisations are carrying out development work in livestock within the confines of double-fenced containment facilities. This, in my view enables New Zealand to assess the potential opportunities or benefits of this technology whilst minimising any potential risks (by containing the genetically modified livestock).

As we enter a new millennium, parallel developments in biological research involving genomics in humans and farm animals, gene transfer strategies to precisely insert or delete specific genes, and nuclear transfer will significantly impact on tomorrow’s animal breeding and livestock industries. Equally important in the commercial exploitation of these technologies will be better public understanding. The exciting potential impact of the biotechnologies currently risks being lost if the public is not better informed about the technologies, the risk-benefit equation, and effects on animal welfare.

REFERENCES


