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Transgenic Cattle Applications: The Transition from Promise to Proof

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Introduction

Progress in the genetic modification of farm animals has been considerably slower compared to that for the mouse. The main reasons for this include the low efficiency of initial transgenic methods, the associated high costs, the long generation intervals compared to the mouse, a lack of understanding as to the genes to productively manipulate and their often poor regulatory control, sometimes leading to inappropriate gene expression, compromising animal welfare (van Reenen *et al.*, 2001). Although the first mammalian transgenic experimentation dates back to 1971 (Brackett *et al.*, 1971), public opinion still considers transgenic animal applications a new and untested technology. The lack of public confidence, partly due to the fact that initial applications failed to offer direct benefits to the consumers, the animals, or the environment, and the associated uncertainty surrounding the acceptance by both the public and regulatory bodies, has contributed to restraining advancement of transgenic livestock applications. While it will be some years yet before the promise is fully delivered, there is convergence from a number of scientific disciplines that will aid progress in this area. These will come from advances in animal genomics (Darvasi, 2003; Suchyta *et al.*, 2003) and the application of new molecular tools, improving the precision and control of the introduced genetic modifications (Clark *et al.*, 2000).

There are a wide variety of applications for transgenic livestock in both

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Abbreviations: AI, artificial insemination; BAC, bacterial artificial chromosomes; BFF, bovine fetal fibroblasts; ERMA, Environmental Risk Management Authority; ES, embryonic stem cells; FAO, Food and Agriculture Organization; GFP, green fluorescent protein; HAC, human artificial chromosomes; ICSI, intracytoplasmic sperm injection; IGF-I, insulin-like growth factor I; *IGHM*, immunoglobulin μ; MAC, mammalian artificial chromosomes; MAF, Ministry of Agriculture and Forestry; MI, microinjection; NT, nuclear transfer; *PRNP*, prion protein; SMT, sperm-mediated transgenesis; WHO, World Health Organization; YAC, yeast artificial chromosomes.

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agriculture and biomedicine, depending upon the particular genes that are manipulated. Described below is a selection of the areas of interest; many projects were initiated with the pronuclear injection methodology, which was, until recently, the preferred method for the production of livestock animals. All are still at the research or testing phases to either further develop the technology, evaluate safety and efficacy of biomedical products, or establish economic benefits of the technology.

ANIMAL AGRICULTURE

As our understanding of the genes that influence livestock production traits improves, so does the knowledge for accurately modifying the appropriate genes to generate new and desired animal products in the future. Agricultural applications of transgenesis are aimed at increasing the quantity and quality of valuable meat, milk, and fibre components, and improving environmental sustainability that will have economic benefits for farmers and processors, or additional health benefits for consumers.

The most efficient means of disseminating a desired genetic modification into the wider population will be through low cost artificial insemination (AI) (or natural mating) from males homozygous for the desired trait at a specific locus. However, animal industries may have to regularly introduce the same transgene (or an improved version) on a contemporary genetic background in order to capture the annual incremental genetic gains from conventional animal breeding. Even then, the economic benefits of a genetic modification affecting a production trait must be sufficiently large to compensate for the lag in genetic gain during the time taken to introduce the transgene and test its performance before wider dissemination.

Many initial livestock transgenic experiments focused on modifying body composition by the introduction of growth hormone or insulin-like growth factor I (IGF-I). However, poor transcriptional regulation of these transgenes in pioneering work resulted in high levels of these hormones in systemic circulation, with animals consequently suffering a number of deleterious side effects (Pursel and Rexroad, 1993). More desirable effects on growth rate and body composition have been achieved, without apparent abnormalities, by restricted expression of the IGF-I transgene to skeletal muscle in pigs (Pursel *et al.*, 1999). An even tighter control of transgene expression can be achieved with inducible control elements that essentially function as on/off switches, such as the metallothionein promoter (Nottle *et al.*, 1999). Rather than attempting to manipulate primary endocrine signals, specific loss-of-function mutations in the myostatin gene or its regulatory sequences might confer an acceptable degree of double-muscling in livestock species similar to that observed from natural mutations in some cattle breeds (Kambadur *et al.*, 1997). Whereas the natural mutation is associated with major calving problems and the resulting welfare concerns, an engineered modification could avoid these problems by targeting the effects of the mutation to stages of major muscle growth in postnatal animals.

There is great appeal in the prospect of using transgenesis in livestock to aid sustainable agriculture. The intention to improve animal health, reduce pollution, and more effectively utilize feed resources might be better received by society.

Although improved disease and pest resistance remains a long-term ambitious goal (Muller and Brem, 1998), the result would be improved animal welfare and reduced reliance on animal remedies. By extrapolation from the mouse (Bueler *et al.*, 1993), inactivation of the *PrP* gene in livestock (Denning *et al.*, 2001) would be expected to produce animals resistant to prion diseases. Animals resistant to transmissible spongiform encephalopathies would also provide improved safeguards for biomedical applications. Possibly, sheep could be made resistant to 'fly-strike' by producing chitinase in their skin to kill larvae (Ward *et al.*, 1993). Transgenic pigs with phytase expressed in their saliva efficiently digest dietary phytate, decreasing levels of phosphate in excrement, providing novel solutions for both improving nutrition and reducing environmental pollution from intensive farming (Golovan *et al.*, 2001). Sows over-expressing bovine α-lactalbumin in their milk increased piglet growth and health (Wheeler *et al.*, 2001). Bacterial genes that encode biochemical pathways that are non-functional in livestock could be introduced to increase the availability of specific nutrients that are rate limiting for production, so long as they did not cause pleiotropic side effects. For instance, increased feed utilization efficiency from dietary roughage might be achieved by introducing a glyoxylate cycle into ruminants, enabling the synthesis of glucose directly from acetate produced in the rumen (Ward, 2000). In another example of modifying intermediary metabolism, attempts have been made to introduce a functional cysteine biosynthetic pathway into sheep so that this rate-limiting, essential amino acid may be synthesised *de novo* to enhance wool growth (Ward, 2000).

Genetic modification of milk composition in dairy cattle has received considerable attention in efforts to improve production, aid human nutrition, and alter various processing properties designed to suit the manufacture of specific food products (Wall *et al.*, 1997; Karatzas, 2003). Manipulations include the over-expression of naturally occurring minor, but valuable, milk proteins or the introduction of a foreign gene producing a novel protein in milk to generate a nutraceutical (medical food). There may also be advantage in over-expressing major milk proteins, even for traditional commodity markets (Zuelke, 1998). Although agricultural applications are of global economic importance, especially dairy farming, with milk and associated dairy products constituting a major food source for the human population, concerns around the new technology have shifted most efforts into biomedical applications. Accordingly, most of the milk-related agricultural applications have not progressed beyond feasibility studies in transgenic mice. We have taken the opportunity to summarize these studies in more detail, and report on current progress and issues of altering bovine milk composition in a dedicated section below.

BIOMEDICAL APPLICATIONS

There has been greater research effort, progress, ethical justification, and economic incentive to generate transgenic livestock for various biomedical applications compared to those for agriculture and food production.

Specific human genes encoding medically important proteins, under the control of mammary-specific promoters, can be introduced into cultured cells and directed to be expressed in the lactating mammary gland, with secretion into the milk, in

resulting cloned-transgenic females (Clark, 1998). These therapeutic proteins are then extracted from the milk, purified, and used in clinical trials to evaluate their safety and effectiveness in treating particular human diseases and disorders before gaining final regulatory approval. Livestock are favoured where functional proteins are difficult to make in sufficient quantities, cost-effectively and safely by other methods. In harnessing the potential of the mammary gland to synthesize heterologous proteins, the choice of species (rabbits through to cows) depends upon the quantities required (Rudolph, 1999). Indeed, other tissue systems may sometimes be preferable; for instance, human polyclonal antibodies produced in the blood of transgenic cattle (Robl *et al.*, 2003), or proteins produced in the egg whites of laying hens (Ivarie, 2003), or the seminal fluid of pigs (Dyck *et al.*, 1999), which offer a more closed system for the production of highly bioactive molecules.

The shortage of human donor organs to treat chronic organ failure and various degenerative tissue diseases could be overcome by targeting specific genetic modifications to generate pathogen-free herds of pigs whose organs would be immunologically compatible with humans following xenotransplantation. Recently, pigs have been produced that completely lack the enzyme α-1,3-galactosyl-transferase (Phelps *et al.*, 2003) to counter hyperacute immune rejection. Subsequent immuno-suppressive drug therapy or additional genetic modifications could be used to manage the body's other rejection processes.

It may be ethically acceptable to genetically modify farm animals to serve as models for various inherited human genetic diseases to aid research and preliminary evaluation of novel therapies. An ovine model of cystic fibrosis, for example, is considered superior to available mouse models because of the greater similarity in lung anatomy and physiology with humans (Harris, 1997).

Generation of transgenic livestock animals

Although most transgenic livestock animals produced in the 20th century have been generated by microinjection (MI), this technology is becoming quickly superseded by new technological developments. Particularly, cell-mediated transgenesis in combination with nuclear transfer (NT), with its unique ability for sophisticated genetic modifications, and more recently, transgenesis with vectors derived from lentiviruses offering high efficiencies have taken the production of transgenic livestock to a new level. For these reasons, we will focus on cell and lentivirus-mediated transgenesis, in addition to a summary of most other technologies commonly used for the generation of transgenic livestock. It is by no means a comprehensive list of all technologies currently available. Major milestones in transgenic animal production applying the discussed methods are summarized in *Table 7.1*.

MICROINJECTION

Although it was, in fact, viral-mediated gene transfer that was first used to produce transgenic mice (Jaenisch and Mintz, 1974; Jaenisch *et al.*, 1975; Jaenisch, 1976), the development of an MI technology which enabled the direct injection of exogenous DNA into the pronucleus of fertilized mouse zygotes was the technology

Table 7.1. Milestones in the production of transgenic mice, livestock and cattle using different technologies

Method	Mouse	Livestock	Cattle
Viral mediated transfer	1974[1]	1991[2] (chicken)	1998[3]
Microinjection	1980[4]	1985[5] (sheep, pig)	1991[6]
Nuclear transfer	2000[7]	1987[8] (sheep)	1998[9]
Sperm-mediated transfer	1989[10]	1991[11] (chicken)	1996[12]

[1](Jaenisch and Mintz, 1974); [2](Briskin *et al.*, 1991); [3](Chan *et al.*, 1998); [4](Gordon *et al.*, 1980); [5](Hammer *et al.*, 1985); [6](Krimpenfort *et al.*, 1991); [7](Rideout *et al.*, 2000); [8](Schnieke *et al.*, 1997); [9](Cibelli *et al.*, 1998); [10](Lavitrano *et al.*, 1989); [11](Gruenbaum *et al.*, 1991) ; [12](Rottmann *et al.*, 1996).

that revolutionized the transgenic animal field and resulted in the widespread application of animal transgenesis. Using MI, it was shown in rapid succession that it was possible to generate mice with integrated copies of the injected DNA construct (Gordon *et al.*, 1980), that such mice were capable of expressing the integrated genes (Brinster *et al.*, 1981; Wagner *et al.*, 1981a), and that the foreign DNA is present not only in somatic tissues but may also be present in gametes, and thus transmitted through the germline to progeny in mice (Costantini and Lacy, 1981; Wagner *et al.*, 1981b). Following these pioneering experiments, a vast number of different lines of transgenic mice have been produced to date, and were used to study the regulation and function of mammalian genes *in vivo*. As the application of the technology for livestock animals offered the prospect to improve the genetics of livestock animals for both agricultural and biomedical applications, it did not take long until the first report of the production of transgenic rabbits, sheep, and pigs by MI was published (Hammer *et al.*, 1985). While still a standard technique for mice, pronuclear MI in livestock was hampered by greater technical difficulty, a low transgene integration efficiency, and high degree of mosaicism. The technology is further limited to the addition of a DNA construct which is randomly integrated into the genome and often affected by the surrounding genomic sequences. Moreover, the low efficiency, coupled with the longer generation intervals in livestock species, rendered the production of transgenic livestock expensive (Wall, 1996). This is especially true for cattle, where typically less than 1% of injected zygotes develop into transgenic animals (Wall *et al.*, 1997). To make matters worse, due to mosaicism and the random nature of the integration process, not all of the transgenic founder animals give rise to transgenic lines expressing the transgene at adequate levels. Surprisingly, a technology intended to introduce site-specific modifications has been shown to increase the efficiency of transgenic livestock production by MI. In both goats and pigs, the use of DNA coated with the bacterial recombinase RecA resulted in a significant increase not only in embryo survival rates (two-fold in goats) but also in transgene integration frequencies (eleven-fold in goats and three-fold in pigs). Although an improvement in production efficiency of transgenic animals, RecA coating could not reduce the high incidence of mosaic founder animals.

SPERM-MEDIATED TRANSGENESIS

Sperm are a natural vector for transmitting DNA in the sexual propagation of all mammalian species, and if it could be loaded with exogenous DNA, it would provide

a simple means to produce transgenic animals. Although the concept was established as early as 1971 (Brackett *et al.*, 1971), the first transgenic mouse produced by sperm-mediated transgenesis (SMT) was not reported until 1989 (Lavitrano *et al.*, 1989). Despite being conceptually very simple and elegant, it proved to be a highly unreliable technique (Brinster *et al.*, 1989). Nevertheless, efforts over many years have created a better understanding of the underlying process, which has now resulted in the demonstration of SMT in numerous species, including cattle. This has been achieved through the refinement of the basic SMT concept by combining it with restriction enzyme-mediated integration (Shemesh *et al.*, 2000), electroporation (Gagne *et al.*, 1991), antibody linkers (Chang *et al.*, 2002), and intracytoplasmic sperm injection (ICSI; Perry *et al.*, 1999). The production of transgenic livestock by SMT has a number of advantages compared to other transgenic techniques (e.g. NT, MI). Both NT and MI techniques are technically demanding and labour intensive. In contrast, SMT (with the exception of its combination with ICSI) can be performed without the need for sophisticated equipment or micro-manipulative skills. Studies have shown that spermatozoa from numerous species, including bovine, can bind and take up foreign DNA and transfer it to the embryo following fertilization (Gandolfi, 2000). In bovine studies, the efficiency of SMT varies widely, depending on both the transgene and the gene transfer method (Sperandio *et al.*, 1996; Rieth *et al.*, 2000). Liposomes have been shown to be particularly effective in transferring DNA into bovine sperm (Shemesh *et al.*, 2000). However, not all embryos derived from transfected sperm contain the transgene, suggesting mechanisms exist which impede SMT. Furthermore, it appears that, after fertilization, the introduced DNA can stay episomic, or can integrate at a late embryonic stage, giving rise to a high proportion of mosaic animals (Gandolfi, 2000).

VIRAL VECTORS

Similar to sperm, a virus is a natural DNA delivery vehicle. The early vectors used to generate transgenic animals were based on retroviral sequences and, with their broad host range, have general applicability to many different species. Retroviruses are RNA viruses that insert a reverse transcribed DNA copy of their genetic material into the host genome. While the process of transgene integration is relatively efficient, it is unfortunately offset by a high degree of mosaicism in the transgenic founder animals. Moreover, the viral sequences of these vectors are often recognized in the animals and inactivated as a response of the host defence mechanism, resulting in the concomitant silencing of the transgene expression (Gilboa *et al.*, 1986; Chan *et al.*, 1998; Wells *et al.*, 1999). Some of these limitations have been overcome with the use of replication defective, adenovirus-based vectors (Tsukui *et al.*, 1996; Kubisch *et al.*, 1997). However, these have now been superseded by the recent development of lentiviral vector systems for the generation of transgenic animals following infection of oocytes or pre-implantation embryos by either injecting viral particles underneath the zona pellucida (Lois *et al.*, 2002) or incubation of zona-free embryos in a virus-containing solution (Pfeifer *et al.*, 2002). In contrast to the retroviral vectors described above, lentiviruses have the unique ability to infect not only dividing but also non-dividing cells, and are thus less prone to create mosaic animals. Lentiviral-mediated transgenesis resulted in the generation of transgenic

mice with unprecedented ease and efficiency; with 80% of the offspring born being transgenic and 90% of those showing high level expression of the transgene (Lois *et al.*, 2002). In addition, lentiviral vectors are not affected by gene silencing, and the resulting animals show stable expression of transgenes following germline transmission (Pfeifer *et al.*, 2002). Although efforts to generate transgenic monkeys by injecting lentivectors into blastocysts failed (Wolfgang *et al.*, 2001), application of the technology to farm animals recapitulated the high efficiencies seen with mice. In the pig, the transfer of 244 virus-infected embryos resulted in 45 live piglets, of which 32 were transgenic (71% of offspring, or 13% of embryos transferred; Hofmann *et al.*, 2003). However, there was considerable variability in the efficiencies between the six established pregnancies in this study with 0, 4, 4, 16, 26, and 30% of the respective piglets in each litter being transgenic. Transgenesis with another construct, using a reduced viral titre, resulted in the production of only two transgenic offspring from 86 transferred embryos (2%; Hofmann *et al.*, 2003). One crucial aspect was, however, highly consistent; namely, a high proportion of transgenic offspring expressed the GFP transgene with the two different constructs (94% and 100%, respectively).

Similarly impressive results have been achieved in cattle with lentiviral vectors harbouring a green fluorescent protein (GFP) reporter gene. Infection of oocytes, followed by *in vitro* fertilization resulted not only in 83% of green fluorescing blastocysts but also the birth of 4 calves from 8 transferred GFP positive blastocysts that were all transgenic (Hofmann *et al.*, 2004). In contrast, a preliminary study showed that although 17% of infected bovine zygotes were shown to express a GFP transgene at the blastocyst stage (Hofmann *et al.*, 2003), the transfer of 17 blastocysts derived from infected zygotes resulted in 4 calves that were not transgenic (Hofmann *et al.*, 2004). This highlights that whereas in mice and pigs infection of zygotes resulted in successful transgenesis, cattle require infection of oocytes that lack a nuclear envelop compared to zygotes.

The technology has, however, a significant intrinsic limitation due to the physical size constraints of the viral particle used to transfer DNA. In the case of lentiviral vectors, the viral particles can only accommodate an RNA genome of about 10 kb in size. Thus, the transgene constructs have to be smaller than 10 kb, which only allows the use of small genes or cDNAs. Transgenesis using lentiviral vectors is therefore unsuitable for applications requiring larger constructs for better expression, correct regulation, or sophisticated genetic modifications, as we will discuss further in the context of cell-mediated transgenesis below.

CELL-MEDIATED TRANSGENESIS USING NUCLEAR TRANSFER

Until recently, the application of cell-mediated transgenesis for the modification of the mammalian genome has been almost exclusively in conjunction with mouse embryonic stem (ES) cells. Due to their pluripotent nature, it is possible to genetically modify these cells and subsequently use them to generate chimeric blastocysts, which give rise to chimeric transgenic animals. As the modified ES cells also contribute to the germline of the chimeric mice, simple breeding results in the production of full transgenic animals. Essentially, cell-mediated transgenesis allows the modification and characterization of a cell, which is then used to generate an

animal with the same genetic make-up. Due to the lack of *bona fide* ES cells being available from livestock species, this technology was restricted to mice. The recent development of NT using somatic cells circumvents the need for such ES cells, and made cell-mediated transgenesis available for livestock species.

Cloning by NT describes a technology in which the nucleus from a donor cell is transferred into the cytoplasm of an oocyte, or a zygote, from which the genetic material has been removed. This reconstructed, one-cell embryo is then stimulated to undergo embryonic development. After transfer into surrogate females for *in vivo* development to term, this results in offspring with the same genomic make-up as the nuclear donor cell. This elegant concept was first described by Spemann in 1938, and was first developed in pioneering experiments with amphibians (Briggs and King, 1952; and reviewed by Gurdon, 1999 and di Berardino, 2001). In mammals, the use of undifferentiated pre-implantation embryonic cells as nuclear donors resulted in the cloning of mice (McGrath and Solter, 1983) and then sheep, as the first livestock species cloned by NT (Willadsen, 1986). The dogma that the nuclei from differentiated donor cells were not totipotent and could not result in the production of live animals following NT was shattered by the ground-breaking experiments of Wilmut and co-workers at the Roslin Institute in Scotland. After firstly demonstrating NT in sheep with differentiated cultures of embryonic cells (Campbell *et al.*, 1996), they extended their studies to the cloning of Dolly, the sheep from an adult mammary gland cell (Wilmut *et al.*, 1997). Once this had been demonstrated, it was only a small step to combine NT with the genetic modification of cultured cells using established transfection methods. In this way, cloning with genetically modified donor cells demonstrated a novel route to produce transgenic sheep (Schnieke *et al.*, 1997). Since then, successful NT has been demonstrated for most livestock species, including cattle (Cibelli *et al.*, 1998), goats (Baguisi *et al.*, 1999), and pigs (Polejaeva *et al.*, 2000), rendering it a transgenic technology with broad applicability. Compared to all other transgenic methods, the NT cell-mediated approach has a number of distinct advantages, including: 1) the ability to introduce specific genetic enhancements to an existing superior background using cells from an animal of chosen genotype and sex (particularly important for agricultural traits); 2) the potential for a more extensive range of genetic modifications to cells cultured *in vitro*; 3) the ability to screen cells for specific genetic modification before producing transgenic animals; 4) all embryos/offspring are transgenic and none should be mosaic (where there is a mixture of transgenic and non-transgenic cells in the same organism); and 5) the generation of small herds from each cell line in the first generation, rather than individual founder animals that need to be subsequently bred.

The process has its limitations, however, with the current methods only resulting in low efficiencies in the production of live cloned offspring, which range between 1 and 5% of the embryos reconstructed by NT (Solter, 2000). This inefficiency is assumed to be predominately caused by an inadequate transition of the gene expression profile of the specialized somatic donor cell to the requirements of a zygote. Consequently, this creates limitations in the silencing of differentiation-specific genes and appropriate reactivation of embryonic genes in the correct tissues, in the correct abundance, and at the correct times for normal development to occur. Collectively, this process is commonly referred to as nuclear reprogramming. There

is increasing evidence of epigenetic errors in reprogramming following NT, leading to abnormal patterns of: DNA methylation (Bourc'his *et al.*, 2001; Dean *et al.*, 2001; Kang *et al.*, 2001); chromatin modification (Santos *et al.*, 2003); X-chromosome inactivation (Xue *et al.*, 2002); and expression of imprinted and non-imprinted genes (Rideout *et al.*, 2001; Wrenzycki *et al.*, 2001; Humpherys *et al.*, 2002). The effects are not limited to high embryonic and fetal losses throughout pregnancy, but extend to post-natal losses and even the production of compromised offspring (Wilmut *et al.*, 2002). The pattern of mortality and clone-specific phenotypes observed presumably reflects the inappropriate expression of various genes whose harmful effects are exerted at different stages of development. Although most of the surviving cloned animals are physiologically normal and healthy (Lanza *et al.*, 2001; Renard *et al.*, 2002), results from mice suggest that clones might have subtle epigenetic differences compared to conventional animals. The microarray analysis of more than 10 000 genes revealed an aberrant expression of around 4% of genes expressed in the placenta and 0.5% of genes expressed in the liver (Humpherys *et al.*, 2002). During gametogenesis, somatic epigenetic modifications are removed and the parental specific modifications established on the gametes, which ensures correct embryonic development following fertilization. If any epigenetic errors exist in cloned animals, experimental evidence indicates that they are corrected during germline transmission. Offspring of mouse clones produced by sexual reproduction were free of obesity (Tamashiro *et al.*, 2002) and other clone-associated phenotypes (Shimozawa *et al.*, 2002) characteristic of the cloned parental mice. This is consistent with our observation of sexually reproduced offspring of cloned cattle, which appear to be phenotypically normal. On the other hand, evidence is accumulating that some epigenetic modifications can escape epigenetic reprogramming during development, and thus could result in the transgenerational inheritance of epigenetic states (Roemer *et al.*, 1997; Lane *et al.*, 2003; Rakyan *et al.*, 2003). Additional molecular evidence showing that the aberrant gene expression profile present in cloned mice is indeed corrected in the offspring will be required to confirm that most, if not all, epigenetic errors that exist in cloned animals are not transmitted to sexually reproduced offspring. Whilst the present NT technology is indeed able to produce a few founder transgenics, currently it is desirable to use assisted sexual reproduction thereafter to further multiply animals and to circumvent or minimize potential epigenetic aberrations in the cloned generation.

Despite the obvious shortcomings of NT as discussed above, the technology still offers a number of unique advantages. With NT there is no practical limitation to the size of the transgene. Since cDNA constructs appear to be less efficiently expressed in transgenic animals, most transgene constructs are based on much longer genomic sequences. Furthermore, long stretches of regulatory sequences at both the 5'- and 3'-ends are usually included to ensure correct expression and to reduce the influence of the neighbouring genomic context of the insertion site, the so-called 'position effect', which can result in the misexpression or silencing of the transgene. Therefore, constructs of 30 kb and more are not uncommon. Furthermore, artificial chromosomes, such as yeast (YAC) and bacterial artificial chromosomes (BAC), have the capacity to carry fragments of more than one megabase. The use of such large fragments in transgenic animals results in position-independent and copy number-dependent expression (Peterson *et al.*, 1997; McCormick and Nielsen,

1998). These, therefore, have the advantage of excluding any position effects, in addition to providing all of the necessary regulatory elements to ensure correct temporal and spatial expression of the transgene. The handling of such large fragments is, however, challenging due to the intrinsic instability of such constructs, which tend to acquire deletions and rearrangements (Monaco and Larin, 1994). The recent development of mammalian artificial chromosomes (MAC) might overcome these limitations due to their ability to become established as independent chromosomes upon introduction into a mammalian cell line (Ikeno *et al.*, 1998). Introduction of a human artificial chromosome (HAC) into mouse ES cells, and use of these cells for the generation of ES cell chimeras, followed by germline transmission, resulted in a so-called transchromosomic mouse with a genome complemented by a HAC (Tomizuka *et al.*, 2000). The feasibility of this approach for livestock has been demonstrated by the stable transfer of a 10 Mb human artificial chromosome (HAC), containing the complete human immunoglobulin heavy and light chain loci, into primary bovine fibroblasts. NT with these so-called transchromosomic cells resulted in calves which had stably maintained the HAC throughout the enormous number of cell divisions of bovine development and expressed functionally rearranged human antibodies (Kuroiwa *et al.*, 2002). At the cellular level, retention of the HAC in the clonal transchromosomic calves ranged from 78% to 100% in peripheral blood lymphocytes, and was much higher than the 30–40% retention observed in transchromosomic mice.

Besides allowing the addition of larger DNA sequences, cell-mediated transgenesis can accommodate more sophisticated genetic modifications by applying homologous recombination in somatic donor cells for site-directed insertions, functional deletions of an undesirable gene on an otherwise favourable genetic background, or subtle alterations of specific regulatory sequences and target genes to improve the encoded functional properties (Piedrahita *et al.*, 1999; Clark *et al.*, 2000). A complementary strategy is the use of recombinase-mediated, site-specific genome modifications. In transgenic animals, the best studied system is based on the bacteriophage P1 derived recombinase Cre and its loxP recognition sites, which allows conditional disruption of genes or targeted integrations of a construct at a pre-determined site (Kolb, 2002). Furthermore, cell-mediated transgenesis is amenable (although not exclusively) to a whole range of methods to knock down the expression of specific genes, such as expression of ribozymes, DNAzymes, or interference RNAs (Scherer and Rossi, 2003). Thus, the cell-mediated approach has the potential to be far more precise, extensive, and rapid in terms of genetic progress than what can be achieved with traditional animal breeding and other available transgenic methods, including conventional pronuclear injection of DNA into zygotes. Initially developed using murine ES cells, which offer an unlimited lifespan, a relatively good homologous recombination frequency, and a high contribution to the germline in chimeras, homologous recombination technology for livestock animals had to be adapted for the use of primary somatic cells due to the unavailability of *bona fide* ES cells from livestock species (Prelle *et al.*, 2002). Compared to ES cells, primary somatic cells have two major shortcomings: a limited lifespan of about 35 doublings; and a two orders of magnitude lower relative frequency of homologous recombination (compared to mouse ES cells) (Sedivy and Dutriaux, 1999; Piedrahita, 2000). The application of a stringent selection strategy using

promoterless selectable marker genes, which only become functional after trapping the target gene promoter, resulted in the generation of the first gene-targeted sheep (McCreath *et al.*, 2000; Denning *et al.*, 2001) and pigs (Dai *et al.*, 2002; Harrison *et al.*, 2002; Lai *et al.*, 2002), and resulted in targeting efficiencies as high as 60% at the collagen locus (McCreath *et al.*, 2000). Application of this promoter trap approach has its intrinsic limitations and is only applicable for expressed genes. The short lifespan of primary somatic cells has been problematic as many cell clones have entered into senescence following progression through the antibiotic selection regime, limiting the number of cell clones available for characterization and NT (Denning *et al.*, 2001). It is, however, possible to rejuvenate cells by re-deriving cell lines from cloned fetuses, which restores their proliferative lifespan (Clark *et al.*, 2003). The potential of somatic NT for the introduction of multiple and complex, site-specific modifications into the genome of animals has been demonstrated recently by a breathtaking study reporting the sequential targeting of immunoglobulin μ (*IGHM*) and prion protein (*PRNP*) genes (Kuroiwa *et al.*, 2004). Using targeting vectors with a positive (antibiotic) and negative (diphtheria toxin A) selection marker, they were able to target both inactive (*IGHM*) and expressed (*PRNP*) genes. The transcriptionally active *PRNP* gene was targeted at a frequency of 6%, with around a 10-fold drop in efficiency for the transcriptionally silent *IGHM* gene. This was still sufficiently high to identify several targeted cell clones in a standard screening protocol. The targeting efficiency for the *PRNP* locus in this study was in a similar range to the targeting of the same locus in sheep, using what is generally considered to be the more efficient promoter trap approach (Denning *et al.*, 2001), and was much higher than was previously reported for targeting of the expressed α (1,3)-galactosyltransferase gene in pigs by positive negative selection with a targeting efficiency as low as 0.07% (Harrison *et al.*, 2002). This indicates that the targeting efficiencies will not only depend on the chosen targeting strategy and species but also on the gene locus, the vector design, and the cell line.

The sequential targeting for the production of double homozygous knockouts described in the study by Kuroiwa and co-workers represents the current state of the art in livestock gene targeting, and is summarized below (Kuroiwa *et al.*, 2004). Starting with a primary somatic fibroblast cell line, sequential targeting of the first and then second *IGHM* allele was achieved by rejuvenation of a selected cell line by re-deriving cell lines from cloned d45 fetuses after each selection step. In addition, healthy calves were produced from the rejuvenated *IGHM* +/- and -/- cell lines at efficiencies of 6 and 8% (calves per implanted recipient), demonstrating that somatic cells can undergo several rounds of gene targeting and NT and are still able to support development of live calves. Applying Cre-mediated recombination, which is widely used in mouse ES cells, the two different antibiotic selection markers inserted at the targeted alleles of the *IGHM* -/- cells were excised in preparation for targeting the *PRNP* locus. After rejuvenation of the markerless homozygous *IGHM* knockout cells, sequential disruption of both *PRNP* alleles was achieved, essentially by repeating the targeting scheme for *IGHM*. Astonishingly, this complex modification generating a Cre-modified, double homozygous knockout cell line took only 12 and a half months. The *in vitro* developmental potential of the cloned bovine embryos appeared to be unaffected by the 5 steps of sequential selection and rejuvenation of the somatic donor cells. Although development to

term was compatible with at least 2 such steps, as demonstrated with the production of healthy calves, it remains to be confirmed that the development of live double homozygous knockout calves is not compromised from the prolonged time in culture and multiple rounds of cloning as this may increase the risk of accumulating genetic and/or epigenetic abnormalities in these donor cells (Oback and Wells, 2002).

THE METHOD OF CHOICE FOR TRANSGENIC LIVESTOCK PRODUCTION

As discussed above, there are a number of different techniques to choose from, but all of them come with their share of disadvantages. MI and SMT *per se* generate no adverse effects for the welfare of the transgenic animals themselves, but are plagued by low efficiency and a lack of consistency, respectively. Furthermore, a major issue with these methods is the generation of mosaic animals, which is especially problematic in livestock species that are mono-ovulatory and have a long generation interval, like cattle. While the development of lentiviral vectors for livestock transgenesis appears to have solved the efficiency problem, this technology generates complex insertion events. Although this has been discussed previously in a positive context, whereby a single founder animal could segregate several transgenic lines during breeding, further increasing the efficiency in the production of transgenic animals (Whitelaw, 2004), in our opinion, the generation of a well characterized transgenic line with a consistent and heritable phenotype is absolutely desirable, or even a requirement, for applications outside the research realm. Given the relatively long generation times in livestock species, the additional breeding steps required for a transgenic founder with a complex insertion event might be prohibitive for any commercial applications. In addition, the use of viral vectors comes with an additional, less defined burden. Although the viruses that are used for the production of transgenic animals are replication deficient, the technology is associated with the potential risk of generating a functional or a new pathogenic agent as a consequence of *in vivo* recombination events with endogenous viral sequences. There is also the concern about its public perception. Genetic modification in animals is already perceived as a 'risky' technology. There is the concern that the use of these defective viruses is an additional risk factor that might swing public perception towards an even lower acceptability of the technology. No doubt, most of the progress with viral vector systems will be made in the applications for human gene therapy (Pfeifer and Verma, 2001; Pages and Bru, 2004). The experience, achievements, discussions, and acceptability of gene therapy, which intervenes to correct a molecular fault in somatic cells and thus has the potential of a radically new therapeutic weapon, will have a major influence on the ultimate acceptability of viral vectors in the generation of transgenic livestock and, most likely, transgenic technology in general.

Similar to the additional ethical burden of viral vectors discussed before, NT is associated with animal welfare concerns due to decreased survival of pregnancies and post-natal calves. The major cause can be attributed to an incorrect epigenetic reprogramming of the donor cell genome. Novel strategies, including the use of cell extracts to support nuclear reprogramming prior to NT (Sullivan *et al.*, 2004), or the use of embryonic or adult stem cells as nuclear donors, which retain an epigenotype more compatible with early embryonic development and therefore require less

extensive reprogramming (Wells *et al.*, 2003b), are likely to improve cloning success significantly, thus lessening the animal welfare concerns associated with NT technology. On the positive side, NT allows the genotype and sex of transgenic founder animals to be pre-determined, and provides almost unlimited access to elite genetics. In contrast to lentiviral vector transgenesis, which, at least in cattle, relies on the limited number of oocytes that can be recovered from elite cows, NT makes use of an essentially unlimited supply of desired genetics in the form of cells derived from an elite fetus or adult. Elite oocytes are not required as their nuclear genetic contribution is removed during the NT process and thus, oocytes can be sourced in bulk from a commercial slaughterhouse. It is a distinct advantage of cell-mediated transgenesis to accommodate complex and precise modifications with the ability to fully characterize the introduced genetic modifications before a transgenic animal is produced. While some of these attributes might not be required for biopharmaceutical applications, it seems likely that the acceptability of the more agricultural applications will depend on introducing and precisely controlling defined genetic modifications. In summary, there is no single dominating strategy and the method of choice has to be tailored to the species specific factors involved and the needs and requirements for a particular application of transgenic livestock animals.

The potential consequences on the welfare of transgenic animals depend upon the specific genetic modification and its effect in either a hemi- or homozygous state. These potential effects form a continuum from poor animal welfare (for example, inappropriate over-expression of growth hormone, Pursel *et al.*, 1989; or over-expression of human erythropoietin in rabbits, Massoud *et al.*, 1996) to neutral (e.g. increased expression of bovine milk proteins in cattle, Brophy *et al.*, 2003) to improved welfare (e.g. pest and disease resistance, Kerr *et al.*, 2001). The consequences on the animal are also affected by the site of integration of the transgene, the degree of control over its expression (correct time, tissue, quantity), exposure of the host animal to biologically active transgene-derived proteins, and the *in vitro* embryo technologies used to produce the transgenic animals. A greater understanding of the regulation of gene expression will improve the predictability of the physiological consequences on the animal. Despite tight regulation, foreign genes expressed in the mammary gland and secreted into milk may still leak into blood and enter the general circulation (Carver *et al.*, 1992). The consequences of this on the physiology and welfare of transgenic animals depends on the specific foreign protein, its biological activities, and the concentration in plasma.

Altering milk composition by transgenic means

Dairy cattle are, and have been for centuries, the subject of intense selection for a complex set of attributes. With the modern animal breeding schemes in the dairy industry, this phenotypic selection, based on the performance of daughters of progeny tested bulls, results in a constant though relatively small annual genetic gain (in New Zealand, presently around 1% for milk solids yield; Lopez-Villalobos *et al.*, 2002). The random nature of this process, the association of phenotypic traits under selection with complex, multigene loci, and the typical trade-off between desirable traits and the simultaneous selection of less desirable traits without the ability to combine the total pool of superior allelic gene variants, limits the natural

selection process to incremental steps. Nevertheless, this is a proven and successful approach that guarantees genetic improvements, of which the value to national dairy industries cannot be underestimated. Now, with the advent of gene and transgenic animal technology, there is the prospect of making precise alterations to the genome of animals, with the potential to directly improve specific traits.

Given the importance of milk and dairy products as a global food source, transgenic strategies for the improvement of milk composition, which is otherwise relatively refractory to change by conventional means, were intensively discussed more than a decade ago (Jimenez-Flores and Richardson, 1988; Wilmut *et al.*, 1990; Boland *et al.*, 1992). Until recently, however, these concepts have been validated only in feasibility studies using mouse models. These studies could demonstrate that it is possible to over-express the major milk proteins from livestock species (Wall *et al.*, 1997) and that transgene-derived caseins were associated with the mouse casein micelles (Persuy *et al.*, 1995; Gutierrez-Adan *et al.*, 1996; Hitchin *et al.*, 1996; Hiripi *et al.*, 2000).

Furthermore, the demonstration of increased antimicrobial properties in the milk of mice through the introduction of either a biologically active form of lysostaphin (Kerr *et al.*, 2001), lysozyme (Maga *et al.*, 1998), or lactoferrin (Seyfert *et al.*, 1996) illustrates the potential of preventing mastitis in dairy cattle or providing passive immunity for people who consume the milk as a nutraceutical. Moreover, different strategies have been tested to reduce the milk sugar lactose, which causes intestinal disorders in more than 70% of the adult human population as a consequence of insufficient lactose digestion after the consumption of milk (Sahi, 1994). Lactose, which is the major osmotic regulator of milk secretion, is synthesized by the lactose synthetase complex. This complex comprises two enzymes, one of which is the milk protein α-lactalbumin. The disruption of the α-lactalbumin gene in mice resulted in lactose-free milk (Stinnakre *et al.*, 1994). The prevention of lactose synthesis, however, strongly affected the osmotic regulation of milk, resulting in the production of a highly viscous secretion, which was unable to support the adequate nutrition of the suckling young. An alternative approach of expressing a lactose hydrolysing enzyme in the mammary gland under the control of the α-lactalbumin promoter was more successful as it dissected the issues of osmolarity and lactose levels (Jost *et al.*, 1999). Here, milk lactose was reduced by 50–85%, without an apparent change in the osmolarity. In contrast to the knockout approach, lactose was still produced and subsequently hydrolysed into the osmotically active monosaccharides glucose and galactose, compensating for the absence of lactose. The results of these transgenic mouse studies are exciting and promising, but given the intrinsic differences in milk composition between mice and the major target species for these applications, bovine, the success of such a transgenic approach can only be assessed when applied to dairy cattle. Therefore, it will be crucial to progress from basic proof-of-principle studies in mice to studies using the relevant dairy species. Only then will we be able to evaluate the consequences of a particular genetic modification, not only for alterations of milk composition but, more importantly, for the functional properties of milk to determine the suitability for processing into dairy products. This is further exemplified by the ruminant specific whey protein, β-lactoglobulin. Here, a targeted gene disruption of both alleles of β-lactoglobulin could explore firstly the fundamental role of this protein in bovine

milk and secondly, whether such milk reduces allergenicity, as human milk does not contain this protein.

Considering the importance of milk protein for the quality and yield of dairy products, there is a strong interest by both farmers and milk processors to improve milk protein content. Cheese, for example, is essentially composed of casein, which accounts for about 80% of the total milk protein, and milk fat. Any increase in the casein content is therefore an improvement for the manufacture of cheese. Moreover, the casein fractions, comprising four different casein proteins, are aggregated into large colloidal micelles, which are a major determinant of the physicochemical properties of milk. One casein protein in particular, κ-casein, which occupies the surface of the micelle, influences the size of the micelle. This has been demonstrated in transgenic mice over-expressing bovine κ-casein, which resulted in a reduction of the size of the casein micelles. In addition, the milk from these transgenic mice has been shown to form a significantly stronger rennet-induced curd, clearly demonstrating that an increase in κ-casein affects the physicochemical properties of milk (Gutierrez-Adan *et al.*, 1996). Encouraged by these transgenic mouse studies, we have applied these findings to cattle. Using a transgenic approach, we have attempted to increase not only the casein content but also to reduce the size of the casein micelles in order to improve cheese yield. The introduction of additional copies of bovine β- and κ-casein genes into cloned dairy heifers resulted in a significant increase in both total protein and total casein, but most strikingly in a twofold increase of κ-casein in milk derived by hormonal induction from the transgenic heifers (Brophy *et al.*, 2003). Based on the mouse experiments described above, we predict that the observed increase in κ-casein reduces the micelle size, which in turn should increase the cheese yield, as smaller micelles can produce a tighter curd able to trap more milk solids. It remains to be determined to what extent the increase in κ-casein particularly affects micelle size and processing properties for cheese manufacture. A first indication that the physicochemical properties have been changed is a marked colour difference that we have observed in the milk of transgenic cows compared to wild-type controls during their first natural lactation.

For the production of these 'caseinplus' animals, we co-transfected the bovine gene for β-casein (CSN2) and a chimeric κ-casein gene (CSN2/3), combining the regulatory sequences of the β-casein locus with the sequences encoding the mature κ-casein for higher expression levels, into female primary bovine fetal fibroblasts (BFF). After the isolation of clonal cell lines with stable insertions of both casein transgenes, we selected four cell lines denoted TG2, TG3, TG5, and TG7, based on low to medium range numbers of transgene copies and a diploid chromosome constitution, for NT to produce founder transgenic females. Because of the low efficiencies of current NT procedures, we decided to evaluate two cell cycle stages (presumptive G0 and G1) of the donor cell to find conditions with improved NT efficiency for the development of live calves at weaning. Whereas NT with serum-starved (presumptive G0), unmodified BFF donor cells resulted in 20% of transferred embryos, resulting in viable calves (7/35), the efficiencies dropped to 3–5%, with the clonal transgenic cell lines TG2, TG5, and TG7, also using serum-starved cells (Brophy *et al.*, 2003). In an independent NT experiment using TG3 donor cells, with two replicates performed 12 weeks apart, the efficiencies for G0 donor cells were similarly disappointing at 0 and 10%, respectively, with an overall efficiency of 5%

(1/22, *Table 7.2*). Surprisingly, the efficiency was at least one order of magnitude greater when we used TG3 donor cells in G1, with 44% and 20% of the embryos yielding viable calves for each of the two replicates, and an overall efficiency of 29% (7/24). Thus, from the 8 transgenic founders generated with the TG3 cell line, 7 were derived from G1 donor cells (29%) and only a single one was the result of NT with a G0 cell (5%). Interestingly, in our experience to date, this is the only occasion where donor cells in G1 resulted in a higher cloning efficiency than serum-starved cells; with unmodified cells, the reverse relationship exists. For instance, with the BFF parental cells, the use of G0 cells for NT resulted in 20% efficiency (7/35) and showed a significant drop in efficiency to 11% (15/157) when G1 cells were used instead (Wells *et al.*, 2003a). This significantly increased cloning efficiency with serum-starved G0 cells compared to G1 cells has consistently been observed with all other unmodified primary donor cell lines that we have evaluated so far (Tucker *et al.*, 2004). Whereas non-modified primary cell lines represent a heterogeneous mixture of cells, specific transgenic donor cells are all descendants from a single cell clone which have undergone a selection step with extensive propagation *in vitro*. Due to this extended time in culture, transgenic cells are much more prone to acquire damage to the genome. By picking proliferating transgenic G1 cells, we may have avoided damaged cells, which would be masked by the serum starvation treatment used to generate G0 cells, and unknowingly selected viable cells. Presently, our results are based solely upon the single transgenic cell line, TG3. To support our assumptions on the intriguing beneficial effect of using donor cells in G1, and to exclude that this is a cell line specific effect due to characteristic mutations, additional transgenic cell lines need to be investigated.

Whilst we could demonstrate transgene expression in all four transgenic lines, high expression equivalent to the endogenous casein levels was only observed in cattle lines, TG2 and TG3. Furthermore, the observed expression was not correlated to the number of transgene copies, indicative of locus-dependent and copy number-independent expression.

To assess the effect of introducing additional casein genes on milk composition, we analysed milk produced after the hormonal induction into lactation in 7- to 9-month-old heifers. Consistent with this genetic modification, the mineral, milk fat, and lactose levels were unaffected, whereas total milk protein was increased by 13%, indicating that, unlike the mouse mammary gland, there might be no set physiological limit to milk protein synthesis and secretion in cattle. With no apparent alterations to the whey protein, β-lactoglobulin, and the major casein, αS1, the observed increase in total protein appears to be exclusively the result of higher κ-casein (twofold) and β-casein (up to 20%) levels due to their increased gene dosage. Thus, the alterations we have observed are consistent with the initial aims of improving milk composition for cheese manufacture. Presently, we are producing milk from natural, calf-induced lactation to gain access to larger quantities to evaluate the implications of these alterations on processibility. Assuming that increased casein and a smaller micelle size translates to the expected gain in cheese yield, this milk will be of value only if the resulting cheese is of equal or superior quality. Any improvements in cheese production also have to outweigh any potential adverse effects limiting the usage of the 'caseinplus' milk for manufacture of other dairy products. This will be only the first hurdle that needs to be cleared for

Table 7.2. Efficiency of transgenic cattle production depending on the cell cycle stage of the donor cell

NT run	Donor cell cycle stage	Live calves at term	Live calves at weaning	Live calves at 2 years
1	G0	10% (1/10)	10% (1/10)	10% (1/10)
	G1	44% (4/9)	44% (4/9)	44% (4/9)
2	G0	0% (0/12)	0% (0/12)	0% (0/12)
	G1	20% (3/15)	20% (3/15)	20% (3/15)
Average	G0	5% (1/22)	5% (1/22)	5% (1/22)
	G1	29% (7/24)	29% (7/24)	29% (7/24)

this milk to be of commercial value. Milk is a commodity product which is processed by high capacity facilities into a range of different products. Particular transgenic milk streams tailored for specific purposes might be unsuitable for general commodity milk products. A speciality milk like the 'caseinplus' milk has to provide either value to other common milk products, such as milk powders, or needs to offer enough value that justifies the extra cost in keeping the milk separate for a specialised product. For instance, the additional lysozyme with its antimicrobial effect (Maga *et al.*, 1998) not only altered the overall microbial level but also the casein micelle size and rennet clotting times (Maga *et al.*, 1995). Would this be beneficial for cheese production or would the added lysozyme interfere with the microbial processes involved in cheese and yogurt manufacture? One possible scenario for the future is the generation of herds possessing specific genetic modifications producing agricultural products for niche markets. In the dairy industry, transgenic milk from specific herds would need to be kept separate for manufacturing purposes, let alone for food labelling compliance. Such a prospect would pose challenges for the current structure of traditional commodity-based dairy industries processing bulk milk. The integration of transgenesis might necessitate regional herds producing milk of a similar type, with specific processing capability available locally. Only recently, a specialized milk product has been introduced to the New Zealand and Australian markets which is based on sourcing milk from selected cows only producing the β-casein variant protein A2. Conventional milk contains the β-casein variant proteins A1 and A2 in similar quantities. Although highly controversial, it is claimed that A2 milk is healthier than conventional milk due to a perceived risk associated with the A1 variant (McLachlan, 2001; Laugesen and Elliott, 2003). Nevertheless, it is an example of a milk stream that is segregated and marketed as a speciality product, and only time will tell if it has enough additional value or benefit, even if only perceived, to make it a commercial success.

Survival and health of cloned transgenic cattle

Based on our experience with cloned-transgenic cattle at AgResearch, a major issue relates to the survival of pregnancies and calves shortly after birth. These welfare issues impact negatively on the acceptability of this method to generate transgenic animals. However, the long-term survival and health of the founder transgenic cattle

and their progeny appears normal. From 379 cloned-transgenic embryos derived from one parental cell line of bovine fetal fibroblasts (BFF; representing 12 independent clonal transgenic strains) transferred into recipient cows at AgResearch, 51 calves have been delivered at term (representing 13% embryonic survival). This is exactly the same average efficiency as with non-genetically modified cell lines used for NT in our hands (133/988; Wells *et al.*, 2004). The majority of cloned calves are delivered *per vaginum* with a corticosteroid therapy used to aid fetal maturation and induce parturition around one week before expected full term (Wells *et al.*, 2004). This has the associated benefit of an improved maternal response from the recipient cow towards rearing the offspring. Nevertheless, there is a high rate of mortality in the post-natal period. One day after calving, 63% of the cloned-transgenic calves delivered at term remained alive. The most common causes of mortality for the 19 calves that died during this period include spinal fracture and retroperitoneal haemorrhage during or following delivery (47%), calves that either died *in utero* or during delivery from dystocia (32%), and those that were euthanized at birth due to musculoskeletal deformity (16%). Of the 32 calves alive one day after birth, 78% of these survived to weaning at around 3 months of age. The mortality factors during this period were variable, but included cases of infection (43%) and gastroenteritis (29%). Overall, at weaning, only 49% of the cloned-transgenic calves that were delivered at term were still alive. This is somewhat less than what we have experienced with somatic cell cloned cattle derived from non-transgenic cell lines, where 67% survived to 3 months of age (Wells *et al.*, 2004); but it is within the range of 47–80% reported as the proportion of cloned cattle that are long-term survivors (Lanza *et al.*, 2001; Heyman *et al.*, 2002; Pace *et al.*, 2002). In our experience with somatic cell cloned cattle, we observe an annual mortality rate following weaning of at least 8% (Wells *et al.*, 2004). In contrast to this, there has been, so far, only a single case of mortality beyond weaning in the 25 surviving cloned-transgenic cattle that currently range in age between 18 months and 4 years. This transgenic cow, aged 3 years and 7 months, died due to mastitis that developed in her second natural lactation. While there is high pre-weaning mortality amongst the founder cloned-transgenic cattle (51%), all of the progeny born from 14 first-calving, cloned-transgenic heifers, following artificial insemination with a conventional bull, have survived to at least one year of age.

Those cloned-transgenic cattle that survive beyond weaning appear to be normal animals. This can be substantiated by the excellent post-weaning viability, normal growth rates and attainment of puberty, normal reproductive function in cloned-transgenic heifers, spontaneous parturition (requiring only conventional levels of husbandry, unlike the founder generation), and normal maternal behaviour towards bonding with their respective offspring. These observations are complemented by an assessment of the overall physiological health status determined by measuring multiple blood parameters at intervals throughout the life of the transgenic cattle. Routine haematology and serum biochemistry for indicators of muscle injury, liver function, serum protein levels, renal perfusion, and metabolic status have shown the transgenic animals to be within the normal ranges of conventional age-matched stock.

Regulatory issues

Research projects involving transgenic livestock animals involve strict controls about the identification and containment of the animals to ensure that neither the animals from species normally used for human consumption, nor any of their products, enter the food chain. In New Zealand, this is controlled by the Environmental Risk Management Authority (ERMA), and the compliance of the various controls monitored by the Ministry of Agriculture and Forestry (MAF). Our transgenic cattle are farmed on pasture like conventional dairy cows in New Zealand, but have to be held in a farm animal containment facility which is electrified, monitored, and surrounded by two, 2-metre high fences. The transgenic cattle, including recipients of transgenic embryos and non-transgenic siblings, generated through breeding from a transgenic cow, are not allowed to leave the facility. Although transgenic technology has made significant advances in recent years, as described above, and appears to be suitable for even demanding agricultural applications, transgenic animals will not be found outside the context of research projects any time soon, as the regulatory situation relating to the risk and safety assessment of the technology is in its infancy and is not making speedy progress. This might be best exemplified by the situation with just cloned livestock animals. Here, a recent report by the National Academy of Sciences in the USA considered cloned animals to be safe, but recommended further evaluation of the composition of food products from cloned animals and their progeny, to minimize any residual food safety concerns (National Research Council, 2002). Genetic modification will add another level of complexity. Every single transgenic line would have to be considered on a case-by-case basis, as the risk is ultimately associated with the particular genetic modification that has been introduced and the method used for its introduction. National bodies are waiting for signals from international organizations such as the FAO/WHO, who are still in the process of broad consultation and defining guidelines for controlling the safety of genetically modified food products (FAO/WHO, 2000, 2001a,b). Only once such guidelines are in place for implementation, and this appears still to be some time away, will transgenic animal technology have the opportunity to prove its promise.

Perspective

Classical animal breeding alters the frequency of many genes in an often unregulated manner. The new technologies of cloning from cultured cells and transgenesis with site-specific integration have the potential to allow a more controlled approach towards animal breeding. Major improvements are still required in these areas, especially improved reprogramming of the donor genome and an increased frequency of gene targeting in somatic cells. Concurrent with these advances, identification of genes and regulatory elements influencing livestock production traits will enable the effective utilization of cloning to duplicate entire genotypes, and for transgenesis to introduce precise genetic enhancements to progress animal breeding in the 21st century. The final completion of the sequencing of the *Bos taurus* genome, with the first draft having been released in October 2004, will be another significant milestone towards this goal.

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