OPPORTUNITIES FOR ANIMAL BREEDING BY CLONING LIVESTOCK FROM CULTURED CELLS USING NUCLEAR TRANSFER

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SUMMARY
It is remarkable that nuclear transfer using differentiated donor cells can produce physiologically normal cloned animals, but the process is inefficient and highly prone to epigenetic errors. Aberrant patterns of gene expression in clones contribute to the cumulative losses and abnormal phenotypes observed throughout development. Any long lasting effects from cloning, as revealed in some mouse studies, need to be comprehensively evaluated in cloned livestock. These issues raise animal welfare concerns that currently limit the acceptability and applicability of the technology. It is expected that improved reprogramming of the donor genome will increase cloning efficiencies realising a wide range of new agricultural and medical opportunities. Efficient cloning potentially enables rapid dissemination of elite genotypes from nucleus herds to commercial producers. Initial commercialisation will, however, focus on producing small numbers of high value animals for natural breeding, especially clones of progeny-tested sires. The continual advances in animal genomics towards the identification of genes that influence livestock production traits and human health increase the ability to genetically modify animals to enhance efficiency and produce superior quality food and biomedical products for niche markets. The potential opportunities for animal agriculture are more challenging because of the greater demands on biological efficiency, reduced cost, consumer acceptance and value of the product in contrast to biomedicine, which has been the main driver for this technology platform. Nevertheless, cloning and transgenesis are being used together to increase the genetic merit of livestock; however, the integration of this technology into farming systems remains some distance in the future.

Keywords: embryo, nuclear transfer, cloning, genetic modification, animal breeding

THE PRODUCTION OF CLONED ANIMALS BY NUCLEAR TRANSFER
Since the pioneering studies conducted by Wilmut and colleagues at the Roslin Institute, Scotland, it is now recognised that (at least some) somatic nuclei of adult animals are totipotent following an embryo manipulation procedure termed “nuclear transfer” (NT) (Wilmut et al. 1997). That is to say, an entire animal can be cloned from the nucleus of a single donor cell.

Donor cells. The cells used for NT may come from a variety of sources. Broadly, these may include early embryos or somatic tissues; hence the terms embryonic and somatic cell NT, respectively. Somatic cells may be obtained from a fetus or small tissue biopsy taken from a chosen adult. Whilst the ideal donor cell type has yet to be found (Oback and Wells 2002), cell cycle stage (Wells et al. 2003b), genotype (Eggan et al. 2001; Wakayama and Yanagimachi 2001) and the degree of cellular differentiation all affect cloning efficiency. Less differentiated embryonic blastomeres and embryonic stem (ES) cells (derived from the inner cell mass of blastocysts) are apparently more amenable to, or requiring less, reprogramming compared to fetal and adult cell types (Wakayama et al. 1999; Wakayama and Yanagimachi 2001; Heyman et al. 2002a). In contrast, terminally
differentiated cells appear to result in very low cloning efficiencies (Hochedlinger and Jaenisch 2002) possibly due to repressive chromatin structures that are difficult to reprogram (Kikyo and Wolff 2000) or DNA rearrangements that preclude nuclear totipotency (Yamazaki et al. 2001). In practice, common cell types used for NT include ill-defined dermal skin fibroblasts and ovarian follicular cells (for females) (Kato et al. 2000). Thus, the identification of suitable, well-characterised cell types for NT from selected animals is needed. In this regard, adult stem cell populations may be ideal candidates because of their inherently greater developmental plasticity (Blau et al. 2001).

Isolated cells may be cultured to establish a primary cell line and are easily cryopreserved enabling ready access to millions of cells. However, there may sometimes be genetic (Olifent et al. 2002) or epigenetic changes (Humpherys et al. 2001) especially in long-term cultured cells that influence clonability. Donor cells may also be genetically modified in vitro and used to produce cloned-transgenic animals (Schnieke et al. 1997). Thus, the core technique of NT can produce either clones or transgenics, depending upon the choice of donor cell.

The nuclear transfer cloning procedure. Although there are many variations in protocols and timing of events for particular species, the general approach for NT typical of cattle in our laboratory using a “zona-free” methodology (Oback et al. 2003), comprising a sequence of seven main steps, is outlined below. Zona-free cloning methods (Peura et al. 1998; Booth et al. 2001; Vajta et al. 2001) have simplified the manipulation procedure, require less technical skill, enabling standardisation between operators and increased laboratory throughput.

1. The zona pellucida is removed from mature unfertilised oocytes arrested at the metaphase II stage of meiosis by pronase digestion. These recipient oocytes may be obtained either a few hours following ovulation in the female animal or, more commonly, after in vitro maturation of oocyte-cumulus cell complexes. These may be recovered either from the ovarian follicles of slaughtered culled cows or selected females following ovum pick-up.

2. The metaphase II chromosomes from the zona-free oocytes are aspirated with finely controlled micro-surgical instruments in a process termed enucleation, to produce cytoplasts.

3. Individual donor cells are then each adhered to a single cytoplast using standard embryological mouth pipetting techniques to push the two cells together in media containing 10 µg/ml phytohemagglutinin.

4. The cytoplast and donor cell couplets are then fused together using direct current electric field-pulses between two parallel electrodes. With the zona-free fusion method (Oback et al. 2003) a high proportion of the couplets align automatically in the fusion chamber when exposed first to an alternating current electric field prior to the direct current fusion pulses. This enables fusion to be performed en masse and is a distinct advantage compared to the conventional zona-intact cloning methods (Wells et al. 1999) where individual manual alignment is necessary. Following successful cell fusion, the chromatin contained within the nucleus of the donor cell is exposed to the various oocyte cytoplasmic factors that provide the opportunity for reprogramming (see below).

5. Reconstructed one-cell embryos are then artificially activated in order to initiate embryonic development (Machaty and Prather 1998). Activation regimes that mimic the repetitive intracellular calcium oscillations that occur during normal fertilisation result in significantly
improved development (Ozil and Huneau 2001).

6. The reconstructed embryos are then cultured in a chemically-defined medium, typically until the blastocyst-stage after seven days in vitro. Advances in bi-phasic embryo culture media that direct embryos towards a glycolytic metabolic pathway at the time of compaction (Thompson et al. 2000) have been especially beneficial for the in vitro development of cloned bovine embryos.

7. Suitable quality embryos are transferred singularly to the reproductive tracts of synchronised multi-parous recipient cows. Some cloned embryos may implant and develop to term for the eventual birth of cloned offspring.

CURRENT EFFICIENCY OF SOMATIC CELL NUCLEAR TRANSFER

The present NT methods are inefficient. Typically, 1-7% of reconstructed one-cell cloned embryos result in viable offspring in a range of species (Wilmut et al. 2002). AgResearch’s experience in cattle with the best methodology available to date shows that the proportion of reconstructed one-cell embryos that develop to transferable quality blastocysts from a variety of cell lines (40%) is comparable to in vitro produced embryos (IVP: i.e. in vitro matured, fertilised and cultured) with abattoir-derived oocytes. Furthermore, the rate of pregnancy establishment on Day 50 following the transfer of single NT embryos (50%) is similar to both artificial insemination (AI) and single IVP embryos. In contrast to fertilised embryos, however, 60% of the cloned bovine fetuses are subsequently lost throughout the remainder of gestation. Moreover, peri-natal and post-natal mortality rates with cloned offspring are greater than normally expected. Ultimately, only 17% of the cloned embryos transferred into recipient cows result in viable calves at weaning. This compares to 45% embryo survival with IVP. These losses raise serious animal welfare issues that currently limit the utility and acceptability of the technology.

REPROGRAMMING DONOR CELLS

There are many factors which affect the success of NT. One of the most critical aspects is reprogramming gene expression in the genome of a differentiated cell. For normal development, the organisation of the chromatin and the resulting pattern of gene expression in a specialised somatic cell must be completely reset to a zygotic state to enable embryonic genes to be reactivated in the correct tissues, in the correct abundance and at the correct times. Clearly this is a highly orchestrated process and is understood poorly. There is, however, increasing evidence of epigenetic errors in reprogramming following NT leading to abnormal patterns of gene expression (Rideout et al. 2001; Fairburn et al. 2002) and these are considered the major cause of the developmental failures. Moreover, sub-lethal aberrations that occur early in embryo or fetal development may impair health in adulthood. Understanding the molecular mechanisms involved in reprogramming will ultimately improve cloning efficiencies.

HEALTH OF CLONED PREGNANCIES AND ANIMALS

Complete reprogramming is apparently rare, with the majority of cloned embryos failing at various stages of development. The main consequence of faulty reprogramming is a failure of the placental membranes to develop and function normally. In cattle, the number of placentomes is approximately halved compared to normal with compensatory overgrowth (Hill et al. 2000). Of concern is hydroallantois where 25% of established cloned bovine pregnancies may succumb to this syndrome.
The volume of allantoic fluid may be four times normal, necessitating elective abortion in mid-gestation to minimise distress to the recipient. Research aims to detect hydroallantois earlier to lessen the welfare burden and ultimately, to prevent the syndrome through improved reprogramming.

Recipients pregnant with clones generally show poor preparation for parturition and prolonged gestation, with an increased risk of dystocia from heavier birthweight offspring often prompting elective caesarean section (Wells et al. 1997, 1999). However, corticosteroid therapy to induce parturition one week before expected full term has successfully aided fetal maturation, (assisted) vaginal delivery and improved the maternal response towards rearing offspring (Wells et al. 2003b).

Post-natal mortality is also greater with clones, especially in cattle and sheep but less so in pigs and goats (Wilmut et al. 2002). The stage of the donor cell cycle at the time of NT has a significant effect, with a higher proportion of calves at term, derived from quiescent G0 donor cells, surviving to weaning (76%) compared to clones derived from G1 cells (57%) (Wells et al. unpublished). Newborn clones have an altered metabolism, possibly due to the in utero placental abnormalities (Garry et al. 1996), and require time to adjust to a normal physiology (Chavatte-Palmer et al. 2002). Most deaths are due to either abnormalities of the cardiovascular system, the skeletal system, brain, or kidney, along with umbilical and lung infections and digestive disorders (Wilmut et al. 2002).

Although there are reports of physiologically normal cloned animals (Lanza et al. 2001; Renard et al. 2002) displaying normal behaviour, growth rates, reproduction, livestock production characteristics and lifespans (Wells 2003), other reports indicate long-term health concerns. These have included obesity (Tamashiro et al. 2002) and shortened lifespan (Ogonuki et al. 2002) in mice and compromised immune systems in cattle (Renard et al. 1999). This emphasises the need for detailed long-term scientific studies on cloned animals. The incidence of these clone-associated phenotypes varies according to the particular species, genotype, sex, cell type or specific aspects of the NT and culture protocols used. In cattle, the proportion of cloned calves born that are longterm survivors ranges between 47-80% (Lanza et al. 2001; Heyman et al. 2002b; Pace et al. 2002). The cloned offspring syndrome is a continuum, in that lethality or abnormal phenotypes may occur at any phase of development depending upon the degree of dysregulation of key genes. Even apparently normal clones may have abnormal regulation of many genes that are too subtle to result in an obvious phenotype (Humpherys et al. 2002).

**TRANS-GENERATIONAL EFFECTS**

Although there are problems in the cloned generation stemming from incomplete reprogramming, the offspring of surviving clones produced following sexual reproduction appear completely normal, even when male and female clones have been mated together (Tamashiro et al. 2002; Wells 2003). This suggests that any epigenetic errors in the clones are corrected during gametogenesis providing initial confidence in those applications of cloning technology that capture the potential of breeding from genetically elite clones. However, additional studies need to exclude the possibility of transmission of differential recessive genetic or epigenetic traits between the two cloned parents. Moreover, detailed molecular studies should determine whether the DNA methylation patterns in gametes, zygotes and embryos are indeed restored to normal.
GENETIC AND PHENOTYPIC IDENTITY OF CLONES
Unlike monozygotic twins, NT-derived animals are not strictly “true clones” and there is the expectation of greater phenotypic differences amongst members of a clonal family (a set of NT clones derived from the same source of donor cells). Nuclear transfer clones might for instance possess: different mitochondrial (mt) DNA derived from the recipient oocyte (if obtained from different maternal lineages) and moreover, mtDNA heteroplasmy with a small contribution from the fused donor cell (Steinborn et al. 2002); possible point mutations or other chromosomal rearrangements in the genomic DNA of individual donor cells; alternative patterns of X-chromosome inactivation in females; various other epigenetic alterations in the patterns of gene expression arising from in vitro culture (of the donor cells or embryos) or perturbations from the NT process; and various environmental influences from the oocyte cytoplasm, maternal uterus in the surrogate female and during the post-natal period. All these factors contribute to potential variations in phenotype (and genotype also in some cases) within a clonal family and deviations from the original founder animal. In practice, however, initial observations from one small set of cloned dairy cows revealed great similarity in milk composition compared to the original donor cow (Wells et al. 2003a).

SAFETY OF FOOD PRODUCTS DERIVED FROM CLONES
A number of international food regulatory agencies are presently addressing issues surrounding the safety of food products derived from clones and their offspring. Although subtle epigenetic errors in surviving clones will contribute to phenotypic variability, it is difficult to foresee that milk or meat from cloned livestock would be outside the normal range of food products consumed by humans. Scientific data is extremely limited at present and needs to be gathered; however, initial results indicate that the composition of milk from cloned dairy cows is within the broad range of milk produced from conventional cows (Wells et al. 2003a).

APPLICATIONS OF CLONING TECHNOLOGY
A wide variety of potential applications of NT technology exist, including:
1. Increasing genetic gain in animal breeding schemes.
2. Dissemination of genetic gain.
4. Animal research models.
5. Production of genetically modified livestock.

If the ethical costs associated with producing the few surviving healthy clones can be justified, some commercial and research applications are possible now. Other opportunities, however, will not be feasible nor tolerated until complete reprogramming results in an efficient and acceptable animal cloning technology with pregnancy and neo-natal survival rates comparable to normal breeding.

1. Increasing genetic gain in animal breeding schemes. A common consensus has been that cloning will have only a marginal effect on the rate of genetic gain, however, if incorporated properly into a breeding scheme it may in fact improve rates of genetic progress without any increase in the rate of inbreeding (Woolliams and Wilmut 1999). Effective breeding programmes require the accurate identification of superior livestock in the population before their subsequent multiplication.
using various assisted reproductive technologies, including cloning. Marker-assisted selection strategies that allow for the identification of favourable genes that correlate with production, will aid in selecting desirable genotypes. However, actual performance may remain uncertain unless markers have exceptional predictive value for polygenic quantitative traits.

Nuclear transfer could be used to directly determine the phenotype of different lines of cloned animals in a variety of environmental conditions and thus, enhance genetic progress by increasing the accuracy of selection and more easily identify genotype x environmental interactions. The evaluation of clonal families requires fewer clones per family compared to the number of offspring per parent in progeny-testing schemes to give the same accuracy of selection. This is because the clones in a family are all of the same genotype and will average out the environmental influences (Woolliams and Wilmut 1999). Villanueva et al. (1998) have calculated that the desirable number of clones to evaluate is 35 per family; however, this depends upon the trait with those of lower heritability benefiting from additional accuracy and hence, larger family sizes. At a fixed number of animals evaluated in a breeding scheme (clones or progeny), clonal testing possibly enables greater selection pressure to be exerted by measuring more cloned families or genotypes (McClintock 1998). Another possibility is the use of lines of cloned dams in sire-proving schemes, to reduce the cost and increase the accuracy of selecting elite males for the AI industry.

The rate of genetic gain would be further enhanced by evaluating clones produced from embryonic cell lines rather than clones of adults to avoid the delay in (one or more) generation intervals. These cell lines could be derived from embryos previously screened as superior by marker assisted selection following matings within nucleus breeding herds. With beef animals, for example, lines of cloned cattle could be generated and specific meat quality characteristics directly measured by slaughtering some clones within each line. In those clonal lines that perform favourably, the remaining cloned animals could be used for breeding. In addition, other clones could be readily produced by thawing the appropriate frozen cells and using NT to release a larger number of the desirable animals to the industry. An extension of this is to identify carcasses with superior meat characteristics shortly after slaughter and to clone animals from recovered cells either for breeding or commercial meat production and so, rescuing these valuable genetics. This has been exemplified by the resurrection of a steer following post-slaughter meat assessment to generate a set of cloned bulls for breeding (Wells 2003).

2. Dissemination of genetic gain. Efficient cloning could enable the rapid and widespread dissemination of superior genotypes from nucleus breeding flocks and herds, directly to commercial producers. Genotypes could be provided that are ideally suited for specific product characteristics or environmental conditions. These genotypes could be disseminated by the controlled release of selected lines of elite live animals for breeding or on a larger scale by the transfer of frozen/thawed cloned embryos. The embryo costs need to be relatively low, although a premium should be expected for high value known genetics of proven performance compared with semen from a progeny-tested sire which only provides half the genes and an unknown performance in the individual offspring. The marketing of cloned embryos would be an alternative to AI but needs to be as equally successful, with the infrastructure and technical expertise required for extensive embryo transfer on farms, and cost-effective to be adopted. It is noteworthy that even less complex
reproductive technologies such as IVP are difficult to implement in low-cost pastoral agriculture systems and require excellent husbandry and management practices to succeed (Smeaton et al. 2003). However, if the cloned genotypes generate either novel or value-added products for which farmers receive a premium then there may be greater economic incentive for technology adoption.

By disseminating the best available clones from nucleus herds directly to producers, the resulting commercial herds would ironically have a greater average genetic merit than the average for the nucleus itself (Woolliams and Wilmut 1999). This strategy would change some industry breeding structures as intermediate multiplier herds/flocks would no longer be required, with profound consequences on those sectors within the farming community (Nicholas 1996). It has been estimated that the implementation of cloning strategies into breeding schemes could result in large increases in genetic gain, up to 15 times the annual genetic response compared to more conventional schemes (Baker et al. 1990). It is important to note that for individual herd owners this represents a substantial one-off genetic lift until the next outstanding individuals are identified for cloning. However, for an animal industry as a whole, it is difficult to foresee that even when biological efficiencies become optimal that it would be either logistically possible or wise to disseminate clones extensively throughout an animal industry in a short time-frame. More likely, smaller herds will be generated by disseminating clones perhaps on a rotational basis to fulfil needs in specialised markets.

The prospect of widespread cloning is often criticised for its potential to decrease genetic diversity. This is in fact true for all forms of assisted reproductive technologies and has been the challenge presented to quantitative geneticists to develop models avoiding unacceptability high levels of inbreeding (Nicholas 1996). Rigorous testing of the genotype before widespread dissemination gives a full appreciation of the benefits and limitations of each clonal family. This knowledge in itself will lessen the risks from any potential epidemics or adverse health effects in the specific environments; for example, susceptibility to lameness in dairy cattle, or susceptibility to regional diseases such as facial excema, ryegrass staggers, etc. As no single genotype will prove superior in all attributes of productivity, health and management for any particular farming system or regional environment, farmers are more likely to select various different lines of cloned embryos to maintain some genetic diversity in their herds. Moreover, different age groups within the herds will likely be of different genotypes further increasing genetic variation on the farm or in the region providing a buffer to counter any potential disease epidemic. Genetic epidemiological models now exist for animal populations (Mackenzie and Bishop 1999) to enable assessment of the risks and develop guidelines concerning the scales of dissemination of single genotypes within individual herds or regions.

With the identification of unique genotypes that provide an opportunity to generate new agricultural products, perhaps meeting specific or changing market requirements, cloning potentially allows for their rapid multiplication to generate large flocks or herds enabling an economic volume to be produced. Despite being genetically identical, however, variability in livestock production traits within a herd of clones will persist, depending upon the broad heritability of the trait in question. In addition to environmental and epigenetic influences on phenotype, more subtle effects on some production characteristics may arise if clones possess different mtDNA compositions (Schutz et al. 1994; Mannen et al. 1998) where cytoplasts are obtained from different maternal lineages.
Given the current (in)efficiencies of NT, a niche opportunity presently exists in the production of small numbers of cloned animals with superior genetics for breeding. Ideally, these would be cloned sires from progeny-tested males for widespread dissemination of their elite genetics following natural breeding or alternatively, increased semen production for AI. If cloned sires are faithful genomic copies of the original donor, this application avoids confounding issues with the transmission of mtDNA (which is only maternally inherited; Sutovsky et al. 2000) and phenotypic differences arising from environmental influences as they only need to transmit haploid copies of the donor’s genome in the form of sperm. Importantly, initial results suggest that any subtle epigenetic errors in the clones are corrected via gametogenesis with resulting offspring being apparently normal. This opportunity in the dairy cattle industry is being currently developed through AgResearch’s involvement with Clone International. However, it remains to be demonstrated that the daughters of cloned dairy sires perform similarly to contemporary progeny of the original bull. In sheep and beef industries, widespread natural mating with cloned sires could substitute for AI, where it is often expensive and inconvenient to disseminate superior genes in these more extensive farming systems.

Cloning could be extremely useful in multiplying outstanding F1 crossbred animals, or composite breeds with otherwise complicated and expensive breeding strategies, to maximise the benefits of both heterosis and greater uniformity within the clonal family. If specific heterozygotes at particular loci were identified as being beneficial it would be possible to disseminate these genotypes reproducibly to commercial producers, without segregation (Woolliams and Wilmut 1999).

3. Conservation of endangered livestock breeds. Cloning can be integrated into assisted reproductive strategies to conserve rare farm animal genetic resources that should not be lost from the global gene pool (Wells et al. 1998). This is a very significant application of cloning technology as most of the genetic variation in a livestock species resides in the various different breeds (Woolliams and Wilmut 1999). Thus, the demise of indigenous or traditional breeds represents a very significant loss of biodiversity and limits any future opportunities to capture as yet unappreciated traits. More important than cloning per se, is the cryo-preservation of somatic cells from rare breeds of livestock. The cryo-banking of this genetic material would provide an insurance policy against further losses of diversity or possible extinction and would be easier than preserving gametes and embryos. Nuclear transfer could then be used to produce a clone of a deceased animal using a previously cryo-preserved cell and thus, re-introduce its genetics back into the live breeding population. Even for conventional agriculture, it might be prudent to cryopreserve cells from genetically elite animals in case of accidental death or disease.

4. Animal research models. Naturally occurring sets of genetically identical twin livestock have been well utilised in animal experimentation (e.g. Auldist et al. 1998). Larger sets of NT-derived clones are now being utilised to reduce genetic variation and allow more stringent analysis of treatment effects on the same genotype in large animal research. This is exemplified in studies aimed at identifying genes and pathways that regulate mucosal immunity (Hein and Griebel 2003).

5. Production of genetically modified livestock. Progress in farm animals has been hindered by 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 and compromised animal welfare (Van Reenen et al. 2001). 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. This will come from advances in animal genomics (Darvasi 2003), genome reprogramming (see above) and gene targeting in somatic cells (Piedrahita et al. 1999).

Nuclear transfer is but one of several of methods available to produce transgenic animals. This approach, however, has a number of distinct advantages, including: (1) the ability to introduce specific genetic enhancements to an existing genetically superior background using cells from an animal of chosen performance and sex (especially important for agricultural applications); (2) more efficient production of transgenic offspring (even with current NT methods); (3) the potential for a more extensive range of genetic modifications to the cells cultured in vitro; and (4) none of the transgenic animals should be germline mosaics. Although it is still commonplace for introduced genes (either from the same species or a different species) to be integrated at a random location within the genome, methods of site-specific recombination enabling "gene targeting" in somatic cells of livestock (Piedrahita et al. 1999) have been successfully used at some loci (McCreath et al. 2000).

Gene targeting also allows for either the functional deletion of an undesirable gene on an otherwise favourable genetic background, or to precisely alter specific base pairs in a particular gene to improve a particular function in the resulting protein. The combination of NT and gene targeting have the potential to be far more precise, extensive and rapid in terms of genetic progress than what can be achieved with traditional breeding and other available transgenic methods, including conventional pronuclear injection of DNA into zygotes. Obstacles that remain, however, include the very low frequency of successful gene targeting events in primary cultures of somatic cells (compared to mouse ES cells) and the need to avoid the use of antibiotic resistance marker genes, commonly used to aid identification of transgenic cells, to alleviate some societal concerns with the technology.

There are a wide variety of applications for transgenic livestock in both agriculture and biomedicine, depending upon the particular genes that are manipulated. Described below is a small sample of the areas of interest; many projects were initiated with the pronuclear injection methodology with the option to now convert to NT where appropriate. 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 understanding of the genes that influence livestock production traits improves, so does the knowledge to accurately modify 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.

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). 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).

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. The introduction of additional copies of bovine β- and κ-casein genes into cloned dairy heifers resulted in a 30% increase in casein protein in milk within one generation (Brophy et al. 2003). It remains to be determined how the increase in κ-casein particularly affects micelle size and processing properties for cheese manufacture. 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.

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 utilise 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 utilisation 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).

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. 1997) or
lactoferrin (Seyfert et al. 1996) illustrates the potential of preventing mastitis in dairy cattle (see www.ars.usda.gov/is/pr/2001/010110.2.htm) or providing passive immunity for people who consume the milk as a nutraceutical. However, particular transgenic milk streams tailored for specific purposes might be unsuitable for general commodity milk products. For instance, 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 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.

The most efficient means of disseminating a desired genetic modification into the wider population will be through low cost AI (or natural mating) from males homozygous for the desired trait at a specific locus. Animal industries may choose to annually introduce the same transgene on a new genetic background using cell lines derived from the most recently selected progeny-tested sires so as to capture the annual incremental genetic gains from conventional animal breeding. 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 in a variety of genotypes (Siewerdt et al. 1999) before wider dissemination.

Biomedical applications. Although the emphasis of this paper is on agricultural applications, it is important to briefly consider the contribution of cloned-transgenic farm animals to future human healthcare. 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 (Brink et al. 2000). 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 regulatory approval. Livestock are favoured were 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 synthesise 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, proteins produced in the egg whites of laying hens (Ivarie 2003) or human polyclonal antibodies produced in the blood of transgenic cattle (Robl et al. 2003).

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-galactosyltransferase (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).

6. Human cell-based therapies. The prospect of human therapeutic cloning (Gurdon and Colman 1999) whereby cloned blastocysts are produced as a source of autologous ES cells to generate histocompatible tissue for transplantation has been exemplified in the bovine (Lanza et al. 2002) and combined with gene therapy in mice (Rideout et al. 2002). Advances in the understanding of reprogramming will ultimately enable the transdifferentiation of cells in vitro (Hakelien and Collas 2002) avoiding the ethical controversy surrounding human cloning and ES cells.

PERSPECTIVES

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 utilisation of cloning to duplicate entire genotypes and for transgenesis to introduce precise genetic enhancements to progress animal breeding in the 21st century.

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