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Cloning Livestock from Cultured Cells Creates New Opportunities for Agriculture

D.N. Wells

AgResearch Ltd., Ruakura Research Centre, PB 3123, Hamilton, New Zealand

ABSTRACT

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 towardsthe identification of genes that influence livestock production traits and human health increase the ability to genetically modify animals to enhance agricultural efficiency and produce superior quality food and biomedical products for niche markets. The potential opportunities inanimal agriculture are more challenging than those in biomedicine as they require greater biological efficiency at reduced cost to be economically viable and because of the more difficult consumer acceptance issues. 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 in 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) (1). That is to say, an entire animal can be cloned from the nucleus of a single donor cell.

Donor cells. The cells used for NTmay 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 (2), cell cycle stage (3), genotype (4, 5) 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 (5–7). In contrast, terminally differentiated cells appear to result in very low cloning efficiencies (8) possibly due to repressive chromatin structures that are difficult to reprogram (9) or DNA rearrangements that preclude nuclear totipotency (10). In practice, common cell types used for NT include ill–defined dermal skin fibroblasts and ovarian follicular cells (for females) (11). 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 (12). Likewise, ES cells isolated from blastocysts cloned from adult cells may be used as an intermediate cell type with inherently greater clonability (13).

Somatic cells may be cultured from biopsy tissue to establish a primary cell line and are easily cryo–preserved enabling ready access to millions of

cells. However, there may sometimes be genetic (14) or epigenetic changes (15) 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 (16). 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 current approach for NT in cattle at AgResearch is a "zona-free" methodology (17), comprising a sequence of seven main steps, outlined below. Zona-free cloning methods (18-20) have simplified the manipulation procedure, requireless 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 g/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 (17) 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 (21) 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 epigenetic reprogramming (see below).

5. Reconstructed one-cell embryos are then artificially activated in order to initiate embryonic development (22). Activation regimes that mimic the repetitive intracellular calcium oscillations that occur during normal fertilisation result in significantly improved development (23).

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 (24) 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 (25). Results from AgResearch in cattle show 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 and single IVP embryos. In contrast to fertilised embryos, however, typically 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 to proceed, it is considered that *the organisation of chromatin and the pattern of gene expression in a* specialised somatic cell must be completely reset to a zygotic state and for 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: DNA methylation (26–28) chromatinmodification (29) X–chromosome inactivation (30) and expression of imprinted and non–imprinted genes (31, 32). Aberrations in these epigenetic mechanismsare considered the major causes of the developmental failures with cloned embryos. Moreover, *sub–lethal aberrations that occur early in embryo or fetal* development may impair health in adulthood of the clones. 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 (33). Of concern is hydroallantois where 28% of established cloned bovine pregnancies at Day 120 of gestation may succumb to this syndrome (Wells *et al.*, unpublished). 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 prolonged gestation, poor preparation for parturition and an increased risk of dystocia from heavier birthweight offspring, often prompting elective caesarean

section (21, 34). 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 (3).

Post-natal mortality is also greater with clones, especially in cattle and sheep but less so in pigs and goats (25). The stage of the donor cell cycle at the time of NThas a significant effect, with a higher proportion of calves at term, derived from quiescent G0donor cells, surviving to weaning (76%) compared to clones derived from proliferating G1 cells (57%) (Wells *et al.* unpublished). Newborn clones have an altered metabolism, possibly due to the *in utero* placental abnormalities (35), and require time to adjust to a normal physiology (36). Most deaths are due to either abnormalities of the respiratory, cardiovascular, skeletal, central nervous or urogenital systems, along with umbilical and lung infections and digestive dysfunction (25).

Although there are reports of physiologically normal cloned animals (37, 38) displayingnormal behaviour, growth rates, reproduction, livestock production characteristics and lifespans (39), other reports indicate long-term health concerns. These have included obesity (40) and shortened lifespan (41) in some cloned mice and compromised immune systems in cattle (42). 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 (43). In cattle, the proportion of cloned calves born that are long-term survivors ranges between 47-80% (37, 44, 45). 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 (32).

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. This has been demonstrated when male and female clones have been mated together (39, 40) and most convincingly, when male and female cloned mice

were derived from XY and XO embryonic stem cells, respectively, obtained from the same cell line (46). This indicates that there are no obvious deleterious recessive genetic orepigenetic traits transmitted by clones. It also implies that any epigenetic differences in gene expression present in the clones are corrected during gametogenesis, providing some confidence in those applications that aim to capture the benefits of breeding from genetically elite clones. However, detailed molecular studies are required to confirm whether the necessary epigenetic modifications 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 differencesamongst 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 mitochrondrial (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 (47) 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 deviationsfrom 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 (48).

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 limited at presen-tand needs to be gathered however, initial results indicate that the com-position of milk from cloned dairy cows is within the broad range of milk produced from conventional cows (48).

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.
3. Conservation of endangered livestock breeds.
4. Animal research models.
5. Production of genetically modified livestock.
6. Human cell-based therapies.

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

1. **Increasing genetic gain in animal breeding schemes.** Effective breeding programmes require the accurate identification of superior live-stock 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 in the future. 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 identifygenotype x environmental interactions (49). 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 of up to three generation intervals (13). 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 (39).

2. **Dissemination of genetic gain.** Efficient cloning would potentially enable the rapid dissemination of elite 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. This could be achieved in the dairy industry through the transfer of selected lines of genetically superior cloned embryos resulting in a large genetic lift in the commercial population of up to 15 times the typical annual genetic response (50), without overly reducing the genetic variation available for future selection in the elite breeding population.

An ideal opportunity exists in the production of small numbers of cloned animals with superior genetics for breeding. Ideally, these would be cloned sires of progeny-tested males for widespread dissemination of their elite genetics following natural breeding or alternatively, increased semen production for artificial insemination. 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 51) 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 (www.cloneinternational.com.au) which aims to market cloned copies of top Australasian bulls indeveloping dairying nations. 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 teams of genetically elite cloned sires could substitute for artificial insemination, which is poorly adopted in these more extensive farming industries, to effectively disseminate genetic gain.

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 possessing the same nuclear genetics, variability in livestock production traits within a clonal family 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 characteristicsmay arise if clones possess different mtDNA compositions (52, 53) where cytoplasts are obtained from different mater-nal lineages.

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 uni-formity within the clonal family. If specific heterozygotes at particular loci were identified as being beneficial it would be possible to disseminate thesegenotypes reproducibly to commercial producers, without segregation (49).

3. **Conservation of endangered livestock breeds.** Cloning can be integrated into assisted reproductive strategies to conserve rare farm ani-

mal genetic resources that should not be lost from the global gene pool (54). 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 (49). 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 cryo-preserve 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. 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 (55).

5. **Production of genetically modified livestock.** Probably the most significant application of nuclear cloning will be in conjunction with genetic modification. Specific genetic enhancements can be stably integrated into the genomes of cultured cells growing in the laboratory and NT then used to generate cloned-transgenic livestock (56). The continual advances in animal genomics towards the identification of genes and their regulatory sequences that influence livestock production traits and human health will increase the ability to genetically modify animals to enhance agricultural efficiency and produce superior quality food and biomedical products for niche markets. Furthermore, gene targeting technology will enable particular genes to be introduced at precise locations in the genome, as well as the subtle modification of endogenous genes and even the functional

removal of unfavourable genes (on an otherwise favourable genetic background), to result in a wide range of desired outcomes altering the production characteristics of livestock (57). 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 techniques. Obstacles that remain, however, include the very low frequency of successful gene targeting events in primary cultures of somatic cells (especially 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. Agricultural applications of transgenesis are aimed at inc-reasing the quantity and quality of valuable meat, milk and fibre com-ponents (58), improving disease or pest resistance (59) resulting in better animal health and welfare and reduced animal remedy costs and reducing environmental pollution to aid sustainable agriculture (60), that will collec-tively have economic benefits for farmers and processors, or additional health benefits for consumers. At AgResearch, the introduction of addi-tional copies of bovine - and -casein genes into cloned dairy heifers re-sulted in a substantial 30% increase in casein protein content in milk within one generation (56). Casein is of value for cheese manufacture and if there is consumer and dairy industry acceptance of transgenic technology in the future, then themost efficient means of disseminating this desired genetic modification into the wider population will be through low cost artificial insemination from males homozygous for the desired trait at a specific locus.

There are many high value biomedical opportunities such as the creation of small specialist dairy herds producing novel high-value human pharma-ceutical proteins in their milk (61) to treat specific diseases following puri-fication and rigorous clinical testing. Additional medical applications inclu-de the production of pigs that completely lack the enzyme -1,3-galactosyl-transferase (62) aimed to counter hyperacute immune rejection

following xenotransplantationand the generation of livestock models of particular human diseases to test newtherapies, where these may be more suitable than available mouse models (63).

6. Human cell–based therapies. The prospect of human therapeutic cloning (64) whereby cloned blastocysts are produced as a source of autologous ES cells to generate histocompatible tissue for transplantantion has been exemplified in the bovine (65) and combined with gene therapy in mice (66). Advances in the understanding of reprogramming willultimately enable the transdifferentiation of cells *in vitro* (67) 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 regula–tory 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.

REFERENCES

1. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., and Campbell, K. H. (1997) Viable offspring derived from fetal and adult mam–malian cells. *Nature* 385, 810–813
2. Oback, B., and Wells, D. (2002) Donor cells for nuclear cloning: many are called, but few are chosen. *Cloning Stem Cells* 4, 147–168
3. Wells, D. N., Laible, G., Tucker, F. C., Miller, A. L.,Oliver, J. E., Xiang, T., Forsyth, J. T., Berg, M. C., Cockrem, K., L'Huillier, P. J., Tervit, H. R., and Oback, B. (2003) Coordination between

donor cell type and cell cycle stage improves nuclear cloning efficiency in cattle. *Theriogenology* 59, 45-59

4. Eggan, K., Akutsu, H., Loring, J., Jackson-Grusby, L., Klemm, M., Rideout, W. M., 3rd, Yanagimachi, R., and Jaenisch, R. (2001) Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc Natl Acad Sci U S A* 98, 6209-6214
5. Wakayama, T., and Yanagimachi, R. (2001) Mouse cloning with nucleus donor cells of different age and type. *Mol Reprod Dev* 58, 376-383
6. Wakayama, T., Rodriguez, I., Perry, A. C., Yanagimachi, R., and Mombaerts, P. (1999) Mice cloned from embryonic stem cells. *Proc Natl Acad Sci U S A* 96, 14984-14989
7. Heyman, Y., Chavatte-Palmer, P., LeBourhis, D., Camous, S., Vignon, X., and Renard, J. P. (2002) Frequency and occurrence of late-gestation losses from cattle cloned embryos. *Biol Reprod* 66, 6-13
8. Hochedlinger, K., and Jaenisch, R. (2002) Monoclonal mice generated by nuclear transfer from mature B and T donor cells. *Nature* 415, 1035-1038
9. Kikyo, N., and Wolffe, A. P. (2000) Reprogramming nuclei: insights from cloning, nuclear transfer and heterokaryons. *J Cell Sci* 113, 11-20
10. Yamazaki, Y., Makino, H., Hamaguchi-Hamada, K., Hamada, S., Sugino, H., Kawase, E., Miyata, T., Ogawa, M., Yanagimachi, R., and Yagi, T. (2001) Assessment of the developmental totipotency of neural cells in the cerebral cortex of mouse embryo by nuclear transfer. *Proc Natl Acad Sci U S A* 98, 14022-14026
11. Kato, Y., Tani, T., and Tsunoda, Y. (2000) Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. *J Reprod Fertil* 120, 231-237
12. Blau, H. M., Brazelton, T. R., and Weimann, J. M. (2001) The evolving concept of a stem cell: entity or function? *Cell* 105, 829-841
13. Wells, D. N., Oback, B., and Laible, G. (2003) Cloning livestock: time to return to embryonic cells. *Trends in Biotechnology* 21,

428–432

14. Olifent, M., Miller, A., Beaton, A., L'Huillier, P., Wells, D. N., and Laible, G. (2002) Karyotyping as an important screen for suitable donor cells to generate cloned and cloned transgenic animals by nuclear transfer. *Proceedings of the New Zealand Society of Animal Production* 62, 199–201
15. Humpherys, D., Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W. M., 3rd, Biniszkiewicz, D., Yanagimachi, R., and Jaenisch, R. (2001) Epigenetic instability in ES cells and cloned mice. *Science* 293, 95–97
16. Schnieke, A. E., Kind, A. J., Ritchie, W. A., Mycock, K., Scott, A. R., Ritchie, M., Wilmut, I., Colman, A., and Campbell, K. H. (1997) Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* 278, 2130–2133
17. Oback, B., Wiersema, A. T., Gaynor, P., Laible, G., Tucker, F. C., Oliver, J. E., Miller, A. L., Troskie, H. E., Wilson, K. L., Forsyth, J. T., Berg, M. C., Cockrem, K., McMillan, V., Tervit, H. R., and Wells, D. N. (2003) Cloned cattle derived from a novel zona-free embryo reconstruction system. *Cloning Stem Cells* 5, 3–12
18. Peura, T. T., Lewis, I. M., and Trounson, A. O. (1998) The effect of recipient oocyte volume on nuclear transfer in cattle. *Mol Reprod Dev* 50, 185–191
19. Vajta, G., Lewis, I. M., Hyttel, P., Thouas, G. A., and Trounson, A. O. (2001) Somatic cell cloning without micromanipulators. *Cloning* 3, 89–95
20. Booth, P. J., Tan, S. J., Reipurth, R., Holm, P., and Callesen, H. (2001) Simplification of bovine somatic cell nuclear transfer by application of a zona-free manipulation technique. *Cloning Stem Cells* 3, 139–150
21. Wells, D. N., Misica, P. M., and Tervit, H. R. (1999) Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol Reprod* 60, 996–1005
22. Machaty, Z., and Prather, R. S. (1998) Strategies for activating nuclear transfer oocytes. *Reprod Fertil Dev* 10, 599–613
23. Ozil, J. P., and Huneau, D. (2001) Activation of rabbit oocytes: the impact of the Ca2+ signal regime on development. *Development*

128, 917–928

24. Thompson, J. G., McNaughton, C., Gasparrini, B., McGowan, L. T., and Tervit, H. R. (2000) Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured *in vitro*. *J Reprod Fertil* 118, 47–55

25. Wilmut, I., Beaujean, N., De Sousa, P. A., Dinnyes, A., King, T. J., Paterson, L. A., Wells, D. N., and Young, L. E. (2002) Somatic cell nuclear transfer. *Nature* 419, 583–587

26. Bourc'his, D., Le Bourhis, D., Patin, D., Niveleau, A., Comizzoli, P., Renard, J. P., and Viegas–Pequignot, E. (2001) Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. *Curr Biol* 11, 1542–1546

27. Dean, W.,Santos, F., Stojkovic, M., Zakhartchenko, V., Walter, J., Wolf, E., and Reik, W. (2001) Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *Proc Natl Acad Sci U S A* 98, 13734–13738

28. Kang, Y. K., Koo, D. B., Park, J. S., Choi, Y. H., Chung, A. S., Lee, K. K., and Han, Y. M. (2001) Aberrant methylation of donor genome in cloned bovine embryos. *Nat Genet* 28, 173–177

29. Santos, F., Zakhartchenko, V., Stojkovic, M., Peters, A., Jenuwein, T., Wolf, E., Reik, W., and Dean, W. (2003) Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. *Curr Biol* 13, 1116–1121

30. Xue, F., Tian, X. C., Du, F., Kubota, C., Taneja, M., Dinnyes, A., Dai, Y., Levine, H., Pereira, L. V., and Yang, X. (2002) Aberrant patterns of X chromosome inactivation in bovine clones. *Nat Genet* 31, 216–220

31. Rideout, W. M., 3rd, Eggan, K., and Jaenisch, R. (2001) Nuclear cloning and epigenetic reprogramming of the genome. *Science* 293, 1093–1098

32. Humpherys, D., Eggan, K., Akutsu, H., Friedman, A., Hochedlinger, K., Yanagimachi, R., Lander, E. S., Golub, T. R., and Jaenisch, R. (2002) Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. *Proc Natl Acad Sci U S A* 99, 12889–12894

33. Hill, J. R., Burghardt, R. C., Jones, K., Long, C. R., Looney, C.

R., Shin, T., Spencer, T. E., Thompson, J. A., Winger, Q. A., and Westhusin, M. E. (2000) Evidence for placental abnormality as the major cause of mortalityin first-trimester somatic cell cloned bovine fetuses. *Biol Reprod* 63, 1787-1794

34. Wells, D. N., Misica, P. M., Day, A. M., and Tervit, H. R. (1997) Production of cloned lambs from an established embryonic cell line: a comparison between *in vivo*- and invitro-matured cytoplasts. *Biol Reprod* 57, 385-393

35. Garry, F. B., Adams, R., McCann, J. P., and Odde, K. G. (1996) Postnatal characteristics of calves produced by nuclear transfer cloning. *Theriogenology* 45, 141-152

36. Chavatte-Palmer, P., Heyman, Y., Richard, C., Monget, P., LeBourhis, D., Kann, G., Chilliard, Y., Vignon, X., and Renard, J. P. (2002) Clinical, hormonal, and hematologic characteristics of bovine calves derived from nuclei from somatic cells. *Biol Reprod* 66, 1596-1603

37. Lanza, R. P., Cibelli, J. B., Faber, D., Sweeney, R. W., Henderson, B., Nevala, W., West, M. D., and Wettstein, P. J. (2001) Cloned cattle can be healthy and normal. *Science* 294, 1893-1894

38. Renard, J. P., Zhou, Q., LeBourhis, D., Chavatte-Palmer, P., Hue, I., Heyman, Y., and Vignon, X. (2002) Nuclear transfer technologies: between successes and doubts. *Theriogenology* 57, 203-222

39. Wells, D. N. (2003) Cloning in livestock agriculture. *Reproduction* Supplement 61, 131-150

40. Tamashiro, K. L., Wakayama, T., Akutsu, H., Yamazaki, Y., Lachey, J. L., Wortman, M. D., Seeley, R. J., D'Alessio, D. A., Woods, S. C., Yanagimachi, R., and Sakai, R. R. (2002) Cloned mice have an obese phenotype not transmitted to their offspring. *Nat Med* 8, 262-267

41. Ogonuki, N., Inoue, K., Yamamoto, Y., Noguchi, Y., Tanemura, K., Suzuki, O., Nakayama, H., Doi, K., Ohtomo, Y., Satoh, M., Nishida, A., and Ogura, A. (2002) Early death of mice cloned from somatic cells. *Nat Genet* 30, 253-254

42. Renard, J. P., Chastant, S., Chesne, P., Richard, C., Marchal, J., Cordonnier, N., Chavatte, P., and Vignon, X. (1999) Lymphoid

hypoplasia and somatic cloning. *Lancet* 353, 1489–1491

43. Ogura, A., Inoue, K., Ogonuki, N., Lee, J., Kohda, T., and Ishino, F. (2002) Phenotypic effects of somatic cell cloning in the mouse. *Cloning Stem Cells* 4, 397–405

44. Heyman, Y., Zhou, Q., Lebourhis, D., Chavatte–Palmer, P., Renard, J. P., and Vignon, X. (2002) Novel approaches and hurdles to somatic cloning in cattle. *Cloning Stem Cells* 4, 47–55

45. Pace, M. M., Augenstein, M. L., Betthauser, J. M., Childs, L. A., Eilertsen, K. J., Enos, J. M., Forsberg, E. J., Golueke, P. J., Graber, D. F., Kemper, J. C., Koppang, R. W., Lange, G., Lesmeister, T. L., Mallon, K. S., Mell, G. D., Misica, P. M., Pfister–Genskow, M., Strelchenko, N. S., Voelker, G. R., Watt, S. R., and Bishop, M. D. (2002) Ontogeny of cloned cattle to lactation. *Biol Reprod* 67, 334–339

46. Shimozawa, N., Ono, Y., Kimoto, S., Hioki, K., Araki, Y., Shinkai, Y., Kono, T., and Ito, M. (2002) Abnormalities in cloned mice are not transmitted to the progeny. *Genesis* 34, 203–207

47. Steinborn, R., Schinogl, P., Wells, D. N., Bergthaler, A., Muller, M., and Brem, G. (2002) Coexistence of Bos taurus and B. indicus mitochondrial DNAs in nuclear transfer–derived somatic cattle clones. *Genetics* 162, 823–829

48. Wells, D. N., Cockrem, K., and Forsyth, J. T. (2003) Composition of milk from somatic cell cloned dairy cows. *Horizons in Livestock Sciences: the impact of the new biology, 25–28th May 2003, Queensland, Australia,* , Abstract 31

49. Woolliams, J. A., and Wilmut, I. (1999) New advances in cloning and their potential impact on genetic variation in livestock. *Anim Sci* 68, 245–256

50. Baker, R. L., Shannon, P., Garrick, D. J., Blair, H. T., and Wickham, B. (1990) The future impact of new opportunities in reproductive physiology and molecular biology on genetic improvement programmes. *Proceedings of the New Zealand Society of Animal Production* 50, 197–210

51. Sutovsky, P., Moreno, R. D., Ramalho–Santos, J., Dominko, T., Simerly, C., and Schatten, G. (2000) Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial

inheritance in mammalian embryos. *Biol Reprod* 63, 582–590

52. Schutz, M. M., Freeman, A. E., Lindberg, G. L., Koehler, C. M., and Beitz, D. C. (1994) The effect of mitochondrial DNA on milk production and health of dairy cattle. *Livestock Production Science* 37, 283–295

53. Mannen, H., Kojima, T., Oyama, K., Mukai, F., Ishida, T., and Tsuji, S. (1998) Effect of mitochondrial DNA variation on carcass traits of Japanese Black cattle. *J Anim Sci* 76, 36–41

54. Wells, D. N., Misica, P. M., Tervit, H. R., and Vivanco, W. H. (1998) Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed. *Reprod Fertil Dev* 10, 369–378

55. Hein, W. R., and Griebel, P. J. (2003) A road less travelled: large animal models in immunological research. *Nat Rev Immunol* 3, 79–84

56. Brophy, B., Smolenski, G., Wheeler, T., Wells, D., L'Huillier, P., and Laible, G. (2003) Cloned transgenic cattle produce milk with higher levels of beta–casein and kappa–casein. *Nat Biotechnol* 21, 157–162

57. Clark, A. J., Burl, S., Denning, C., and Dickinson, P. (2000) Gene targeting in livestock: a preview. *Transgenic Res* 9, 263–275

58. Murray, J. D., Anderson, G. B., Oberbauer, A. M., and Mc–Gloughlin, M. M. (1999) Ed. In Transgenic Animals in Agriculture. CABI Publishing, Oxon, UK, 290 pp.

59. Muller, M., and Brem, G. (1998) Transgenic approaches to the increase of disease resistance in farm animals. *Rev Sci Tech* 17, 365–378

60. Golovan, S. P., Meidinger, R. G., Ajakaiye, A., Cottrill, M., Wie–derkehr, M. Z., Barney, D. J., Plante, C., Pollard, J. W., Fan, M. Z., Hayes, M. A., Laursen, J., Hjorth, J. P., Hacker, R. R., Phillips, J. P., and Forsberg, C. W. (2001) Pigs expressing salivary phytase produce low–phosphorus manure. *Nat Biotechnol* 19, 741–745

61. Brink, M. F., Bishop, M. D., and Pieper, F. R. (2000) Developing efficient strategies for the generation of transgenic cattle which produce biopharmaceuticals in milk. *Theriogenology* 53, 139–148

62. Phelps, C. J., Koike, C., Vaught, T. D., Boone, J., Wells, K. D.,

Chen, S. H., Ball, S., Specht, S. M., Polejaeva, I. A., Monahan, J. A., Jobst, P. M., Sharma, S. B., Lamborn, A. E., Garst, A. S., Moore, M., Demetris, A. J., Rudert, W. A., Bottino, R., Bertera, S., Trucco, M., Starzl, T. E., Dai, Y., and Ayares, D. L. (2003) Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science* 299, 411-414

63. Harris, A. (1997) Towards an ovine model of cystic fibrosis. *Hum Mol Genet* 6, 2191-2194

64. Gurdon, J. B., and Colman, A. (1999) The future of cloning. *Nature* 402, 743-746

65. Lanza, R. P., Chung, H. Y., Yoo, J. J., Wettstein, P. J., Blackwell, C., Borson, N., Hofmeister, E., Schuch, G., Soker, S., Moraes, C. T., West, M. D., and Atala, A. (2002) Generation of histocompatible tissues using nuclear transplantation. *Nat Biotechnol* 20, 689-696

66. Rideout, W. M., 3rd, Hochedlinger, K., Kyba, M., Daley, G. Q., and Jaenisch, R. (2002) Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell* 109, 17-27

67. Hakelien, A. M., and Collas, P. (2002) Novel approaches to transdifferentiation. *Cloning Stem Cells* 4, 379-387