Future opportunities in livestock production and biomedicine from advances in animal cloning

D.N. WELLS, P.M. MISICA AND H.R. TERVIT

Dairy and Beef Division, AgResearch Ruakura, Private Bag 3123, Hamilton, New Zealand.

ABSTRACT

Recent international advances in animal cloning technology have enabled the production of offspring from cultured cells derived from either embryos, foetuses or adults. When the current nuclear transfer process becomes more efficient, there will be three main applications for agriculture. These include: (1) the rapid dissemination of animals with the desired genetic characteristics; (2) the use of cloning for phenotypic evaluation of animals; and most significantly, (3) the production of genetically modified animals. Nuclear transfer will be combined with gene targeting technology to introduce precise genetic modifications to the cultured cells in the laboratory, resulting in the production of cloned transgenic farm animals. It is anticipated that this will herald a new era in biotechnology, with opportunities to generate animals for various biomedical applications and with modified livestock production characteristics.

Keywords: nuclear transfer; cloning; transgenics; sheep; cattle.

INTRODUCTION

Plant breeders are able to propagate large numbers of genetically identical plants simply by taking cuttings from desirable specimens. This is cloning; and for many years we have been attempting to perform a similar feat with desirable farm animals.

Artificially, or experimentally, there are a number of ways to produce genetically identical animals by manipulating early embryos. A few days after fertilisation the early embryo is simply a small ball of identical cells which have not yet differentiated or specialised into the particular cell types of the subsequent foetus. It is therefore during these stages that manipulation of the embryo to produce cloned animals is easiest. So the production of genetically identical animals can be simply achieved by gently prizing apart the cells of a 2-, 4- or 8-cell embryo so that each individual cell (or smaller group of cells) can potentially form a foetus. Alternatively and more commonly, identical twins can be produced by bisecting an early stage embryo (up to 7 days after fertilisation) into two equal halves with a microsurgical knife. This embryo splitting technology is now used commercially to increase the number of lambs or calves born following routine embryo transfer or *in vitro* embryo production.

A potentially more powerful method of generating clones involves the use of another embryo manipulation procedure termed nuclear transfer (outlined in materials and methods). The technique was first used by researchers studying frogs nearly 50 years ago. Nuclear transfer has also been used during the last decade to clone sheep (Willadsen, 1986) and cattle (Prather *et al.*, 1987) using embryo cells, commonly from the 32-cell stage (5 days following fertilisation) , to create small numbers of genetically identical animals from original founder embryos.

The major breakthrough in the field of nuclear transfer is the ability to clone animals from cultured cells which are more differentiated than those previously used. These differentiated cells have come from cell cultures obtained from either embryos, foetuses, or adult animals (Campbell *et al*., 1996; Wells *et al*., 1997ab; Wilmut *et al*., 1997b). Under suitable *in vitro* culture conditions, these cell lines can be grown for many months in culture dishes in the laboratory. Furthermore, samples can be readily frozen and stored in liquid nitrogen and thawed for use at any desired time. With the cultured cell lines there is ready access to millions of cells and so, in theory at least, a large number of cloned animals could be produced.

Here, we summarise experiments conducted by AgResearch to investigate the developmental potential of two sheep embryonic cell lines by nuclear transfer and discuss the potential applications of this technology.

MATERIALS AND METHODS

Cell lines were established from two Day 8 ovine embryos. One cell line (REF38b) was male and possessed an epithelial-like cellular morphology and the other (RDB) was female and with a fibroblastic cell type. Both cell lines have remained stable in culture for relatively long periods of time and can be frozen and thawed as required.

The nuclear transfer process comprised a sequence of four main steps. First, unfertilised metaphase II-arrested sheep oocytes, obtained either a few hours following ovulation or after *in vitro* maturation of abattoir-derived oocytes, were enucleated. This involved manipulation of oocytes with finely controlled micro-surgical instruments to physically aspirate the metaphase II chromosomes and first polar body. Thus, the genetic material of the oocytes was removed, resulting in what is termed a cytoplast (a cell

containing only cytoplasmic material). Secondly, single donor cells obtained from the cell line were injected underneath the outer zona pellucida (a protective membrane surrounding the egg and early embryo before implantation) and adjacent to the cytoplast membrane. Thirdly, the cytoplast and the donor cell were then fused together utilising a minute electrical pulse. Thus, the genetic information contained within the nucleus of the cultured donor cell enters the cytoplast. Fourthly, the pulse of electricity also artificially activated the reconstructed 1-cell embryos to begin development. Because of the ready availability of cells, this nuclear transfer process could be repeated many times.

The reconstructed embryos were then cultured in a chemically-defined medium for 6-7 days *in vitro*. After this time, embryos that developed into blastocysts of suitable quality were surgically transferred to the uteri of synchronised recipient ewes for development to term. Embryo survival was monitored throughout gestation by a combination of plasma progesterone (Day 15), pregnancy-specific serum proteins (Days 21 and 28) and ultrasonography (Day 35 onwards). Lambing was either allowed to occur naturally, following induction or following Caesarian section.

The experiments described herein were approved by both the Ruakura Animal Ethics Committee and the AgResearch Ruakura Biosafety Committee.

RESULTS

Embryo Development

Table 1 shows the proportion of embryos reconstructed with *in vivo*-derived oocytes and either RDB or REF38b donor cells that firstly fused and then subsequently developed into blastocyst-stage embryos following culture.

TABLE 1: Effect of ovine cell line on the fusion of reconstructed nuclear transfer embryos and the resulting development to the blastocyst-stage (data from Wells *et al*., 1997ab; Misica *et al*., 1998).

Cell Line	reconstructed embryos	% Fused	% Blastocysts
REF38b	258	64%	24%
RDB	489	57%	28%

Cytoplast Source

Cytoplasts obtained from in vivo-derived oocytes tended to be superior to *in vitro* matured cytoplasts, both in terms of development of fused embryos to blastocysts (24% vs. 17%) and survival of embryos to term (7% vs. 2%)(Wells *et al*., 1997a).

Embryo Survival

Data were complied from three separate experiments (Wells *et al*., 1997ab; Misica *et al*., 1998) to summarise the typical survival of cloned embryos and foetuses during gestation following nuclear transfer, with *in vivo*-derived oocytes. Eight days following embryo transfer, all recipient ewes analysed were biochemically pregnant. A high proportion of embryos were lost over the attachment phase, resulting in 22% embryo survival on Day 35. Very few foetuses were lost during mid-gestation (3%). However, 43% of foetuses present at around Day 100 were subsequently lost during the last trimester, resulting in 11% (12/112) of embryos transferred surviving to full term (all born as singletons). In addition, one lamb derived from nuclear transfer with an *in vitro* matured cytoplast was born (2% embryonic survival to term). DNA analyses using microsatellite markers were consistent with the cloned lambs having originated from the respective cell lines.

The average birthweight of the cloned male lambs (Romney x East Friesian genotype) was 5.5 ± 1.6 kg (n=3) and for the females (Romney x Poll Dorset), 4.9 ± 0.3 kg (n=10). Five lambs survived, with one dying of misadventure and the others dying between 10 mins and 2 days of birth, with a kidney defect diagnosed as hydronephrosis and lung dysfunction being common abnormalities.

Regular health tests have shown the surviving cloned sheep to be normal. Furthermore, natural mating of two cloned rams at seven months of age to eight mature ewes resulted in a total of 15 progeny born. Microsatellite analyses were consistent with the offspring being sired by these two cloned rams.

DISCUSSION

We have confirmed that it is biologically possible to produce normal, fertile cloned sheep from cultured cell populations using nuclear transfer technology. In addition, we have established cloned pregnancies in cattle from both foetal and adult cell lines (Wells *et al*., 1998; Wells *et al*., unpublished). However, the current nuclear transfer process is inefficient in four major areas, namely: cell fusion, blastocyst development, embryo survival and neonatal mortality. Cell fusion with cytoplasts is technically demanding given the small size of the donor cells (~20µm) and is possibly complicated due to an incompatibility between the two cellular membranes. The developmental rates and quality of cloned embryos are influenced by oocyte cytoplasm, the *in vitro* culture system and the reprogramming of differentiated cell nuclei. Recent experiments in cattle suggest that up to 50% of foetal cells fused with *in vitro* matured oocytes develop to the blastocyst-stage (Wells *et al*., 1998). The poor rates of pregnancy establishment and foetal and neonatal losses are presumably a consequence of improper nuclear reprogramming. The abnormalities encountered by us in some cloned lambs and the loss rates, are similar to those reported elsewhere (Wilmut *et al*., 1997ab). Further research is required to reduce these problems.

Once somatic cell cloning technology becomes efficient there are three main agricultural applications. These include: (1) the rapid dissemination of desirable or genetically superior animals; (2) utilising cloning for phenotypic evaluation; and (3) the production and multiplication of transgenic animals.

Cloning for Gene Dissemination

Cloning could enable the rapid dissemination of superior genotypes from breeding flocks and herds, directly to commercial farmers. This could be achieved either through the mass release of a large number of clones or in the case of the sheep industry, providing an alternative to artificial insemination; whereby a number of elite, genetically identical rams could be used for widespread natural mating, in commercial lamb production. It has been estimated that the implementation of cloning strategies could result in large one-off increases in genetic gain, equivalent to 15-17 years of traditional animal breeding (Nicholas and Smith, 1983; Baker *et al*., 1990). The rate of genetic progress would then slow to that of elite herds in which the animals are being multiplied by natural means, until new desirable genes are identified for a further round of cloning.

Cloning is probably best used to generate commercial animals or integrated into a specialised sector of the sire breeding herds, so as to not overly reduce the genetic variation in the breeding population from which to select superior stock in future generations. It is conceivable, however, that within herds there could be small groups of elite clones and following natural breeding from them, the genetic variation available for selection amongst their progeny would be increased. Cloning could also be integrated into breeding strategies aimed at multiplying endangered indigenous breeds of livestock, in order to conserve the genetic diversity of animals adapted to specific environments.

Somatic cell cloning would allow the rapid production of large herds or flocks enabling an economic volume of product (meat or milk) to meet specific and/or changing market requirements. In the situation whereby all animals in a particular herd are genetically identical, there is the novel opportunity to produce more uniform products. However, since livestock production traits depend upon environment influences as well, some variability in animal performance will remain, depending on the heritability of the trait. Also, during the process of nuclear transfer each donor cell is fused to a separate oocyte, many with differing mitochondrial DNA, which may modify some production traits (Gibson *et al*., 1997). Nevertheless, contemporary beef clones all in the same herd, raised especially in a feedlot situation, could well be expected to have more similar meat characteristics than present and provide more consistent, quality products for consumers.

Cloning for phenotypic evaluation

The effective utilisation of cloning, be it from embryos, foetuses or adults, requires the accurate identification of superior livestock in the population. In the future, marker assisted selection strategies that allow for the identification of favorable genes that correlate with production, will aid in selecting desirable genotypes. However, actual performance will remain uncertain. Cloning could in fact be used to directly determine the phenotype of animals in a variety of environmental conditions, as described by Bennett and Clarke (1984) and thus, enhance genetic progress. 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 favorably, 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 nuclear transfer to release a large number of the desirable animals to the industry.

Cloning for transgenic applications

In the near term, the greatest impact of using nuclear transfer to clone animals from cultured cells will probably be in the production of transgenic animals. While the cells are in culture, very precise modifications can be made to specific genes by procedures such as “gene targeting” (Hooper, 1992) to alter the production characteristics of animals in a controlled and desired manner. Following the selection of the cells that have been genetically altered, nuclear transfer could be used to produce cloned transgenic animals. This will be a more efficient means of producing transgenics than conventional pronuclear injection of DNA where typically only 0.1% of injected embryos develop into transgenic animals and, as there is no control over the site of integration nor the number of copies introduced, there have been highly variable outcomes in each line of transgenics.

There are a wide range of new opportunities available for genetically modifying livestock both for the biomedical and agricultural sectors of society. It is possible to introduce into the cultured cells, human genes which are directed to be expressed only in the mammary gland of lactating sheep or cattle, in order to produce pharmaceutical proteins in the milk which can be purified and used for human therapy. Alternatively, bovine milk could be made more nutritious for human infants or general human health. The area of xenotransplantation could benefit from targeted genetic changes to cultured pig cells to lessen the antigenicity of animal organs following human transplantation. Animals could also be generated as models for research on human genetic diseases such as cystic fibrosis. Such animals could be used to test new drugs and treatments for various diseases before evaluation in humans.

Our research is encouraged by the prospects of modifying farm animal production characteristics. As understanding of the genes that control production traits increases in the future, so too will our ability to accurately modify the appropriate genes to generate new and desired products from farm animals. Specifically, we are interested in altering the milk protein composition of dairy cows. Additionally, it may be possible to improve the feed conversion efficiency of livestock animals and improve disease or pest resistance, resulting in better animal welfare and reduced remedy costs.

Summary

The ability to clone livestock animals from cultured somatic cells from elite animals of proven performance provides exciting new opportunities for agriculture in the future. Although the technology is currently inefficient and not yet commercially viable, it possesses the potential to rapidly multiply animals with desired genetic character-

istics to improve the value and market potential of farm animal production. The production of genetically modified animals by combining gene targeting and nuclear transfer technologies is also imminent. Undoubtedly, further research over the coming years will improve the efficiency of the nuclear transfer process. As a consequence of this research it is anticipated that somatic cell cloning in livestock species will provide enormous future benefits to farmers and society.

ACKNOWLEDGEMENTS

We wish to thank staff of the Reproductive Technologies Group, AgResearch Ruakura, for their support and assistance throughout this project and particularly to John Lange for care of experimental animals. We are especially grateful to Tony Day and Robyn Wells for help in the delivery of these animals, Alan Crawford and his colleagues for microsatellite analyses and to Neil Clarke and Phil L'Huillier for reviewing the manuscript. This research was supported by the Foundation for Research Science and Technology, New Zealand (contract number C10 402).

REFERENCES

Baker, R.L.; Shannon, P.; Garrick, D.J.; Blair, H.T. and Wickham B.W. 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.

Bennett, G.L. and Clarke, J.N. 1984. Expected selection responses in lamb carcass composition and weight. *Proceedings of the New Zealand Society of Animal Production* **44:** 243-247.

Campbell, K.H.S.; McWhir, J.; Ritchie, W.A. and Wilmut I. 1996. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380:** 64-66.

Gibson, J.P.; Freeman, A.E. and Boettcher, P.J. 1997. Cytoplasmic and mitochondrial inheritance of economic traits in cattle. *Livestock Production Science* **47:** 115-124.

Hooper, M.L. 1992. *In:* Evans, H.J. (ed.), Embryonal Stem Cells: Introducing Planned Changes into the Germline. Switzerland:Harwood Academic; 147pp.

Misica, P.M.; Peterson, A.J.; Day, A.M. and Wells, D.N. 1998. Co-transfer of trophoblastic vesicles with cloned sheep embryos is unable to improve survival to term. *Theriogenology* **49:** 247.

Nicholas, F.W. and Smith, C. 1983. Increased rates of genetic change in dairy cattle by embryo transfer and splitting. *Animal Production* **36:** 341-353.

Prather, R.S.; Barnes, F.L.; Sims, M.L.; Robl, J.M.; Eyestone, W.H. and First, N.L. 1987. Nuclear transfer in the bovine embryo: assessment of donor nuclei and recipient oocyte. *Biology of Reproduction* **37:** 859-866.

Wells, D.N.; Misica, P.M.; Day, A.M.; and Tervit, H.R. 1997a. Production of cloned lambs from an established embryonic cell line: a comparison between in vivo- and *in vitro*-matured cytoplasts. *Biology of Reproduction* **57:** 385-393.

Wells, D.N.; Misica, P.M.; Day, A.M. and Tervit, H.R. 1997b. Generation of cloned lambs from an established embryonic fibroblast cell line. *Proceedings of Transgenic Animals in Agriculture Conference*, Abstract 18.

Wells, D.N.; Misica, P.M.; McMillan, W.H. and Tervit H.R. 1998. Production of cloned bovine fetuses following nuclear transfer with cells from a fetal fibroblast cell line. *Theriogenology* **49:** 330.

Willadsen, S.M. 1986. Nuclear transplantation in sheep embryos. *Nature* **320:** 63-65.

Wilmut, I.; McWhir, J. and Campbell K. 1997a. Nuclear transfer from cultured cells: a new opportunity in animal breeding? *In:* Welch, R.A.S.; Burns, D.B.; Davis, S.R.; Popay, A.I.; Prosser, C.G. (eds.), Milk Composition, *Production and Biotechnology*. CAB International, Oxfordshire; 389-396.

Wilmut, I.; Schnieke, A.E.; McWhir, J.; Kind, A.J. and Campbell, K.HS. 1997b. Viable offspring derived from fetal and adult mammalian cells. *Nature* **385:** 810-813.