6

Embryonic Stem Cell Chimeras and Somatic Cell Nuclear Transplantation for Production of Transgenic Cattle

J.M. Robl[2], J.B. Cibelli[1,2], P.G. Golueke[1], J.J. Kane[1], C. Blackwell[1], J. Jerry[2], E.S. Dickenson[1], F.A. Ponce de Leon[2] and S.L. Stice[1]

[1]*Advanced Cell Technology, Inc. and* [2]*Department of Veterinary and Animal Sciences, Paige Laboratory, University of Massachusetts, Amherst, Massachusetts, USA*

Introduction

Genetic modification of cattle could be useful in increasing the efficiency of meat and milk production. Small numbers of transgenic cattle have been made using traditional pronuclear microinjection (Pursel and Rexroad, 1993). However, no transgenic lines have yet been commercialized for agriculture. At least part of the reason for this slow progress is the limitations and inefficiency of the transgenic production technology.

One of the limitations of pronuclear microinjection is that the gene insertion site is random. This typically results in variations in expression levels and several transgenic lines must be produced to obtain one line with appropriate levels of expression to be useful. Because integration is random, it is advantageous that a line of transgenic animals be started from one founder animal, to avoid difficulties in monitoring zygosity and potential difficulties that might occur with interactions among multiple insertion sites (Cundiff *et al*., 1993). Furthermore, if inbreeding is to be avoided, starting a transgenic line from one hemizygous animal with a random insert would require breeding several generations and significant time for introgression of the transgene into the population before breeding and testing homozygotes (Cundiff *et al*., 1993). Even without concern for inbreeding, it would take 6.5 years before reproduction could be tested in homozygous

 Transgenic Animals in Agriculture
(eds J.D. Murray, G.B. Anderson, A.M. Oberbauer and M.M. McGloughlin)

animals (Seidel, 1993). Finally, the quality of the genetics of a homozygous transgenic line would lag behind that of the general population because of the reduced population within which to select future generations of transgenic animals and the difficulty of bringing new genetics into a population in which the transgene is fixed.

A second limitation of the pronuclear microinjection procedure is its efficiency, which ranges from 0.34 to 2.63% of the gene-injected embryos developing into transgenic animals and a fraction of these appropriately expressing the gene (Pursel and Rexroad, 1993). This inefficiency results in a high cost of producing transgenic cattle because of the large number of recipients needed and, more importantly, unpredictability in the genetic background into which the gene is inserted because of the large number of embryos needed for microinjection. For agricultural purposes a high-quality genetic background is essential; therefore, long-term backcrossing strategies must be used with pronuclear microinjection.

An ideal system for producing transgenic animals for agricultural applications should be highly efficient and use small numbers of recipient animals to produce transgenics. It should allow the insertion of a transgene into a specific genotype. The insertion would preferably be into a predetermined site that would confer high expression and not affect general viability and productivity of the animal. Furthermore, the identification of a locus for insertion should allow multiple lines to be produced and crossed to produce homozygotes, and new genetic background could easily be added to the transgenic line by the production of new transgenics at any time. Therefore, the ideal system would likely require the transfection and selection of cells that could be easily grown in culture yet retain the potency to form germ cells and pass the gene to subsequent generations.

One such system for producing transgenic animals has been developed and widely used in the mouse. This approach involves the use of embryonic stem (ES) cells. Mouse ES cells are relatively easy to grow as colonies *in vitro*. The cells can be transfected by standard procedures and transgenic cells clonally selected by antibiotic resistance (Doetschman, 1994). Furthermore, the efficiency of this process is such that sufficient transgenic colonies (hundreds to thousands) can be produced to allow a second selection for homologous recombinants (Doetschman, 1994). Mouse ES cells can then be combined with a normal host embryo and, because they retain their potency, can develop into all the tissues in the resulting chimeric animal, including the germ cells. The transgenic modification can then be transmitted to subsequent generations. One of our objectives for the production of transgenic cattle has been to use a similar approach and develop pluripotential embryonic cell lines (PEC) from cattle that could be transfected and selected *in vitro* and, after association with a host embryo, contribute to various tissues, including the germline, in the resulting offspring.

Another system for producing transgenic animals has been demonstrated by the recent studies of Wilmut *et al*. (1997) in the sheep. In this approach

somatic cells grown *in vitro* are reprogrammed by fusion to a recipient egg. The resulting embryo is then capable of developing into a full-term offspring. Because somatic cells such as fibroblasts can be easily grown, transfected and selected *in vitro*, this would be an ideal system for producing transgenic animals. Furthermore, because the entire animal would be derived from the transgenic donor cell it would not be necessary to breed one generation to produce a pure transgenic, as with the chimeric approach, and all the offspring would be genetically identical. Cloning would also be an advantage in that the offspring could be of a predetermined sex so sex-limited traits, such as milk production, could be tested in females produced in the first generation. A second objective of our work, therefore, has been to develop a system for using somatic cell nuclear transplantation to produce transgenic cattle.

Production of Transgenic Cattle PECs

The defining requirements we used for designating cells as ES cells were: (i) the cells should be derived from the inner cell mass (ICM) of a blastocyst stage embryo; (ii) they should be capable of dividing in culture without showing signs of morphological differentiation; and (iii) they should contribute to cells of the germline and endodermal, mesodermal and ectodermal tissues when combined with a host embryo to form a chimera. In addition, cells were evaluated in relation to mouse ES cells for morphology, several cytoplasmic markers and growth characteristics.

Morphologically, the colonies that were established from bovine ICMs maintained distinct margins, had high nuclear to cytoplasmic ratios, generally maintained a high density of lipid granules (Fig. 6.1) and were cytokeratin- and vimentin-negative as in the mouse but, contrary to the mouse, the cells grew as a single layer with individual cells being visible and were not positive for alkaline phosphatase. Another difference between mouse ES cells and bovine PECs was that bovine PECs were much slower growing than mouse ES cells, indicating a much longer cell cycle (estimated to be about 40 h).

Two methods were used to establish PEC colonies from day-7 *in vitro*-produced bovine blastocysts. The method involved isolating the ICM immunosurgically. Antisera were developed against bovine spleen cells in mice. The zona pellucida was removed using 0.5% pronase until the zona thinned and could be removed by pipetting. The blastocysts were exposed to a 1:100 dilution of anti-bovine mouse serum for 45 min then washed and treated with guinea pig complement. The lysed trophectodermal cells were removed by pipetting. For the second method the ICM was isolated mechanically using two 26-gauge needles. The needles were crossed and brought down on the zona-intact blastocysts, which were cut using a scissor action. Some of the trophectodermal cells remained with the ICM

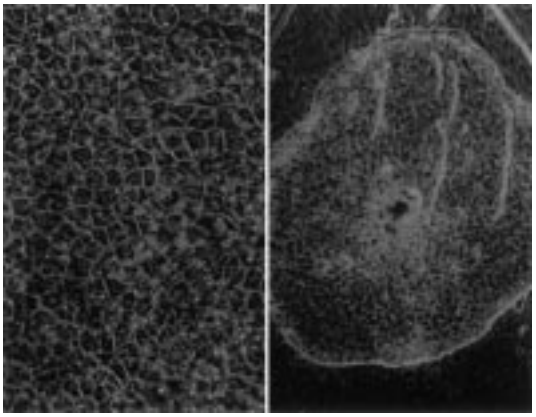

Fig. 6.1. Bovine PEC colony showing distinct margins with cells containing a high nuclear to cytoplasmic ratio and a high density of lipid granules.

and inevitably disappeared following plating and passaging. A PEC colony was considered as established after the third passage without signs of differentiation. For the immunosurgically isolated ICMs 5/9 (55%) formed PEC colonies and for the mechanically isolated ICMs 6/12 (50%) formed colonies. Because no difference was detected between these methods, the mechanical method was adopted for the advantage of simplicity.

Establishment of PEC colonies and maintenance of the undifferentiated state depend on an intimate contact between the ICM and the mouse fibroblast feeder layer. In an attempt to increase the contact during the initial establishment, day 7 *in vitro*-produced ICMs were placed either beneath or on top of mouse fetal fibroblast feeder layers. As above, a PEC colony was considered as established after the third passage without signs of differentiation. In agreement with previous results, 5/9 (55%) ICMs plated on top of the feeder layer produced colonies but only 4/11 (36%) of those placed beneath the feeder layer provided less appropriate interaction to inhibit differentiation of the ICMs.

Several methods of passaging bovine PEC colonies were attempted. Because it is beneficial to clonally propagate PECs following transfection and is necessary for homologous recombination, many attempts were made to trypsinize colonies to produce single cells and establish new colonies from these cells. To summarize, all attempts at clonally propagating bovine PECs were unsuccessful. Therefore, the routine method of passage that was established was to mechanically cut the colony into pieces that contained at least 50 cells and plate the clumps of cells on new feeder layers.

Following the development of methods of establishing and passaging bovine PECs and the identification of limitations in clonally propagating the cells, we turned to pursuing methods of transfecting and selecting for transgenic cells. The construct that was used contained a human cytomegalovirus

promoter and β-galactosidase/neomycin resistance fusion gene (Friedrich and Soriano, 1991; β-GEO). Selection was based on treatment with Geneticin (G418, Sigma, St Louis, Missouri) to kill non-expressing cells. The β-GEO gene was used to verify incorporation and expression.

Prior to transfecting cells, it was necessary to determine the sensitivity of non-transgenic cells to G418. Colonies from three different embryos were challenged with 0, 50, 100 and 150 μg ml^{-1} G418. A colony was considered dead when it completely lifted from the feeder layer. Survival varied among lines of cells with the first line surviving an average of 9 days at 100 μg ml^{-1} and 7 days at 150 μg ml^{-1}. The second survived 12, 10 and 7 days at 50, 100 and 150 μg ml^{-1}, respectively and the third line survived 8, 7 and 5 days at 50, 100 and 150 μg ml^{-1}, respectively. To ensure death of all non-transgenic colonies, 150 μg ml^{-1} G418 was chosen as the dose for subsequent transfection experiments.

Because it was not possible to trypsinize and produce a cell suspension of bovine PECs, the method of transfection was limited to either microinjection or lipofection. Various lipofection protocols were tested and found to be effective on fibroblast and Comma D cell cultures but were not effective on bovine PECs. Therefore, microinjection was used. PECs from three different lines were microinjected into the nucleus with a linearized version of the construct described above. At 1 day following microinjection the colonies were treated with 150 μg ml^{-1} G418 continuously for 30 days (four or five passages). For the three lines 3753, 3508 and 3502 cells were injected and five, two and zero colonies, respectively, survived selection in G418. Some cells within each of these colonies expressed β-galactosidase activity and samples of cells were positive for the transgene when amplified by PCR (35 cycles) and analysed by Southern blot hybridization to the amplified product. Because the colonies essentially disappeared during selection it is likely that the transgenic lines were of clonal origin, although this was not confirmed. Variation in expression in cells within a colony was probably due to cell-to-cell variation in factors such as cell cycle stage, position effects and others.

Potency of the cells was tested by producing chimeras with host embryos. Prior to evaluating the incorporation of PECs into embryos, the relationship between the number of PECs injected into morulae and the rate of development to the blastocyst stage was investigated. As shown in Table 6.1 either four, eight or 12 cells were injected following isolation by trypsinization. Rate of development to the blastocyst stage decreased with increasing number of ES cells used. As an injection control, fibroblasts (either four, eight or 12 cells) were injected into morulae, and as a non-injection control, a group of non-treated embryos were cultured to the blastocyst stage. There were no differences in development rate due to the number of cells injected, but manipulation, or the injection of cells, did appear to have a detrimental effect on development. Although it was found that increasing the number of PECs injected decreased the rate of development it was also

Table 6.1. Effect of cell injection on development of bovine morulae to the blastocyst stage.

Type of cell	Number of cells injected	Number of morulae injected	Number of blastocysts (%)
PEC	4	62	15 (24)[a,b]
PEC	8	65	10 (15)[a]
PEC	12	67	9 (13)[a]
Fibroblast	4	54	16 (30)[a,b]
Fibroblast	8	58	11 (19)[a,b]
Fibroblast	12	36	10 (28)[a,b]
Control	0	46	19 (41)[b]

[a,b] Percentages with different superscripts are significantly different ($P<0.05$).

believed that decreasing the number of cells would decrease the level of chimerism in the embryos. A compromise position of injecting eight cells was chosen for further experiments.

Incorporation of PECs into bovine blastocysts was evaluated to determine if the PECs could interact with the host embryo and be incorporated into the inner cell mass of the blastocyst. PECs were labelled with 100 μg ml^{-1} of the fluorescent carbocyanine dye, DiI (Sigma, St Louis, Missouri), then injected into morula stage embryos. Four days later the resulting blastocysts were observed under the fluorescent microscope. Incorporation of labelled PECs into both the ICM and the trophectoderm was detected in all blastocysts. To further verify that the cells had been incorporated into the ICM, the trophectoderm was removed by immunosurgery and the isolated ICM was observed. In all cases labelled cells were detected in the ICM. This indicated that the PECs could be incorporated into the compacted morula and ICM and form the early precursors of the fetus.

The next step in examining the potency of the PECs was to test chimerism in fetuses recovered at 40 days of gestation. Eighteen day-7 blastocysts, injected with eight to ten PECs were transferred into six recipient cows. Forty days after transfer the fetuses were recovered by Caesarean section. The total number of fetuses recovered was 12, with six being normally developing and six dead and in the process of being resorbed. Of the six normal fetuses, the β-GEO transgene was detected, by PCR (35 cycles) and Southern analysis of the PCR product, in some tissues in all of them (Table 6.2). Of the abnormal fetuses it was possible to analyse some tissues in one and it, too, was transgenic. In addition to analysing somatic tissues, preparations enriched in PGCs were isolated and analysed in the normal fetuses and two showed evidence of having transgenic cells. The results of this experiment indicated that the PECs did have the capacity to differentiate into many different kinds of tissues and survive at least 40 days *in vivo*. Further work is in progress on alternative methods of generating transgenic PECs and evaluating survival of PECs to term in chimeric animals.

Table 6.2. Contribution of transgenic PECs to various tissues in 40-day bovine fetuses.

	Fetus number[a]					
Tissue	1	2	3	4	5	6
Heart	+	+	–	+	+	+
Muscle	+	+	n.d.[b]	–	n.d.[b]	+
Brain	–	+	+	–	+	+
Liver	n.d.[b]	–	+	–	+	+
Gonads	–	+	+	+	+	+
PGC[c]	+	–	+	–	–	–

[a] Fetal tissues were analysed by 35 cycles of PCR followed by Southern hybridization of the amplified product.
[b] Not determined.
[c] PGCs were isolated from a trypsin digest of the genital ridge, then individually

These results are promising for the development of a highly efficient method of producing PECs in the bovine. Bovine PECs may be very useful as a source of *in vitro*-produced cells for transplantation into humans. However, the use of bovine PECs for gene targeting does not appear to be practical. Generally, it is expected that between 100 and 1000 transgenic colonies need to be produced and screened to find one or more colonies with the correct insert (Doetschman, 1994). Our highest level of efficiency in transgene incorporation was five colonies from 3753 cells injected. To produce 100 transgenic colonies would require injecting about 75,000 cells. This would require 25 days of injection if one person injected 3000 cells per day, which is possible with experience. Producing more than 100 transgenic colonies is probably unrealistic without significant increases in efficiency.

Production of Transgenic Cattle Somatic Cell Nuclear Transplant Embryos

Fibroblasts were chosen as the donor cell because of their ease of isolation, growth and transfection. Bovine fetal fibroblasts were produced from 30–100 mm crown rump length (approximately 40–80 days of gestation) fetuses obtained from the slaughterhouse. Fetuses were shipped by overnight express mail on ice. In some cases, when a 2-day shipment was used, healthy fibroblast lines could still be produced. After propagation for three passages, fibroblasts were transfected by electroporation with a closed circular construct of β-GEO. Following electroporation, transfected cells were selected on 400 μg ml^{-1} of G418. After 3 weeks of selection, single colonies were isolated, propagated and used for nuclear transfer experiments.

Nuclear transplant blastocysts and fetuses were produced from fibroblasts using standard procedures. Basically, *in vitro*-matured oocytes were obtained from Trans Ova Genetics, Inc. by overnight express mail. Oocytes were enucleated following fluorescent labelling of the DNA to verify enucleation. Trypsinized fibroblast cells were transferred to the perivitelline space and fused to the oocyte cytoplast by electroporation. Activation was induced by a combination of calcium ionophore and 6-dimethylaminopurine. The rate of development to the blastocyst stage was about 10% (353/3625) for nuclear transfer embryos and 14% (106/758) for activated controls. Some blastocysts were shipped to Ultimate Genetics, Inc. for transfer into recipient cows. Two blastocysts were transferred into each recipient. Fetuses recovered at day 40 were morphologically normal and fibroblast cells recovered from these fetuses expressed β-galactosidase at a high level. Development to term is in progress.

The results indicate that fibroblast nuclear transplantation may be an ideal method of producing transgenic cattle. Transfection, selection and clonal propagation are relatively easy in primary fibroblasts. The CMV promoter, along with several other constitutive promoters, drive gene expression at a high rate in fibroblasts allowing for routine antibiotic selection. These factors have allowed us to produce a number of transgenic lines with high-expressing random gene inserts. Our preliminary results also indicate that fibroblasts can be grown for a sufficient number of passages *in vitro*, without becoming senescent, to allow a second round of selection for a targeted insert. The fibroblast nuclear transplant system may be a method that will finally allow the commercial production of transgenic livestock for improved agricultural production.

References

Cundiff, L.V., Bishop, M.D. and Johnson, R.K. (1993) Challenges and opportunities for integrating genetically modified animals into traditional animal breeding plans. *Journal of Animal Science* 71 (Suppl. 3), 20–25.

Doetschman, T. (1994) Gene transfer in embryonic stem cells. In: Pinkert, C. (ed.) *Transgenic Animal Technology: a Laboratory Handbook*. Academic Press, New York, pp. 115–146.

Friedrich, G. and Soriano, P. (1991) Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes and Development* 5, 1513–1523.

Purcel, V.G. and Rexroad, C.E., Jr (1993) Status of research with transgenic farm animals. *Journal of Animal Science* 71 (Suppl. 3), 10–19.

Seidel, G.E., Jr (1993) Resource requirements for transgenic livestock research. *Journal of Animal Science* 71 (Suppl. 3), 26–33.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H.S. (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature* 385, 810–813.