Transgenics and Dairy Animal Reproduction: Current Status and Potential

Carol L. Keefer
Nexia Biotechnologies, Inc.
Ste. Anne de Bellevue, QC H9X 3V9
Canada

© Copyright American Association of Bovine Practitioners; open access distribution.

Abstract

Application of transgenic technology to domestic animals has been limited in the past. Improvements in reproductive techniques, including in vitro embryo production, and economic incentives have lead to the implementation of transgenic programs by commercial groups. Transgenic technology incorporates molecular and reproductive techniques in order to direct and harness the tremendous protein synthetic capacity of the mammary gland of dairy animals. Transgenic animals (animals which have exogenous DNA stably integrated into their genome) can be used to express value-added exogenous proteins in their milk for subsequent purification or to increase milk protein and calcium concentration in their milk for increased efficiency of production of processed dairy foods.

Introduction

Reproductive techniques in dairy animals have been moving steadily from the research laboratory to commercial use (Table 1). The pace may seem slow as researchers can visualize practical applications long before efficiencies reach the level required for commercial application. However, within a few decades of initial research, the techniques of artificial insemination (AI) with frozen semen and embryo transfer (ET) with both fresh and frozen embryos have become standard tools in the cattle industry. Newer techniques of *in vitro* maturation (IVM), in vitro fertilization (IVF), embryo sexing, and transvaginal oocyte retrieval (TVOR also known as oocyte pick-up (OPU)) are in a transitional phase, moving from the research laboratory into commercial use. The feasibility of semen sexing has been proven, but it has not yet become a commercial tool. While production of transgenic mice has become a common research tool to study gene function, application of transgenic technology to domestic animals has been limited owing to the cost of running such a research program. Long generation intervals, maintenance cost of recipient herds, and technical difficulties all combine to make the production of transgenic dairy animals a major undertaking. **The estimated cost for producing one transgenic calf is over $500,000 US dollars (Seidel, 1993; Wall, 1996). Smaller dairy animals, goats and sheep, with their shorter generation interval are less costly, but still expensive programs.**

Even with the current low efficiencies and high cost of production of transgenic dairy animals, commercial interest in the production of pharmaceutical proteins in the milk of dairy animals is high (Rudolph, 1995; Velander *et al.*, 1997). This is due in part to the high market value of pharmaceutical proteins and to the fact that current protein production methods using fermentation facilities are over a thousand fold more expensive than using transgenic animals (Bremel, 1996).

What are transgenic animals?

A transgenic animal has a piece of foreign DNA, usually a construct containing a promoter region and the gene coding for a protein, stably integrated into its genome. This foreign DNA is called a transgene. It can be derived from another animal of the same species, from a different species, even from bacteria or plants. Tissue specificity, i.e. expression of the transgene protein in a specific tissue, is conferred by the promoter elements of the transgene. Promoter elements derived from the caseins of the whey protein family could be used to direct expression and secretion of the transgene protein in the milk. In order to get the transgene incorporated into the animal's genome, many copies of the gene construct are injected directly into a pronucleus of a recently fertilized oocyte (Figure 1). The pronuclear DNA is thought to be more receptive to incorporating extra pieces of DNA than nuclei at other stages of development. If the transgene is incorporated at this pronuclear stage, then all cells of the resulting animal (the founder animal for a transgenic line) will contain the transgene. However, in reality, the transgene does not always in-

Presented at the AABP / Society for Theriogenology joint meeting in Montreal, Quebec, Canada, September 18-20, 1997.

Table 1. Transfer of reproductive technology from the research laboratory to the cattle industry.

Procedure	Initial research success	Current Commercial Use	Commercial application
Frozen semen for AI	Polge & Rowson (1952)	widespread	propagation of superior genetics
Embryo transfer	fresh: Willet *et al.* (1951) frozen: Wilmut & Rowson (1973)	widespread	propagation of superior genetics
IVF	Brackett *et al.* (1982)	limited commercial use	propagation of superior genetics, production of transgenic animals
IVM-IVF	Liebfried-Rutledge *et al.* (1986)	limited commercial use	propagation of superior genetics, production of transgenic animals
OPU-IVM-IVF	Pieterse *et al.* (1991)	limited commercial use	propagation of superior genetics, production of transgenic animals
embryo sexing using DNA probes	Bondioli *et al.* (1989)	limited commercial use	propagation of superior genetics
semen sexing	Cran *et al.* (1993)	research applications	
nuclear transfer (cloning)	embryos: Prather *et al.* (1987) embryonic cells: Campbell *et al.* (1996)[sheep]	research applications	
transgenic domestic animals	Hammer *et al.* (1985) [rabbit, sheep, pig] Bierry *et al* (1988) [bovine] Clark *et al,* (1989) [milk-specific in sheep]	limited commercial development	production of pharmaceutical and nutraceutical proteins

© Copyright American Association of Bovine Practitioners; open access distribution.

corporate at this stage but incorporates one or more cleavage divisions later. The result can be a mosaic animal with some cells containing the transgene and others not. Usually multiple copies of the transgene are incorporated into one site on one chromosome, although multiple sites are possible (Pursel & Rexroad, 1993). As long as the germ cells contain the transgene, then approximately half of the founder animal's offspring will be transgenic. However, there is no guarantee that the founder animal or its offspring will express high amounts of the desired protein because of the random integration of the transgene.

Due to the inefficiencies in the technique of pronuclear microinjection, over 1,000 bovine oocytes, 300 sheep and 200 goat PN oocytes must be injection in order to produce one founder transgenic animal (Seidel,1993; Pursel & Rexroad, 1992; Wall, 1996). The larger number of bovine oocytes required is probably due to the use of *in vitro* matured and fertilized (IVM-IVF) oocytes which have a lower developmental rate than that of *in vivo* fertilized oocytes which are commonly used to produce transgenic goats and sheep. Even so, the number of recipients required is large. Improvements in techniques can be expected to reduce the number of oocytes and recipients required.

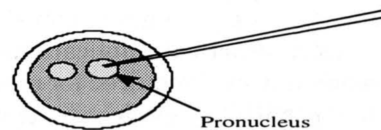

Figure 1. Multiple copies of the DNA constructs are injected directly into the pronucleus of a zygote. In most domestic species, the zygote must be centrifuged in order to visualize the pronuclear structures.

Why make transgenic dairy animals?

Dairy animals have the capacity to synthesize large amounts of milk proteins. Transgenic technology incorporates molecular and reproductive techniques in order to direct and harness this tremendous synthetic capacity. Transgenic animals (animals which have exogenous DNA stably integrated into their genome) can be used to express value-added exogenous proteins in their milk. Alteration of milk through transgenic techniques falls into three general categories; 1) value-added products, e.g. increased casein levels for cheese production, 2) improved nutritional value, e.g. humanized milk for infant formula, and 3) production of proteins for treatment or diagnosis of human disease, e.g. alpha-1-antitrypsin (Karatzas & Turner, 1997). The largest economic incentives are found in the third category, production of proteins for treatment or diagnosis of human disease. Pharmaceutical proteins are products with much higher profit margins than those found in traditional agricultural products. Currently, human pharmaceutical proteins are either isolated from human fluids (e.g., blood-clotting factors) or produced as recombinant proteins in fermentation systems. The first method involves the risk of contamination (e.g., HIV, Creutzfeldt-Jakob disease (CJD)). The second, production of recombinant proteins through mammalian cell culture and bacterial fermentation systems, is very expensive (Bremel, 1996). Production of these proteins in transgenic dairy animals provides significant advantages in areas of health risk and production costs. Transgenic dairy animals capable of secreting human proteins in their milk have been produced in research programs supported by commercial companies (Rudolph, 1995). Genzyme Transgenic Corporation (GTC, Farmingham, MA, USA) has produced transgenic goats which express human antithrombin III, a plasma protein that helps prevent blood clotting, in their milk. Phase II clinical trials for this protein are underway. PPL Therapeutics (Edinburgh, Scotland, UK) has production flocks of sheep for alpha-1 antitrypsin and protein C (Rudolph, 1995). Other human proteins under development in the milk of transgenic animals include collagen, Factor IX and VIII, and human serum albumin (Rudolph, 1995). **While cows produce higher volumes of milk, goat and sheep are the primary dairy animal used in these programs, as they produce sufficient protein levels to meet the market demand and are less costly to maintain.**

Reproductive Technology in the Production of Transgenics

Recent progress made in reproductive technologies has permitted the application of transgenics to dairy animals. Almost all of the currently used reproductive techniques, including AI, ET, OPU, IVM, IVF, and sexing, have a role in the building of transgenic herds. As large numbers of pronuclear staged oocytes must be injected, slaughterhouse derived oocytes which are matured, fertilized and cultured *in vitro* are the most economic source of oocytes. If genetic background and disease status must be controlled then either TVOR for collection of oocytes or tagging of ovaries during slaughter can be utilized. With high value genetics TVOR would be preferred. In sheep and goats *in vivo* fertilized oocytes are generally used, owing to the limited availability of slaughterhouse derived ovaries, at least in the US and Canada. These are collected by a surgical procedure which is relatively simple in the smaller dairy animals. Culture of injected zygotes *in vitro* allows selection of viable embryos prior to transfer. In cattle programs, embryos are transferred at the morula-blastocyst stage by standard ET practices. In sheep and goats, injected zygotes can be transferred surgically to the oviduct immediately after injection or cultured *in vitro* for several days prior to transfer by surgical or laparoscopic procedures to the uterus (Ebert & Schindler, 1993). In our own goat program, we have achieved acceptable pregnancies rates (33-60%) with transfer 48 to 144 hr after initial collection and injection. Although embryo biopsies can be taken prior to ET to determine sex, this technique can not yet be used accurately to determine whether the embryo is transgenic. Improvements in molecular techniques should resolve this problem and allow transfer of only viable, transgenic embryos (Wall, 1996). This selection would greatly decrease the cost of transgenic animal production, as the recipient herd contributes a large portion to the expense.

At birth the offspring must be tested for the presence of the transgene. As an animal may be mosaic, its offspring must also be tested. If the founder has the transgene stably integrated into its genome and correctly expresses the gene (foreign protein secreted into the milk), then the transgenic line can be extended rapidly through the use of semen from a male founder or by ET from a female founder. Embryo splitting and nuclear transfer (embryo cloning) may also be used to increase the number of animals in a transgenic line.

One of the key issues in production of pharmaceutical proteins in milk is whether the foreign protein will have any effect on physiology and lactation of the transgenic animal. Although many proteins of biomedical importance have been expressed successfully in the mammary glands of transgenic animals, several examples illustrate the adverse effects of the "newly expressed protein." Transgenic goats that express human tissue plasminogen activator in their milk experienced premature shut down of milk production, which has been attributed to the high levels and interactions of the newly expressed protein with its casein

© Copyright American Association of Bovine Practitioners; open access distribution.

components (Ebert *et al.*, 1994). In another example, transgenic rabbits that expressed low levels of erythropoitin in their milk showed leakage of the protein into their blood. These animals were infertile and had abnormally viscous blood with a high haematocrit (Massoud *et al.*, 1996). Appropriate expression can be obtained with careful design of DNA constructs and prescreening of constructs through cell lines and transgenic mice. In addition to producing a transgenic line which secretes acceptable levels of the targeted protein in the milk without affecting the physiology of the animal, issues of purification of the protein, clinical trials, and regulatory approval must also be considered.

Application of developing reproductive technologies

How will the developing technologies of semen sexing, nuclear transfer, and embryonic stem cells be utilized in a transgenic program? Semen sexing would be utilized in the same manner as it will be in the dairy and beef industries. Semen from transgenic males will be sorted and used to produce offspring of the desired sex, most likely, females. Nuclear transfer and embryonic stem cells could be used to produce transgenic animals much more efficiency. Unlike direct injection of DNA into pronuclear oocytes, embryonic cell lines could be transfected *in vitro* using standard techniques. This may include chemical (lipids, calcium phosphate), physical (electroporation, gene gun bombardment, direct injection) or retroviral transfection. Cells could be selected for appropriate integration into the genome. In this manner, a cell line could be derived which would have a stably integrated transgene. Any offspring produced by nuclear transfer using cells from the line would be transgenic. This would represent a tremendous increase in efficiency over the low percentage (<10%) of transgenic offspring currently expected. Furthermore, with appropriate selection and screening of the cell lines, most of the transgenic animals produced should also appropriately express the transgene. Embryonic stem (ES) cells are commonly used to produce transgenic mice (Robertson, 1987). The technique used in mice is that of chimera formation in which ES cells are injected into a host embryo. The resulting chimeric animal has cells derived from both the transgenic embryonic stem cells and from the host embryo. Due to difference in the developmental biology of mice and domestic animals, chimera production with germ line contribution by the ES cells has not been achieved in domestic animals. Chimeric pigs have been produced, but germ line chimeras have not been reported (Wheeler, 1994). However, lambs have been produced by nuclear transfer using embryonic and even adult-derived cell lines (Campbell et al, 1996: Wilmut *et al.*, 1997). As offspring produced by nuclear transfer are derived from the single donor cell (Figure 2), then all cells, including the germ cells, will be identical genetically and the problems associated with chimeras can be avoided.

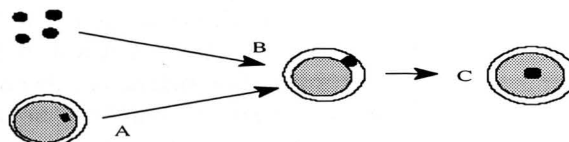

Figure 2. In the step A of the nuclear transfer procedure the recipient oocyte is enucleated (metaphase chromosomes removed). In step B the isolated donor cell is transferred under the zona pellucida of the enucleated oocytes. In step C, the donor cell and oocyte are fused by an electrical pulse resulting in an activated zygote containing the DNA of the donor cell and the ooplasm of the recipient cytoplasm.

Summary

While application of transgenic technology to domestic animals has been limited, improvements in reproductive and molecular techniques and economic incentives have lead to the implementation of transgenic programs by commercial groups. While costs for producing a transgenic calf can be as high as 500,000 US dollars, fermentation costs for production of pharmaceutical proteins can be even higher. Smaller dairy animals, goats and sheep, with their shorter generation interval are even less costly and can produce sufficient protein to meet the market demand of most pharmaceutical proteins.

References

Bierry KA, Bondioli KR, DeMayo FJ. Gene transfer by pronuclear injection in the bovine. *Theriogenology* 1988; 29:224. Bondioli KR, Ellis SB, Pryor JH, Williams MV, Harpold MM. The use of male-specific DNA chromosomal fragments to determine sex of bovine implantation embryos. *Theriogenology* 1989; 35:95-104. Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF, Dressel MA. Normal development following *in vitro* fertilization. *Theriogenology* 1982;27:147-158. Bremel RD. Potential role of transgenesis in dairy animal production and related areas. *Theriogenology* 1996; 45:51-56. Campbell KHS, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 1996; 380:64-66. Clark AJ, Bessos H, Bishop JO, *et al.* Expression of human anti-hemophilic factor IX in the milk of transgenic sheep. *Biotechnology* 1989;7:487-492. Cran DG, Johnson LA, Miller NG, Cochrane D, Polge C. Production of bovine calves following separation of X- and Y-chromosome bearing sperm and in vitro fertiliztion. *Veterinary Rec* 1993; 132:40-41. Ebert KM, DiTullio P, Barry CA, Schindler JE, Ayres SL, Smith TE, Pellerin LJ, Meade HM, Denman J, Roberts B. Induction of human tissue plasminogen activator in the mammary gland of transgenic goats. *Biotechnology* 1994;12:699-702. Ebert KM, Schindler JES. Transgenic farm animal: Progress report.

© Copyright American Association of Bovine Practitioners; open access distribution.

Theriogenology 1993; 39:121-135. Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL. Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* 1985; 315:680-685. Karatzas CN, Turner JD. Toward altering milk composition by genetic manipulation: Current status and challenges. *J Dairy Sci* 1997: in press. Leibfried-Rutledge ML, Critser ES, Eyestone WH, Northey DL, First NL. Developmental potential of bovine oocytes matured *in vitro* or *in vivo*. *Theriogenology* 1986;25:164. Massoud M, Attal J, Thepot D, Pointu H, Stinnakre MG, Theron MC, Lopez C, Houdebine LM. The deleterious effects of human erythropoietin gene driven by the rabbit whey acidic protein gene promoter in transgenic rabbits. *Reprod Nutr Dev* 1996; 36:555-563. Pieterse MC, Vos P, Kruip T, Wurth YA, Beneden TH van, Willemse AH, Taverne M. Transvaginal ultrasound guided follicular aspiration of bovine oocytes. *Theriogenology* 1991; 35:19-24. Polge C, Rowson LEA. Fertilizing capacity of bull spermatozoa after freezing at -7°C. *Nature* 1952; 169:626-627. Prather RS, Barnes FL, Sims MM, Robl JM, Eyestone WH, First NL. Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient cytoplasm. *Biol Reprod* 1987; 37:859-866. Pursel VG, Rexroad CE, Jr. Status of research with farm animal. *J Anim Sci* 1993; 71 suppl. 3:10-19. Robertson EJ. Embryo-derived stem cell lines. In EJ Robertson (ed): "Teratocarcinomas and Embryonic Stem Cells: A practical approach" *Oxford: IRL Press*, 1987; 71-112. Rudolph NS. Advances continue in production of proteins in transgenic animal milk. *Genetic Engineering News* 1995; October 15, 1995 issue, 8-9. Seidel GE Jr. Resource requirements for transgenic livestock research. *J Anim Sci* 1993;71 suppl.3: 26-33. Velander WH, Lubon H, Drohan WN. Transgenic livestock as drug factories. *Scientific American* January 1997:70-74. Wall RJ. Transgenic livestock: Progress and Prospects for the future. *Theriogenology* 1996; 45:57-68. Wheeler MB. Development and validation of swine embryonic stem cells: a review. *Repeod Fertil Dev* 1994: 6:563-568. Willet EL, Black WG, Cadida LE, Stone WH, Buckner PG. Successful transplantation of a fertilized bovine ovum. *Science* 1951; 113:247. Wilmut I, Schnieke AE, McWhir J, Kind AJ, KHS Campbell Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997; 385:810-813. Wilmut I, Rowson LEA. Experiments on the low-temperature preservation of cow embryos. *Veterinary Record* 1973; 92:686-690.

Abstract

Use of physiologic variables to predict milk yield after clinical mastitis in dairy cattle

W. M. Sischo, *et al*
J. Am. Vet. Med. Assoc., 1997; 211:470-475

Eighty-two cows with clinical mastitis (22 coliform, 22 Streptococcus or Staphylococcus sp, 38 without bacterial growth) were used in an observational study of the association between clinical variables and mature equivalent 305-day (ME305) milk production. For cows with coliform mastitis 5 days after clinical detection, higher serum sorbitol dehydrogenase activity and WBC values were associated with higher values for ME305 milk production, whereas PCV values had an overall negative association. For streptococcal and staphylococcal mastitis, higher PCV values were associated with lower ME305 milk production. Positive response in milk production associated with an increasing number in of WBC suggested that cows responding to infection by mobilizing WBC are better able to neutralize mammary gland infections, resulting in better production. The negative response in milk production associated with an increase in PCV suggested that maintaining hydration in affected cows may be a critical aspect of treating cows with mastitis.

© Copyright American Association of Bovine Practitioners; open access distribution.