Reproductive Technologies and Transgenics

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Introduction

Astounding advances have occurred over the past 12 years (1985–1997) in our ability to control, manipulate or change the reproduction and genetics of cattle. These new techniques are the focus of this chapter. The techniques include the following:

1. The ability to produce, culture, study and use commercially *in vitro* produced embryos. The embryos can be biopsied for preimplantation diagnosis of gender and genetic traits. The oocytes can be retrieved in large numbers from valuable cows via biweekly transvaginal follicular retrieval or obtained from an abattoir.

2. The new techniques also include the production of identical twins by splitting embryos or the production of multiple copies of valuable embryos by transferred nuclei from blastomeres, cultured embryonic cells or fetal cells, and

 The Genetics of Cattle (eds R. Fries and A. Ruvinsky)

in three cases even adult cells, into enucleated oocytes. This technique is being modified in order to improve efficiency.

3. Lastly, to have the ability to introduce deoxyribonucleic acid (DNA) into the genome of animals, including cattle. This technique was developed following the first gene transfers in mice, which resulted in transgenic mice (Gordon *et al.*, 1980; Brinster *et al.*, 1981).

The technologies of *in vitro* embryo production, gene transfer, genetic analysis and diagnosis and embryo cloning have the potential to be used synergistically in cattle breeding and improvement. Both gene transfer and cloning by nuclear transfer (NT) require the ability to culture embryos and preserve them *in vitro* and often the ability to produce the embryos themselves *in vitro*. The efficiency of making or multiplying transgenic animals is improved by the use of NT and subsequent cell-culture techniques. For example, NT allows multiplication of valuable transgenics or the transfer of genes into culture-multiplied cells, of which each has the potential to become an embryo and offspring after use in NT. The use of cultured cells for gene transfer also allows the possibility for gene deletion by DNA homologous recombination transfer (Koller *et al.*, 1989; Joyner, 1991; Melton, 1994; Cibelli, *et al.*, 1998a).

The *In Vitro* Production of Bovine Embryos

The ability to produce embryos of cattle *in vitro* began with studies of Iritani and Niwa (1977) and Brackett *et al.* (1982). Iritani and Niwa (1977) were the first to attempt to fertilize *in vitro*-matured bovine oocytes, while Brackett *et al.* (1982) were the first to produce a live calf from *in vitro* fertilization (IVF). However, this calf was developed from an oocyte matured *in vivo* and an embryo that developed *in vivo*. The first efficient system for maturing bovine oocytes, capacitating sperm, fertilizing *in vitro* and defining the variables was published by Ball *et al.* (1983). Soon thereafter, the first offspring from IVF of *in vitro*-matured oocytes were produced by Critser *et al.* (1986) and by Hanada *et al.* (1986).

In early experiments, such as Brackett *et al.* (1982), embryos resulting from IVF were developed *in vivo* by surgical transfer into the oviducts of cows immediately after fertilization. This *in vivo* culture was replaced by culture in sheep oviducts (Willadsen, 1986; Eyestone and First, 1989), then by co-culture of embryos with oviduct epithelial cells or oviduct-conditioned media (Eyestone and First, 1989) and finally by culture in minimal-composition media supplemented with amino acid(s) (Rosenkrans and First, 1994), media that mimic the composition of the oviduct (Tervit *et al.*, 1972; Takahashi and First, 1992) or media designed to be used in a two-stage sequence to meet embryo nutrient requirements and to reduce cell damage (Gardner *et al.*, 1997; Gardner, 1998). The applications, components, influencing variables, problems and success rates of bovine IVF are the subject of the following discussion.

The *in vitro* production of embryos is a technique that is gaining use in: (i) commercial embryo production from *in vivo*-recovered oocytes of valuable cows (Hasler *et al.*, 1995; van Wagtendonk-de Leeuw *et al.*, 1998); (ii) production of calves from cows not responding to conventional, superovulation and embryo-transfer techniques (Looney *et al.*, 1994); and (iii) commercial production of low-cost embryos from oocytes harvested from abattoir-recovered ovaries, a system called beef production without brood cows (Agca *et al.*, 1998). Collectively, considering all applications, there are at least ten commercial companies producing more than 10,000 calves per year. The process is less than perfect and of lower efficiency than *in vivo* production of embryos (van Wagtendonk-de Leeuw *et al.*, 1998). From some, but not all, laboratories, there are reports of increased frequency of failed or late delivery and larger than normal calves of lower survival rate.

The best success rates are approximately 70–80% of oocytes completing maturation, 70–80% completing fertilization and the first cleavage division, 30–60% of cleaved embryos developing to blastocyst (a stage compatible with non-surgical uterine embryo transfer), 50–60% of these achieving pregnancy and 80% of the pregnancies completing gestation with a live calf. This results in a post-fertilization offspring production efficiency of approximately 20%.

The critical steps in the process are *in vitro* maturation of oocytes, preparation of sperm for participation in fertilization (sperm capacitation and the acrosome reaction), the fertilization process and embryo culture. More and more, it is being recognized that these are not independent events, with later events such as embryo development in culture, highly influenced by variables affecting oocyte maturation and independent of the frequency of oocytes matured. Some of the variables affecting development competence of oocytes (reviewed by Gordon, 1994) are: (i) the age of the females supplying the oocytes; (ii) their health and environmental stress, such as heat stress; (iii) the size and maturity of follicles; (iv) the size of the oocyte (Arlotto *et al.*, 1996); (v) the presence and interaction of cumulus cells with the oocyte; (vi) the conditions of oocyte maturation, such as temperature, pH and gas environment (Lenz *et al.*, 1983); (vii) the number of cohort oocytes; and (viii) the presence of cumulus cells and cell growth factors in the culture media (reviewed by Bavister, 1990; Leese, 1991; Gordon, 1994; Gardner *et al.*, 1997; Leibfried-Rutledge *et al.*, 1997; Gardner, 1998).

Preparation of sperm to bind, penetrate and fertilize oocytes is dependent on preparation of sperm to undergo the acrosome reaction (sperm capacitation) and involves changes in the sperm plasma membrane, allowing the fusion of the outer and inner acrosomal membrane (acrosome reaction). This allows the escape of acrosomal enzymes involved in sperm–oocyte interaction and the passage of sperm through the zona pellucida of the egg (Parrish and First, 1993). The process of sperm capacitation is accomplished at least in part in the oviduct by using heparin sulphate; this compound is the most commonly used agent for causing capacitation of bovine sperm *in vitro* (Parrish *et al.*, 1988, 1989).

The fertilization process and its completion are influenced by conditions of fertilization, such as temperature and media composition (Lenz *et al.*, 1983), by the age of the metaphase II oocyte and by the time kinetics of the sperm–egg interaction (Rose and Bavister, 1992; Dominko and First, 1997). It is also influenced by the sire contributing the sperm (Hillery *et al.,* 1990).

Development of embryos to blastocyst stage, a non-surgical transfer stage, is heavily influenced by conditions of oocyte maturation and the age of the oocyte (Dominko and First, 1997; Leibfried-Rutledge *et al.*, 1997), the sire contributing the sperm (Leibfried-Rutledge *et al.*, 1989; Hillery *et al.*, 1990) and the sex of the embryo (Bredbacka and Bredbacka, 1996). Embryo development is also influenced by conditions of embryo culture, such as media composition, oxidative and peroxidative damage, gas and temperature conditions of incubation and growth factors (Gardner *et al.*, 1997; Leibfried-Rutledge *et al.*, 1997).

The steps in *in vitro* production of embryos are continuously under study. A better understanding of the mechanisms is being achieved and the efficiencies are slowly improving. For greater detail, the reader is referred to several recent reviews (Shamsuddin *et al.*, 1996; Leibfreid-Rutledge *et al.*, 1997; Sirard *et al.*, 1998; Yang *et al.*, 1998).

A modification of the *in vitro* embryo production procedure that has considerably extended its application to oocyte retrieval from live cows and especially cows of high genetic value is transvaginal, ultrasound-guided oocyte recovery (Kruip *et al.*, 1991; Pieterse *et al.*, 1991) as depicted in Fig. 14.1. This

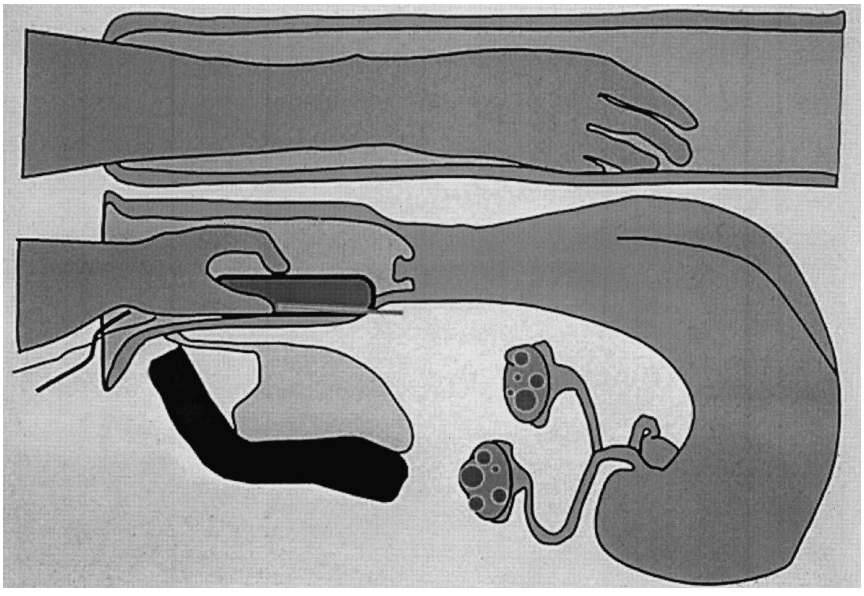

Fig. 14.1. Transvaginal ultrasound-guided retrieval of bovine oocytes. The hand in the rectum places each ovarian follicle on an aspiration needle attached to the ultrasound imaging transducer. The needle penetrates the vaginal wall. Oocytes can be retrieved from unstimulated follicles twice a week. (Redrawn from Kruip *et al.*, 1991.)

is used on a commercial basis to recover primary oocytes from antral follicles, which will be matured, fertilized and cultured to the blastocyst stage using *in vitro* procedures (Looney *et al.*, 1994; Hasler *et al.*, 1995). The procedure requires no superovulation, is minimally invasive and can be done twice per week to recover 15 to 18 oocytes per cow per week (Kruip *et al.*, 1994; Looney *et al.*, 1994; Hasler *et al.*, 1995).

In vitro production of embryos is constantly becoming a more useful tool for maximizing the number of offspring from a valuable cow, producing calves from infertile cows and producing commercial beef cattle in a programme for beef production without brood cows. An important application is the provision of oocyte and embryo culture systems facilitating the practice of gene transfer and animal cloning.

Production of Calves of a Predetermined Sex

The literature is rich in the history of methods attempting to produce offspring exclusively of one sex or the other, as reviewed by Johnson (1994) and Reubinoff and Schenker (1996).

Currently two methods appear feasible for the production of sex-specified cattle and each has limitations concerning how it is applied. The first is the sorting of X and Y chromosome-containing spermatozoa. The only reliable method to date is separation of X and Y by flow-cytometric sorting. This technique is based on determination of the difference in fluorescently labelled DNA of individual spermatozoa in a small flow of medium. Using filtered laser light to illuminate a fluorescent DNA label that spectrophotometrically discriminates between the chromosomes, the sorter gates the spermatozoa according to quantitative fluorescent characteristics of X or Y. The X and Y purity rates range from 85 to 90% (Johnson *et al.*, 1994; Johnson, 1996).

The number of sperm sexed from one ejaculate is adequate for several IVFs but is, however, of quality and quantity insufficient at present for commercial artificial insemination. In spite of this, calves have been born of the predicted sex after artificial insemination with sexed sperm (Seidel *et al.*, 1996). The sexed sperm are of reduced longevity and cannot at present survive freezing. Best success has been with the use of IVF within approximately 6 h after sorting.

The exposure of Hoechst 33342 DNA-labelled sperm to laser light has the potential to cause chromatin damage. Although no deformed or chromatin-damaged offspring have thus far occurred across several species for offspring derived from this sexing procedure, there is a high rate of early embryonic loss, which is suspected to be due in part to chromatin change (Johnson and Schulman, 1994; Reubinoff and Schenker, 1996).

The high-speed flow cytometers in use sort at a rate of 10,000–13,000 sperm s^{-1}. Sorters of four to five times greater speed are becoming available. With higher sort speed and greater sort efficiency, this method may become commercially useful for cattle artificial insemination.

The second reliable method for producing sex-specified offspring is the sexing of cells biopsied from preimplantation embryos, using polymerase chain reaction (PCR) amplification of DNA sequences specific for the Y or for the X chromosome. This method relies on the availability of well-characterized primers which enable amplification of sex-specific regions or single-copy sequence in a multiplex reaction with a positive amplification control. This has been developed and used successfully in several laboratories and with different X or Y chromosome-specific sequences and method variations of the procedure (Avery *et al.*, 1992; Kirkpatrick and Monson, 1993; Hyttinen *et al.*, 1996).

A limitation of the cell biopsy and X–Y PCR method is poor post-freeze survival and subsequent developmental capacity of the previously biopsied embryos.

The Production and Uses of Transgenic Livestock

History, applications and limitations

The ability to make transgenic animals was initially developed in mice. In 1974, Jaenisch and Mintz showed that injection of Simian Virus 40 (SV40) DNA into the blastocoele cavity of mouse embryos resulted in mice carrying the SV40 sequence. Early studies also demonstrated the ability of other viruses to infect their DNA into cells; however, non-replicating viral vectors for introduction of exogenous DNA had not yet been developed when Gordon and colleagues showed that transgenic mice could be developed from mouse eggs microinjected with DNA (Gordon *et al.*, 1980). Within the next few months, this and four other groups reported similar success in stably microinjecting foreign DNA into the genome of the mouse (Brinster *et al.*, 1981; Costantini and Lacy, 1981; Gordon and Ruddle, 1981; Wagner, E.F. *et al.*, 1981; Wagner, T.E. *et al.*, 1981). Some of the foreign genes were expressed in somatic cells (Brinster *et al.*, 1981; Wagner, E.F. *et al.*, 1981; Wagner, T.E. *et al.*, 1981) and in the germ line (Costantini *et al.*, 1981; Gordon and Ruddle, 1981), with offspring of founder mice continuing to express the foreign DNA (Palmiter *et al.*, 1982). Gordon and Ruddle gave the resulting animals expressing the new gene the name 'transgenic'. The experiments most noted by animal scientists were those of Palmiter *et al.* (1982, 1983), wherein a metallothionein-promoter growth-hormone fusion-gene construct was injected into mouse pronuclei, resulting in mice growing much faster and sometimes two times larger than normal non-transgenic mice. Animal scientists quickly attempted to repeat these studies in attempts to greatly increase animal growth rate in pigs, sheep and cattle. Unfortunately, efficiencies of producing transgenics were low in domestic animals (Table 14.1; reviews in Rexroad, 1992; Brem and Muller, 1994; Eyestone, 1994; Colman, 1996; Wall, 1996). Additionally, domestic species with determinant growth characteristics sometimes produced leaner

Table 14.1. Efficiencies of producing transgenic offspring from microinjected zygotes in livestock.

Species	Number of transgenic offspring/number of zygotes injected (%)		Number of injected zygotes required/ transgenic offspring
Cow*	8/5,030	(0.16%)	625
Sheep	45/5,394	(0.80%)	125
Goat	12/985	(1.20%)	83
Pig	182/22,429	(0.80%)	125

**In vivo*-produced zygotes only, from Eyestone (1994).

carcasses but without the expected improvements in growth rate or growth efficiency (Rexroad, 1992).

More efficient methods for making transgenic cattle

In spite of the inefficiency of pronuclear microinjection of DNA, a few transgenic sheep, goats and cattle of extremely high commercial value have been made for the purpose of production of valuable pharmaceuticals in the milk. Companies devoted to this effort include Pharming, Protein Products Limited (PPL), Genzyme Transgenics (GTC) and Gala Design. Some of the products include blood-clotting factor IX, antitrypsin, tissue plasminogen activator and hepatitis antigen. The value in expressing a gene for a pharmaceutical product in milk appears to be in quantitative yield of the protein, especially with cattle and in the ability of mammary cells to provide authentic and proper post-translational processing, such as glycosylations of the protein to be produced (for review, see Bremel, 1996; Ziomek, 1998). In spite of several years' effort and considerable financial support for the production of new proteins in milk, none of the new milk-produced proteins has yet reached the market. The first two products, recombinant human antithrombin III (GTC) and alpha I antitrypsin (PPL), are in or have completed phase II human clinical trials, respectively. They are headed towards or are in phase III trials, respectively, and may reach the marketplace around the turn of the century (year 2000) (Ziomek, 1998). The majority of the products are being made in goats and sheep, respectively, in which transgenic efficiencies from DNA microinjection are more than four times greater than in cattle. The major advantage for use of cattle is the higher milk and protein yield (approximately 8000 l $year^{-1}$, and 10–20-fold greater than in sheep or goats). A disadvantage in cattle is the extended period between the microinjection of DNA into the embryo and the time that the cows first lactate (cows lactate beginning at 30–33 months, approximately twice as late as sheep and goats). The long generation interval is also a disadvantage when producing offspring to be derived from a founder animal.

Critical limitations to transgenesis in cattle are inefficiency of embryo DNA microinjection, and long generation interval (Ziomek, 1998). These limitations

are overcome for pharmaceutical milk production by transomatic gene transfer, wherein the DNA is injected into the mammary gland of lactation-induced females (Bremel, 1996). This injection can be by recent, efficient, pseudotyped, viral-vector methods of gene transfer (Chan *et al.*, 1998) and by the ability to selectively produce female offspring as described on pp. 415–416 or by DNA transfer into female cells used to make offspring by NT or embryonic cell–embryo chimeras (Cibelli *et al.*, 1998a, b).

While this transgenic application is a growing industry in itself, the range of products possible for transgenic production are severely limited by the costs associated with the process. Indeed, the range of possibilities as the transgenic process becomes more efficient appears to include, in an economic hierarchical order, pharmaceuticals, antigens, nutraceuticals and new food products, such as sweeteners, new cheeses, etc., specific disease-resistant animals, animals of changed meat quality and increased milk or wool production or more efficient growth.

In spite of the present cost limitations of transgenic cattle for use in animal breeding, there are emerging possibilities for vastly improved efficiencies in transgenics. One approach has been the introduction of DNA into oocytes or zygotes by replication-defective viral vectors. Replication-defective retroviral vectors were developed based on the discoveries of reverse transcription and the helper-cell principle by Temin (Temin, 1987; Varmus and Brown, 1989; Kim *et al.*, 1993; Haskell and Bowen, 1995; Chan *et al.*, 1998). Replication-defective ribonucleic acid (RNA) viral vectors are usually restricted to a DNA insert of 10–15 kb. The virions cannot be concentrated and the vector efficiency is dependent on the virion titre achieved.

A particularly useful modification of the retroviral vector is a pseudotyped or hybrid vector, in which the retroviral envelope is replaced with a vesicular stomatitis virus envelope (VSVG), as developed by Burns *et al.* (1993). Because of its VSVG, this vector can be concentrated to high infectivity, with titres of 10^9–10^{11} virions. This vector combines the VSVG with a common Maloney murine leukaemia retroviral vector (MoMLV).

Chan *et al.* (1998) used this vector to introduce DNA into the perivitelline space outside the plasma membrane of bovine metaphase II oocytes. Metaphase II is a stage wherein chromatin is exposed to cytoplasm after nuclear envelope breakdown and wherein membrane–viral vesiculation transfers the viral and gene DNA sequences of interest into the ooplasm.

When genes for neomycin resistance and β-galactosidase were included in the vector construct, 57% of the resulting embryos were positive for β-galactosidase expression. The MoMLV–VSVG pseudotyped vector was also constructed to contain the gene for hepatitis B surface antigen, as well as neomycin resistance (PLSRNL) and infected into oocytes, as above, or into pronuclear zygotes. Of 836 oocytes and 584 zygotes infected, 21% and 33%, respectively, became embryos or blastocysts. Ten blastocysts resulting from oocyte injection and 12 blastocysts from zygote injection were randomly selected for embryo transfer into five or six cows, respectively. Four calves were born from each group. All four of the oocyte-infected calves expressed

the hepatitis B antigen and were transgenic, whereas one of the four zygote infected calves was transgenic.

Pseudotyped viral vector infection of DNA into the metaphase II oocyte immediately prior to fertilization appears to be an efficient method for gene transfer. When used to introduce DNA into oocytes, there is an overall efficiency of approximately 8–10% in producing offspring, as compared with approximately 0.02% for conventional pronuclear injection of DNA, as shown in Table 14.1 for cattle. The principal limitation was not in the viral transgenic methodology but in the efficiency of reproductive steps, wherein only 20% of oocytes became blastocysts and 40% of blastocysts transferred into cows became offspring. The same vector is also being used to infect DNA directly into the mammary gland to produce new proteins in milk. This process is called transomatic infection (Bremel, 1996).

Improvements in the *in vitro* production of embryos should enhance the efficiency of the oocyte–pseudotyped viral-vector method. Two limitations of the method are the small size of the DNA construct that can be built into the vectors (approximately 15 kb) and the fact that some viral DNA could be germ-line-transmitted with the gene of interest. Positive aspects are the potential for high-efficiency transfer of genes into oocytes, cultured cells or embryos.

The pseudotyped viral vector infection of DNA into cells can also be an efficient method for the introduction of DNA into cultured embryonic or fetal cells (Chan *et al.*, 1998), which are then used in NT to produce offspring (Cibelli *et al.*, 1998a, b; Schnieke *et al.*, 1997). Other, but less efficient, methods for the introduction of DNA into cultured cells, are ballistic, electroporation and liposome methods. The advantages of transferring DNA into cultured cells are several. Homologous recombination can be used to target a known sequence of the gene construct to the same known sequence in the genome hierarchy, thereby accomplishing site-specific gene transfer or gene deletion. Additionally, embryos can be screened for gene expression when an expressed and selectable gene marker at an embryonic stage is used in the construct. Only the embryos that are expressing the transgene are transferred into cows. The transgenic embryos can also be used to make embryonic cell lines. The cell line can be used either by NT (Cibelli *et al.*, 1998a) or by chimerization of cells (Cibelli *et al.*, 1998b) into a normal embryo to produce bovine blastocysts and ultimately offspring. If the cells are multiplied to large numbers in culture, gene deletion can be accomplished in the culture by the technique of homologous recombination (Koller *et al.*, 1989; Joyner, 1991; Melton, 1994; Cibelli *et al.*, 1998a). The production of transgenic fetal fibroblasts, which are then used as nuclear donors in NT to produce transgenic sheep (Schnieke *et al.*, 1997) and transgenic cattle (Cibelli *et al.*, 1998a, b) has recently been reported (reviewed by Stice *et al.*, 1998).

A variation of site-specific gene transfer is to utilize DNA sequences that can enhance homologous recombinations between foreign DNA and the genome (Wall and Seidel, 1992). A version of this technique that has been used and tested in the mouse is the cre-lox system, in which known lox sites are built by transgenesis into a mouse strain and then reduced to a small

number of insertion sites by titrated cleavage of lox sites with the Cre protein. This lox site containing recipient strain is then used in gene transfer with constructs designed to target the lox sites (Rucker and Piedrahita, 1997). The negative aspect of this system for site-specific gene transfer in cattle is the high cost of building and maintaining a lox strain of cattle as recipients for gene transfer.

A third and highly controversial approach to the transfer of genes into animals or birds is the introduction of DNA into spermatozoa by co-incubation, transfection, gene gun or liposome, with the expectation that the introduced DNA will be carried to fertilization of an oocyte, integrated into the zygotic genome and later expressed. This method was developed initially by Lavitrano *et al.* (1989). Transgenic mice resulted from its use. Numerous other laboratories attempted to repeat the process. Most found the DNA bound to spermatozoa, but transgenic offspring or embryos did not result (Brinster *et al.*, 1989). More recently, it has been shown that interaction of sperm and DNA is dependent on a molecular mechanism involving cooperation of specific protein factors (Bird *et al.*, 1992; Lavitrano *et al.*, 1992; Zani *et al.*, 1995). This DNA binding and uptake process is inhibited by a factor in seminal plasma (Lavitrano *et al.*, 1992). Treatment of the DNA with liposomes enhances DNA uptake in DNA liposome–sperm co-culture (Bachiller *et al.*, 1991). It has been suggested that DNA molecules first bind to sperm and are then integrated into the plasma membrane and migrate into the nucleus (Francolini *et al.*, 1993).

Recently, Shemish has reported liposome introduction of a restriction enzyme–green fluorescent protein (GFP) DNA construct into chicken sperm and claims that lymphocytes of 17 of 19 resulting chickens express the GFP, as do lymphocytes of their offspring (M. Shemish, personal communication). Scientists await confirmation of these studies, but, if repeatable, the latter represents a highly efficient method for producing transgenic birds and perhaps cattle.

Another approach to sperm-mediated DNA transfer is the *in vitro* introduction of DNA into the cells of the testes. The transgenic testis cells are then injected into sterilized seminiferous tubules of an animal to repopulate the tubule and become spermatozoa expressing a new gene. This approach was sparked by Brinster and Zimmerman (1994) when they transplanted testis cells into sterilized seminiferous tubules of mice and showed that spermatozoa resulted from the transferred cells. Subsequently, Kim *et al.* (1997) transferred a liposome Lac-Z DNA preparation into seminiferous tubules of mice and pigs after the native population of spermatocytes had been destroyed by busulphan treatment. The DNA was introduced into a spermatogonial stem-cell population, which eventually became Lac-Z DNA-containing spermatozoa (Kim *et al.*, 1997).

Considering the transgenic chicks (M. Shemish, personal communication) and the DNA-carrying spermatozoa of mice and pigs (Kim *et al.*, 1997), it seems that we may be very near a time when transgenic animals could be efficiently made from spermatozoa carrying exogenous DNA. The promising results with viral introduction of DNA, engineering animals from transgenic cells and perhaps sperm-mediated DNA transfer suggest that animal geneticists

and breeders may soon consider transgenic approaches to genetic change to be possible and affordable. The specificity of gene introduction into a population as compared with gene selection techniques could have an impact on the speed and precision with which genetic change is made and excites the intellect with possibilities for the introduction of genes that are foreign to a population. This could be especially useful in meat animals, such as pigs, cattle and poultry, to capitalize on the muscle development potential realized by suppression of the myostatin gene (Mcpherron and Lee, 1997; Grobet *et al., 1998).*

Cloning Cattle by Nuclear Transfer

Overview, biological mechanisms and constraints

The cloning of animals received much attention in 1997 and 1998, with the production of lambs and calves by nuclear transplantation of differentiated or immortalized fetal and adult cells into enucleated metaphase II oocytes (Schnieke *et al.*, 1997; Wilmut *et al.*, 1997; Cibelli *et al.*, 1998a, b). Although not yet highly efficient, the ability to clone animals from differentiated cells has challenged our understanding of the irrevocable nature of cell differentiation and germline totipotency, based largely on early amphibian studies. Essentially, cloning from differentiated cells rests in the ability of a cell nucleus to retain and retranscribe the complete array of messages previously turned on and turned off with cell differentiation, as well as the ability of a properly timed and prepared metaphase II oocyte to completely erase the differentiation repertoire of the donor cell. This has to be done in such a way that the introduced nucleus is capable of re-expressing its entire genome. The efficiency of the NT process depends on the completeness with which all donor-cell chromatin is captured within the new zygotic nucleus, the cell-cycle matching of donor cell and oocyte and the ability of the oocyte to demethylate or dedifferentiate all DNA of the donor nucleus. It depends on the appropriate incorporation of oocyte proteins, such as nuclear lamins, into the nucleus of the new cell. It also depends on the re-establishment of normal-length chromosome telomeres and the ability of the first one or two zygotic cell cycles to ensure chromatin normality through exercise of cell-cycle checkpoints and DNA repair mechanisms. Historically, research leading to this point was focused on the use of embryonic or germline lineage cells, expected to be totipotent. The ability to clone animals from differentiated fetal or adult cells has scientific value, in causing re-examination of issues such as the differentiation and totipotency of cells, the ageing of cells and gene expression.

The principal application of cloning cattle appears to be in propagation of valuable transgenic cattle or high-performance cattle. Although the genes of clones are identical, geneticists estimate from twin studies that environmental influences will modulate expressed phenotype, leaving clones approximately 75% alike in performance (G. Shook, personal communication).

Cloning of animals began in the 1950s and resulted in the dogma that offspring could be produced from NT using non-differentiated totipotent cells of the germline but not from differentiated somatic cells (reviewed by Di Bernardino, 1997). The first attempt to clone mammals was in mice, in which Illmensee and Hoppe (1981) produced three mice from surgical transplantation of nuclei from blastocyst inner cell mass into recently fertilized mouse eggs. Other scientists have not been able to repeat these results, and development to young in mice has been primarily from use of donor nuclei no later in development than the four-cell stage (reviewed by Di Bernardino, 1997).

The ability to perform the NT procedure was greatly enhanced when McGrath and Solter (1983) showed that the procedure could be done by fusing rather than injecting a nucleus into an oocyte. This procedure resulted in a superior cell-survival rate compared with microinjection and has been used in most subsequent studies with mammals.

The next milestone in cloning research was the use of electrofusion to introduce 8–16-cell-stage donor blastomeres into enucleated metaphase II oocytes, from which live sheep were produced by Willadsen (1986). Eventually sheep were produced from cell stages as late as the blastocyst inner cell mass (Smith and Wilmut, 1989) and cultured cells from inner cell mass (Campbell *et al.,* 1996; Wells *et al.,* 1997; Wilmut *et al.,* 1997). Goats have also been cloned and recloned from embryonic blastomeres (Yong and Yuqiang, 1998).

In cattle, the first calves from NT involved transfer by electrofusion of donor nuclei from the 2–32-cell stage into enucleated metaphase II bovine oocytes (Prather *et al.*, 1987). A model for cloning cattle and sheep from embryonic cells is shown in Fig. 14.2. Here, as in most pre-1994 cattle and sheep NTs, aged oocytes were used because metaphase II oocytes could not be activated by means other than by spermatozoa until aged (Ware *et al.*, 1989). Recently, the combined use of 6-dimethylaminopurine (6-DMAP) to inhibit oocyte kinases with calcium (Ca^{2+}) ionophores in order to induce activation (Susko-Parrish *et al.*, 1994) has allowed activation of oocytes at any post-metaphase II age. Distinct differences exist between activation of the oocyte early after metaphase II vs. late. With early NT-activated oocytes, nuclear envelope breakdown occurs and donor chromatin is exposed to egg cytoplasm. Chromatin is decondensed and then condensed and incorporated totally, along with oocyte proteins, such as nuclear lamins, into a new nuclear envelope, which proceeds normally through the first cell cycle. Use of such early-activated metaphase II oocytes requires cell-cycle synchrony of donor nucleus and oocytes, usually at G_0–G_1 stages in the cell cycle (Fulka *et al.*, 1996, 1997b, 1998; Poccia and Collas, 1997; Collas and Poccia, 1998). Asynchrony can result in more than one nuclear organizing region and ploidy problems in resulting embryos (Navara *et al.*, 1994), as well as failure to complete DNA replication and passage of cell-cycle checkpoint screening at the first mitotic cell cycle.

When aged oocytes are used, donor-cell nuclear envelope breakdown does not occur. Rather, this oocyte proceeds directly to cleavage and first exposes the donor nucleus to new cytoplasm at the two-cell stage. Aged

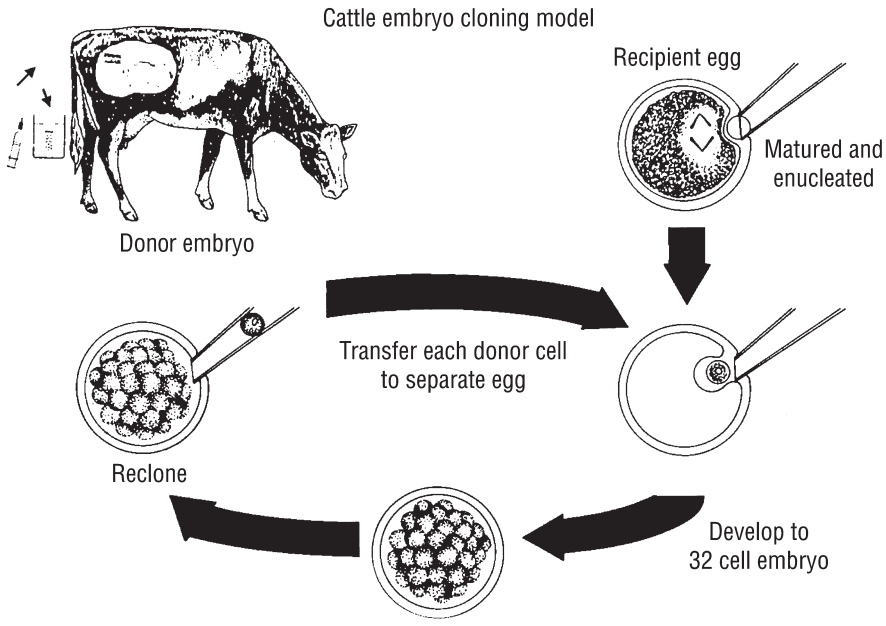

Fig. 14.2. Procedures used to multiply embryos by nuclear transfer. Valuable morula-stage embryos are recovered by non-surgical flush from the uterus of an inseminated cow, the individual cells or blastomeres are removed from the morula and transferred into enucleated oocytes and the new embryos are developed to the morula stage, when the process can be repeated. (Presented originally as a report to W.R. Grace and Company by N.L. First.)

ooplasm is not very competent for dedifferentiation and redifferentiation of differentiated or differentiation-committed nuclei. With aged oocytes, cell-cycle synchrony is not necessary because chromatin is not exposed to cytoplasm until the two-cell stage (First *et al.*, 1992; Leibfried-Rutledge *et al.*, 1992; Campbell *et al.*, 1993; Fulka *et al.*, 1996, 1997b, 1998). Both systems produce offspring when non-differentiated embryonic cells are used as the nuclear donor. However, differentiated cells require long-term exposure of chromatin to ooplasm and synchrony of cell cycles.

Use of oocytes at an age period between early metaphase II and aged oocytes usually results in failed embryo development, because chromatin is often incompletely returned to the nucleus and because ploidy problems occur (Leibfried-Rutledge *et al.*, 1992; Poccia and Collas, 1997).

To date more, than 1000 calves have been produced from NT using embryonic cells as nuclear donors. Recloning has been accomplished (Stice and Keefer, 1993) and frozen donor cells and *in vitro*-matured oocytes (Barnes *et al.*, 1993) have been used. At best, 20–50% of the NTs result in blastocysts for transfer into cows, of which approximately 50% become pregnancies. However, the pregnancy losses are greater than normal, with as many as 20% failing to deliver and requiring induction of labour with larger than normal calves resulting (Wilson *et al.*, 1995). Several aberrations of pregnancy and

parturition have been reported. Failure in placental development, with fewer than normal cotyledons, has been observed, with some evidence of a monorather than a bilayered chorioallantoic placenta (Stice *et al.*, 1996). The prolonged gestation and large offspring syndrome has been shown to exist, but at a lower frequency for some cultured or *in vitro*-produced embryos of cattle and sheep (Thompson *et al.*, 1995; Walker *et al.*, 1996; Kruip and den Daas, 1997). Even though delivery may be induced at term, some of the calves are larger than normal (Behboodi *et al.*, 1995; Farin and Farin, 1995) and a higher frequency of postnatal deaths occurs (Kruip and den Daas, 1997). It has been reported that some calves derived from IVF or NT, even though not larger than normal initially, suffer from delivery-related metabolic problems such as hypoxia, hypoglycaemia, hypothermia and abnormalities in metabolic hormones (Avery and Greve, 1995; Garry *et al.*, 1996; Kruip and den Daas, 1997). If given excellent postnatal care, such calves survive and appear normal by 2 days after birth (Wilson *et al.*, 1995; Garry *et al.*, 1996). There are at least two schools of thought regarding the higher than normal incidence of pregnancy loss and parturition failures. One hypothesis is that faulty *in vitro* culture conditions allow formation of products of amino acid degradation, which damage the cells, causing failure of embryonic and trophoblast (preplacental) cells. Antioxidant media, which reduce oxidative damage, are available, and this school of thought also advocates elimination of serum proteins from the media. The second school hypothesizes that embryo culture, IVF and NT cause failed or inappropriate gene expression.

Nuclear transfer provides opportunities for failures of many kinds (Fulka *et al.*, 1996, 1997b, 1998), including abnormal chromatin of the donor cells (the oocyte does not provide cell-cycle checkpoint screening), inappropriate or incomplete nuclear or cytoplasmic DNA and protein reprogramming or incorporation within the new nuclear envelope, leading to failed gene expression, damage from the transfer, visualization or culture processes, and probably other cell-damaging factors, including timing of events. It is reasonable to expect multiple causes of pregnancy losses. The answers require time and research. Due to placental loss and problems of failed parturition, attempts to bring NT into use for commercial production of cattle or other domestic species have not reached application. Applications such as combinations of gene transfer and NT to produce high-value founder or speciality cattle are feasible and beginning to be used.

Use of nuclear transfer to make animals from cultured embryonic stem cells

The ability to culture embryonic cells to large numbers or to immortalize the cells could allow massive up-front production of clones, which might be selected before transfer to eliminate those destined for pregnancy loss or parturition failure.

Cultured embryonic or adult cells also provide the ability to study cell lineage, cell differentiation, cell ageing and, when genetically engineered or

selected, specifically differentiated cell lines for cell transplants or tumour therapy. For the later uses requiring genetic engineering, the cells are amenable to gene transfer or deletion by use of homologous DNA recombination.

Mice were first produced from embryonic stem (ES) cells derived from the inner cell mass (ICM) of embryonic blastocysts by Evans and Kaufman (1981). Because NT has not been successful for late-stage mouse embryos, the offspring in this and later studies have been obtained by chimerization of the NT product with normal mouse embryonic cells and then selection, after a backcross or sibling mating of the mice that are true-breeding, for a genetic marker. From this early work and the pronuclear transgenics based on microinjection of DNA into the pronuclei of the one-cell zygote, more than 10,000 strains of transgenic mice have now been made, and different cell lines have been used to answer questions concerning genomic control of specific traits, cell lineage, cell differentiation, gene expression and genetic diseases in virtually every major research institution of the world. In some cases gene-deletion 'knockout mice' have been made for every sequenced gene in a few gene families, such as the transforming growth factor β (TgFβ) superfamily. Here the mouse product is used to identify the function of that particular sequence of the gene family.

Initial attempts to culture bovine ICM cells and presumed ES cells, using mouse ES technology, resulted in long-term culture and passage and demonstrated pluripotency, but not totipotency (reviewed by Anderson, 1992; Stice *et al.*, 1994, 1996; Strelchenko and Stice, 1994; Polejaeva *et al.*, 1995; Strelchenko *et al.*, 1995), as tested by NT or chimerism. Offspring have been produced from bovine ICM cells cultured in microdrops for 4 weeks, with approximately four microdrop passages (Sims and First, 1994), from sheep embryonic-disc cells cultured for up to 13 passages (Campbell *et al.*, 1996; Wells *et al.*, 1997; Wilmut *et al.*, 1997), from immortalized primordial germ cells of cattle (American Breeders Service, press release) and from primordial germ cells of pigs (Piedrahita *et al.*, 1998) and transgenic chimeric pigs from cultured embryonic cells (Golueke *et al.*, 1998), as well as chimeric calves from long- term passaged (tenth passage) bovine embryonic stem cells (Cibelli *et al.*, 1998b).

Biologically, ES cells are totipotent cells, capable of differentiating into the precursor cells for any body cell, including functional germ cells. For animal multiplication and gene transfer, these cells must also be totipotent after extensive multiplication and passage in culture, and this may require immortalization of the cell line. Primordial germ cells are the germ cells populating the undifferentiated gonad and are derived from ES cells, one or two cell divisions earlier. The reason why ICM-derived ES cells have been totipotent only after short-term passage is probably due to increased chromatin abnormalities with advanced passages and telomere shortening. This may relate to individual laboratories and their methods of cell derivation and culture or to changes in totipotency after numerous passages. There is evidence in mice that totipotency of ES cells can be reduced after extensive passage or regained by passaged primordial germ cells for a methylation-inactive gene (Stewart *et al.*,

1994). It has been suggested that one change that could affect totipotency of cultured cells is a progressive demethylation of genes of cultured cells to reduce totipotency or a post-mature demethylation, resulting in return to expression of a methylation-inactivated gene, such as Xist (Stewart *et al.*, 1994). Based primarily on mouse studies (Brandeis *et al.*, 1993; Surani *et al.*, 1993), both sperm and egg enter fertilization with DNA moderately methylated, although sperm are hypomethylated relative to oocytes. The DNA of cleavage-stage embryos is poorly methylated but becomes extensively methylated at late blastocyst, the time at which ES cells are derived from ICM cells (Brandeis *et al.*, 1993; Surani *et al.*, 1993). Cells of long-term passaged cell lines suffer chromatin damage and deletion, most often due to telomere shortening with culture age and passage. This problem is avoided by immortalizing the cell line (Counter, 1996; Bodnar *et al.*, 1998). Theoretically, the precursor cells of the totipotent ICM cells should also be totipotent, unless their genome has not acquired a mature methylation pattern sufficient to allow totipotency.

We have cultured, passaged and made reaggregated blastocysts from cells of pooled embryos from the four-cell, eight-cell and 16-cell stages, as well as from inner morula cells and ICM cells (First *et al.,* 1994; Mitalipova *et al.*, 1997; M. Sims, unpublished).

As seen from the data of Table 14.2, none of the above studies with cattle or sheep ES cells are of efficiencies sufficient for commercial livestock production. The efficiencies are adequate for use in producing and multiplying high-value transgenic animals. For this purpose, the ES-cell transgenic efficiencies are higher than pronuclear DNA microinjection in cattle. A successful recent approach is the use of NT to produce ES cells from transgenic fetal fibroblast nuclei. The resulting ES cells can then be used to make offspring by chimerization into normal embryos or by NT (Cibelli *et al.*, 1998a, b).

Cloning from differentiated cells

For several years and since testing of the Spemann hypothesis in amphibians in the 1950–1960s, scientists have considered that embryonic germline cells, such as embryonic cells, epiblast cells, primordial germ cells and possibly gonocytes, are the totipotent cell lineage and that each is capable of becoming any other cell type. Differentiated cells only occasionally show evidence of totipotency (Di Bernardino, 1997). This idea has been supported by the fact

Table 14.2. Cloning from cultured embryonic cells.

	Cell type	Number of offspring	Year	Authors
Cattle	ES*	4	1993	Sims and First
Cattle	PGC	1	1997	ABS (press release)
Sheep	ES	3	1996	Campbell *et al.*
Sheep	ES	4	1997	Wilmut *et al.*

*Not immortalized ES cell line but four- to five-passage cultured cells.
ES, embryonic stem cells; PGC, primordial germ cells.

that the totipotent cells show the presence of the receptor for the cKit oncogene, whereas differentiated cells do not, but differentiated cells, when passed through long-term exposure to oocyte cytoplasm, regain expression of the cKit receptor (Mitalipova *et al.*, 1997). At the time of cell polarization, differentiation-committed (20–30-cell stage) outer and polarized cells of bovine embryos have a low frequency of development to morula or blastocyst stage after use in NT (7%), whereas the inner and non-polarized cells develop after NT to morula or blastocyst at a high frequency (47%) (Navara *et al.*, 1992). The statistically significant difference was interpreted to mean that polarized or differentiation-committed cells had essentially lost totipotency.

The dogma of totipotent cells vs. differentiated cells was challenged for mammals initially when epithelial cells differentiated from ES cells produced offspring after use in NT (Campbell *et al.*, 1996). This challenge became well known with the birth of lambs from transfer of either bovine fetal fibroblast cells, or in the case of the lamb Dolly, cultured mammary epithelial cells into enucleated sheep oocytes (Wilmut *et al.*, 1997). To date, the production of offspring from differentiated fetal epithelial cells has been confirmed (Wells *et al.*, 1997), as has the use of fetal fibroblasts (sheep (Schnieke *et al.*, 1997) and cattle (Cibelli *et al.*, 1998a, b)). The production of offspring from adult differentiated cells has now occurred for the sheep Dolly (Wilmut *et al.*, 1997) and recently mice (Wakayama *et al.*, 1998) and cattle (Kato *et al.*, 1998).

Bovine oocytes, when used in the NT process, have been shown capable of reprogramming nuclei of five species so that they proceed through embryonic development after NT and on a schedule commensurate with the species of the nucleus. These interspecies NTs result in pregnancies with failed early development (Dominko *et al.* 1998; Mitalipova *et al.* 1998). Failure may in part be due to a species mismatch in mitochondria.

It is significant to note that the procedures used in successful production of offspring from differentiated cells have differed from earlier failed attempts, both in the preparation of the donor cells for cell and genomic reprogramming and in the timing and activation of the oocyte events of the reprogramming.

Much research now needs to be done to understand why and how differentiated cells can sometimes be reprogrammed to again express their entire genome. The successful production of transgenic lambs (Schnieke *et al.*, 1997) and calves (Cibelli *et al.*, 1998a, b) from culture-multiplied genetically engineered fetal fibroblasts and subsequent NT or embryonic-cell chimerization, as well as production of calves from fetal muscle cells (Vignon *et al.*, 1998), indicates that methods for producing transgenic founder animals or transgenic animals for production of valuable products are available for use.

Applications of cloning in agriculture

Applications of cloning in animal agriculture or biofarming may be arranged in a hierarchy of efficiency tolerance. These applications range from the production of valuable pharmaceuticals in transgenic milk to multiplication of lesser-

Table 14.3. Cloning from fetal cells.

	Cell type	Number of offspring	Year	Transgenic	Authors
Sheep	Epithelial	2	1996	–	Campbell *et al.*
Sheep	Epithelial	3	1997	–	Wells *et al.*
Sheep	Fibroblast	3	1997	–	Wilmut *et al.*
Sheep	Fibroblast	6	1997	3	Schnieke *et al.*
Cattle	PGC	1	1997	–	ABS (press release)
Cattle	Fibroblast	3	1998a	3	Cibelli *et al.*
Cattle*	Fibroblast	7	1998b	6	Cibelli *et al.*

*Calves produced by chimerization of cells into normal embryos – chimeric calves resulted.

value transgenic animals or extremely high-producing animals for traits such as milk production. For use in improving milk production, the pregnancy loss with clones *in utero* must be no greater than normal to get farmer acceptance of the technology. Additionally, clones, while genetically identical, are not phenotypically identical. This is largely due to different *in utero*, postnatal and lactation environments. Dairy-cattle geneticists, using data from identical-twin studies, estimate that milk production of cloned offspring will be 70–74% of that of the high producing animal whose cells were used to make the clones (G. Shook, personal communication).

Developing efficient systems for cloning from adult cells of cattle has improved since the sheep, Dolly, was produced from a mammary cell line. The present hope comes from a more efficient adult cell cloning process using cumulus cells and shown by Wakayama *et al.* (1998) to result in > 30 genetically identical mouse clones. Successful application using ovarian cumulus cells in cattle (Kato *et al.*, 1998) makes possible the production of clones from the highest milk-producing cows.

Also, on the positive side, the ability to make genetically-engineered cell lines and transgenic animals from culture-multiplied and genetically-engineered fetal fibroblasts (Schnieke *et al.*, 1997, Cibelli *et al.*, 1998a, b) provides opportunities for genetically engineering founder animals for traits deemed important in animal breeding or biofarming. Application of the high-efficiency sperm-mediated gene transfer as demonstrated in chickens could make gene transfer feasible for ordinary cattle breeding and challenge our thinking for ways to engineer cattle with improved meat and milk quality and quantity or for improved, specific disease resistance or environmental adaptation. For further review of these applications see Anderson and Seidel (1998), First and Thomson (1998) and Stice *et al.* (1998).

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