韓國受精卵移植學會誌(1995) 第 10 卷 第 1 號
Korean J. Emb. Trans. (1995) Vol. 10, No. 1, pp. 1~13

Application of Animal Biotechnology to the Beef Industry†

M. E. Westhusin[1,3] and J. A. Piedrahita[2,3]

[1] *Department of Veterinary Physiology and Pharmacology*

[2] *Department of Veterinary Anatomy and Public-Health, College of Veterinary Medicine*

[3] *Center for Animal Biotechnology, Institute of Bioscience and Technology,*

Texas A & M University College Station, Texas 77843, USA

SUMMARY

In conclusion, tremendous potential exists for the application of animal biotechnology to the beef industry, especially with the utilization of embryo cloning to produce genetically identical animals and genetic engineering to modify animal genomes to improve and/or create new phenotypes for many economically important traits. Research involving embryo cloning and genetic engineering of animals has been continuous now for over a decade, however inefficiencies in techniques have prevented large scale application. Large numbers of identical cattle will some day be produced and producers will be utilizing transgenic cattle in their beef production programs.

The development and utilization of animal biotechnology offers tremendous opportunities for livestock producers. Technologies including *in vitro* embryo production, embryo splitting, sexing, cloning, and genetic engineering, when combined with artificial insemination, embryo transfer and selective breeding offer the possibility of rapidly propagating and tailoring genetically superior animals(First, 1990). Genetic engineering and embryo cloning, stand out as having the most potential for effecting significant genetic and economic gain. Unfortunately, these have also proven to be the most difficult (and expensive) to develop, and much research is required before the commercial application of these technologies is possible. A third technology, *in vitro* embryo production plays an integral part in the development of efficient methods for cloning and genetic engineering. Many laboratories now have the capability of producing large numbers of bovine embryos *in vitro*, however commercial utilization ot this point has been limited primarily to overcoming reproductive ineffciency in problem cows.

Besides the commercial applications, animal biotechnologies are powerful research tools and the opportunity to use them as such should not be overlooked. Successful programs involving the production of embryos *in vitro* provide a means by which many important biological and developmental questions can be addressed, including the requirements for normal oocyte growth and maturation, fertilization, and early embryonic development. For instance, nuclear transplantation can be utilized to create genetically identical animals which can be used as models for research. It can also be used to answer important questions involving nuclear/cytoplasmic interactions during embry-

† 1995년도 춘계 한국수정란이식학회 강연요지임.

onic development, and to evaluate the effects of cytoplasmic inheritance. Similarly, genetic engineering of animals, can be utilized to create research models by manipulating genes involved in normal and abnormal(diseased) biological processes. Genetic engineering can also be utilized to conduct experiments designed to study gene function and regulation.

This presentation shall update the present status of genetic engineering in livestock, embryo cloning for the production of identical offspring, and *in vitro* embryo production. Emphasis will be placed on work involving cattle, and identifying the problems that must be overcome prior to wide-spread commercial application of these technologies. The approaches being taken to address these problems in our laboratory and others will be reviewed.

Production of Bovine Embryos *In Vitro*

It has been over ten years since the first calf was born as a result of *in vitro* fertilization(Brackett *et al.*, 1982), and many laboratiories and now actively involved with producing bovine embryos *in vitro*. The production of bovine embryos using *in vitro* technologies has both commercial and research applications. From a practical point of view, *in vitro* embryo production can be utilized to produce multiple offspring from valuable cows with various infertility problems, and/or cows that do not respond to normal procedures involving superovulation and embryo collection/transfer(Looney *et al.*, 1994). This technology can be utilized to produce offspring from valuable animals that are terminal or have recently died(Whitesell *et al.*, 1992). *In vitro* embryo production can also be used in combination with breeding programs involving superovulation and embryo transfer to increase the production of offspring from valuable females.

With only small improvements in the current procedures available for producing embryos *in vitro*, it is possible that this method for propagating superior genetics will replace superovulation and non-surgical embryo collection. Recent studies involving Holstein heifers have indicated that perhaps 4 times as many embryos can be produced when ultrasound-guided follicular aspiration and *in vitro* fertilization are utilized vs superovulation and embryo collection(Looncy *et al.*, 1994; Matthews, 1992). Additionally, one of the most exciting commercial applications of *in vitro* embryos production is that of producing offspring from prepubertal calves. It is now possible to superovulate calves that are only a few weeks old, then collect unfertilized oocytes using laparoscopy. These can be fertilized *in vitro* and the resulting embryos transferred into mature recipient females to produce offspring from heifers that are too young to reproduce naturally(Kajihara *et al.*, 1991; Armstrong *et al.*, 1991; Armstrong *et al.*, 1992; Irvine *et al.*, 1993). Utilization of such a breeding scheme results in a significant decrease in generation interval, thus genetic progress occurs much more rapidly than with conventional breeding programs.

As for research, the production of embryos *in vitro* offers tremendous benefits as large numbers of oocytes and embryos can be produced from ovaries obtained at a local abattoir. This resource allows for factorial experiments to be designed and carried out at relatively little cost. Thus a variety of experiments can be conducted involving the study of oocyte growth and maturation, processes of fertilization and requirements for normal early embryonic development, without the need and expense of live animals. To date, the majority of the research involving the development of successful methods for pro-

ducing transgenic cattle, and embryo cloning to produce identical animals has utilized *in vitro* produced embryos.

In light of all the benefits of producing embryos *in vitro*, there are still several basci problems with present procedures that need to be addressed before the full potential of this technology is realized. The efficiency of embryo production is not satisfactory with various laboratories reporting only 25% to 40% of the oocytes developing to the compact morula or blastocyst stage(Barister *et al.*, 1992). In addition, pregnancy rates of *in vitro* developed embryos are lower than that of embryos developed in utero in cows(Looney *et al.*, 1994). Embryo survival following cryopreservation has also been a problem with embryos produced *in vitro*(Voelkel and Hu, 1992). Finally, the repeatability of producing embryos *in vitro* has been unsatisfactory with unexplained, periodic highs and lows in production efficiency.

There are currently two areas of research which provide the best opportunity for improving the efficiency of embryo production *in vitro*. These are 1) *in vitro* maturation (IVM) and 2) *in vitro* embryo culture (IVC). A variety of methods involving many different culture media have been utilized for IVM and IVC(Bavister *et al.*, 1992; Voelkel and Hu, 1992; Aoyagi *et al.*, 1990; Fukuda *et al.*, 1990; Ellington *et al.*, 1990; Kim *et al.*, 1990; Brackett and Zuelke, 1993). However, none has been completely satisfactory. We do not yet understand all the requirements for normal oocyte growth and maturation nor the requirements for early embryonic development. This is at least partly due to the use of undefined supplementation of media used for oocyte maturation and embryo culture. It is routine to use gonadotrophin preparations and blood serum from a variety of sources along with co-culture of embryos with somatic cells. The need exists to develop defined methods and mediums for IVM and IVC so that consistent results can be obtained and data from different laboratories compared. This will help elucidate the metabolic and nutritional requirements of bovine oocytes and embryos along with increasing the usefulness of *in vitro* embryo production for both basic and applied research and for commercial application(Bavister *et al.*, 1992).

A few laboratories have now reported the development of *in vitro* mautred and *in ivtro* fertilized embryos to the morula and/or blastocyst using defined culture media without serum supplementation and co-culture with somatic cells (Bavister *et al.*, 1992; Kim *et al.*, 1993; Garadner *et al.*, 1994; Rosen Krans and First, 1991). However, it is unclear whether these embryo culture systems are completely cell free, as cumulus cells which remain attached to the zona pellucida following *in vitro* fertilization may be contributing to embryo development. Also, though embryo development to the blastocyst stage can be achieved with these systems, more studies are needed to determine whether or not they are as effective as other systems that involve co-culture with somatic cells. In our laboratory we have been successful in culturing embryos to the blastocyst stage using cell free culture systems, however, the addition of oviductal cells or cumulus cells to embryo culture drops always seems to enhance embryo development, with a higher percentage of embryos reaching the morula/blastocyst stage when compared to culture without cells(Applewhite and Westhusin, 1994).

Embryo Cloning to Produce Identical Bovine Offspring

The production of large numbers of genetically identical bovine offspring offers the potential

for tremendous genetic and economic gain for livestock agriculture. Use of breeding programs involving the production of genetically identical animals would result in much greater genetic progress than is possible with standard programs involving artificial insemination and embryo transfer(Nicholas and Smith, 1983; Robl and Stice, 1989). Performance and production costs could be predicted more accurately by decreasing the great variability among animals, and accurate studies of interactions between genotype and environment would be possible when using identical animals. Especially important in today's society with concerns of animal rights and welfare, far fewer animals are required for research when they are genetically identical.

The first promise of successful methods for producing large numbers of genetically identical animals came in 1986 when Willadsen reported the production of genetically identical sheep using the process of nuclear transplantation(Willadsen, 1986). This event set into motion a rash of research activity both at the corporate level and in academic institutions throughout the world, with the objective of impoving the efficiency of producing identical livestock by nuclear transplantation. Millions of dollars have been spent, and biotech companies have come and gone trying to capitalize on producing genetically identical livestock by nuclear transplantation. However the dreams of producing large numbers of identical animals are yet to be realized.

The process of nuclear transplantation for producing genetically identical livestock consists of dissociating a multicellular embryo into separate blastomeres and then fusing them to enucleated recipient ova. This effectively transfers the nucleus of the donor blastomere into the recipient cytoplasm where, if sucessful, it is reprogrammed and subsequently instructs development of a new embryo which is genetically identical to that from which the blastomeres were acquired. This new nuclear transfer embryo may now be transferred into a surrogate mother for development to term, transferred to a temporary surrogate, or cultrued *in vitro* to the morula stage. Also, blastomeres from this embryo may be used as donor of nuclei to create even more genetically identical embryos. In theory, with multiple generation nuclear transfer, an infinite number of identical animals could be created by transferring the embryo clones into recipient females for gestation and birth of offspring.

The efficiency of nuclear transfer has improved somewhat since 1986 and scientists now have a clearer understanding of the biological processes involved with nuclear transplantation. Synchronization of the cell cycle in both the recipient ova and donor blastomere has resulted in improved development to the blastocyst stage following nuclear transfer(Campbell *et al.*, 1994; Barnes *et al.*, 1993; Cheong *et al.*, 1993). Improved methods for enucleating unfertilized oocytes have increased the efficiency of the process (Westhusin *et al.*, 1992) and the utilization of oocytes collected from ovaries obtained at local abattoirs has dramatically reduced the costs involed(Barness *et al.*, 1993). However, no corporate or academic institution is yet producing large numbers of identical bovine offspring.

Moreover, research activity involving nuclear transplantation seems to have decreased in recent years with many problems still remaining to be solved before the techniques become cost effective and repeatable. In cattle, where the majority of the research has been conducted, the percentage of embryos which develop to the compact morula or blastocyst stage following 7 days culture either *in vitro* or *in vivo* is lower in embryos produced by nuclear transplantation (Westhusin *et al.*, 1992; Westhusin *et al.*, 1991; Bondioli, 1994; Keefer *et al.*, 1994) when com-

pared to normal embryos. Also, when these embryos are transferred into surrogate mothers for development to term, pregnancy rates are significantly lower, and abortion rates are highter than those resulting from the transfer embryos collected from cows or produced by *in vitro* fertilization(Bondioli, 1994; Bondioli *et al.*, 1990). Problems with low pregnancy rates and high abortion rates seem to worsen with multiple generation cloning(Bondioli, 1994). Finally, a significant proportion of the offspring produced from nuclear transfer embryos are extremely large at birth and much larger than normal calves produced by *in vitro* fertilization and/or embryo transfer(Wilson *et al.*, 1994).

Continued efforts to improve the efficiency of nuclear transplantation have taken several pathways. A considerable amount of work has now been conducted involving the synchronization of cell cycles of both donor nuclei and recipient oocyte, and it is now clear that cell cycle synchrony is critical to the successful development of embryo clones(Campbell *et al.*, 1994; Barness *et al.*, 1995). Campbell et al(1994) recently reported the frequency of development to blastocyst was greater in embryos reconstructed during the presumptive s-phase of enucleated activated oocytes than in embryos reconstructed at the time of oocyte activation (55.4% vs 21.3%). Presumably, this increase in development was due to a high proportion of both donor nuclei and recipient oocyte being in the s-phase of the cell cycle at the time of electrofusion.

Many previous attempts to produce identical mice using donor embryos beyond the 2-cell stage of development have failed; and the cause of this failure was thought to be related to the timing of maternal zygotic transition which in the mouse occurs at the 2-cell stage. However, recent work involving the manipulation of cell cycles of donor nuclei and recipient ova have now made it possible to produce identical mice by transferring nuclei of 8-cell embryos into enucleated oocytes(Cheong *et al.*, 1993).

Other laboratories have chosen to work on the problems related to multiple generation cloning which is needed to produced large numbers of identical animals. The majority of the work in this area has involved attempts to develop embryonic stem(ES) cell lines which can be used as nuclei donors thus circumventing problems related to multiple generation cloning. Progress in this area has been encouraging and one recent study reported the birth of calves following transfer on nuclei from cultured inner cell mass (ICM) cells into enucleated oocytes(Sims and First, 1994). To date, no additional calves have been produced using this approach, however a number of pregnancies have been established that subsequently aborted(Stice *et al.*, 1994). Whether or not these cultured ICM cells can be classified as true ES cells awaits further research(Stice *et al.*, 1994; Strelchenko and Stice, 1994). None-the-less, the possibility of using ES cells as donors for nuclear transplantation and the progress made in this area thus far is quite exciting, not to mention the impact true bovine ES cell lines will have on the production of transgenic cattle(see below).

In our laboratories we are attempting to determine the cause(or causes) of abnormal fetal development in embryo clones which results in large birth weight calves. We have taken two approaches to address this problem. Our first approach is to investigate the effects of cytoplasmic inheritance on calf birth weight. Evidence from cross breeding studies has suggested perhaps cytoplasmic inheritance may effect birth weight of calves(Baker, 1990). During the process of nuclear transplantation nuclei are transferred into a wide variety of different oocytes from different breeds and crossbreeds of

cows and perhaps the recipient oocyte cytoplasm is effecting fetal growth and birth weight. In order to test the hypothesis that cytoplasmic inheritance effects birth weights in calves we have begun experiments involving the transfer of pronuclei from one bovine zygote to another. While pronuclear transfer can not be used to create identical animals, it is a powerful tool which can be utilized to define the effect of maternally inherited cytoplasmic constituents on embryogenesis, and the expression of phenotypic traits in the resulting offspring. By using pronuclear transfer to study cytoplasmic inheritance, instead of nuclear transplantation involving the transfer of nuclei from multicellular embryos, we can eliminate factors involved with nuclear reprogramming which may also be effecting birth weights, and test for only cytoplasmic effects. This technology has already been used successfully in mice to show expression of a specific cell surface antigen, SEAA-2, is controlled by cytoplasmic inheritance(McGrath and Solter, 1983). To date we have completed preliminary experiments involving pronuclear transfer using embryos derived completely *in vitro*. The percentage of embryos that develop to the compact morula or blastocyst stage following pronuclear transfer is approximately 25%, and we are currently preparing to transfer these embryos into recipient cows for production of offspring.

The second approach we have taken to try and understand the cause of large birth weights in calves produced by embryo cloning is to analyze the expression of genes thought to be important for early embryonic development of bovine embryos. Previous studies in mice have shown that abnormal expression of genes coding for insulin-like growth factors (IGF-II) during embryonic development can have dramatic effects on birth weights of offspring(Dechiara *et al.*, 1990). No doubt, the expression of other genes also effects fetal growth, and abnormal expression of these may result large birth weights of offspring. We are currently analyzing gene expression in normal embryos collected from superovulated cows, embryos produced using *in vitro* methods, and embryos produced by nuclear transplantation, then comparing the gene expression profiles among embryo types to determine if they are different. Two methods are currently being employed in our laboratories to analyze gene expression. The first method involves the utilization of reverse transcription and polymerase chain reaction (RT-PCR). With this technique mRNA in the embryos is reverse transcribed into cDNA then the expression of specific genes is detected by using PCR with primers designed to amplify only the genes of interest(Watson *et al.*, 1992). Experiments using this technology have indicated differences in the expression patterns of IGF-II and IGF-I receptor between normal embryos and embryos produced by nuclear transfer. Although preliminary, the data indicates that cloned embryos are expressing higher levels of transcript.

A second method we are using to analyze gene expression involves reverse transcription followed by antisense RNA amplification(Eberwine *et al.*, 1992). With this method, mRNA is again reverse transcribed into cDNA, however, an oligo (dT24)-T7 RNA polymerase promoter primer is utilized, thus the resulting cDNA contains a recognition site for T7 RNA polymerase. The aRNA amplification is carrried out using T7 RNA polymerase, ATP, GTP, UTP, and radiolabeled CTP. The RNA synthesized can be utilized as a riboprobe to screen slot blots containing equimolar concentrations of the cDNAs of interest. The advantage of this method over RT-PCR is that expression for a large number of different genes can be analyzed simultaneously

without the need of designing PCR primers for each gene of interest(Paquin *et al.*, 1994). Moreover, studies involving quantitative analysis are easier to carry out. We have not yet used this technique to analyze gene expression in embryos produced by nuclear transfer but have analyzed gene expression in embryos produced by *in vitro* methods. Preliminary results are extremely encouraging as we have been able to detect the expression of several different genes using single embryos for analysis. We have analyzed embryos for expression of c-fos, c-jun, homeobox (Hox) 7.0, Hox 3.1, basic fibroblast growth factor(bFGF), transforming growth factor α(TGF α), TGFβ, IGF-Ⅰ, IGF-Ⅱ, nerve growth factor(NGF), nerve growth factor receptor(NGFR), heat shock protein(hsp) 68, hsp 70, GABA, Na^+ channel, Ca^+ channel, brain derived nerve factor(BDNF), and AW10. Hybridization signals were detected at all stages of embryonic development and varied in intensity. Signals for NGF, NGFR, and Ca^+ channel were very strong while the signal intensity for hsp 68 was very low(Jones *et al.*, 1994).

Genetic Engineering in Cattle

Genetic engineering, or the production of transgenic animals, may be defined as the modification of the genetic composition of an organism by introduction of foreign genes into their genome. The genetic modification can be such that only the animal being treated is modified (somatic changes), or the animal and all future generations derived from it are modified(germ line changes). Similarly, the term transgenic can be used to describe a variant of a species into which a new gene has been inserted(Gordon and Ruddle, 1983).

The first report of genetic engineering in mammals involved work in mice and occurred over ten years ago(Gordon *et al.*, 1980). Shortly thereafter, a number of laboratories reported the successful production of transgenic mice. However, the most significant studies were those of Palmiter et al(1982, 1983) who reported the production of transgenic mice expressing growth hormone genes from rats and humans. These mice grew almost twice the normal size and were more feed efficient than their littermate controls.

As with embryo cloning, this technological breakthrough caused an immediate surge in research at both academic and corporate institutions thrughout the world. Animal scientists became extremely excited about the possibility of producing livestock that grew nearly twice as fast. Since then, not only have thousands of transgenic mice been produced but, in addition, transgenic animals have been produced in amphibians, rats, rabbits, sheep, goats, swine, cattle, poultry and fish.

To date, transgenic animals have been utilized for the study of gene regulation, for production of animal models of human diseases, for understanding basic biological processes such as cell transformation and differentiation, and for production of biologically active compounds in the mammary gland. Most of this work has been carried out in mice with relatively little progress being made in improving farm animal production. This is in part due to the inefficiency of the techniques utilized for producing transgenic animals along with the lack of accurate regulation of the randomly incorporated gene. The current status of genetic engineering in livestock has been recently reviewed by several authors and to do so again here would merely be an exercise in redundancy(First *et al.*, 1991; Ebert and Schindler, 1993). However, with the theme of these meetings being directed towards cattle production, a brief discussion of genetic

engineering in this species seems appropriate.

Of all the livestock species in which genetic engineering has been attempted and live animals born, the least amount of information exists concerning cattle. Likely because cattle are expensive and the costs of research are simply too high. However it is important to keep in mind that as improvements in technology occur, cattle will become a primary target for application of this technology. Thus, with the advent of production of embryos *in vitro* and the ability to identify potential transgenic prior to transfer by PCR(Krimpenfrot *et al.*, 1991; Hyttinen *et al.*, 1994) there has been a resurgence of research on the production of transgenic cattle.

There are currently three methods utilized for genetic engineering in animals; pronuclear injection, utilization of retroviral vectors, and the use of transgenic embryonic stem cells. Pronuclear injection is by far the most widely used method for producing transgenic animals and the only method utilized to date for producing transgenic cattle. Pronuclear injection is based on the introduction of multiple copies of an "artificial" or recombinant gene into the pronucleus of a recenthly fertilized zygote. This exogenous or recombinant DNA is integrated into the chromosomes of the embryo and once integrated is processed in the same manner as the endogenous DNA. The end result of this procedure is a permanent genetic change affecting not only the individual manipulated but also all future generations. The technique of pronuclear injection suffers from several problems, among them; the low efficiency of insertion of the exogenous DNA, with an average of only 1% to 4% of the injected embryos resulting in a transgenic animal; the inability to control the site of insertion of the injected DNA with the result that some of the DNA can insert itself in an area where it can affect the function of genes required for the normal function of the organism; and the inability of the exogenous gene to function in the expected manner due to problems with accurate regulation of gene expression.

In order to gain some appreciation of the amount of work effort required to produce a transgenic calf using pronuclear injection, one only needs to review the available data. In a study reported by Krimpenfort et al(1991), 2470 bovine oocytes were collected from ovaries at a local abattoir. These were matured and fertilized *in vitro* prior to injection with a bovine casein-human lactoferrin transgene. The embryos were then cultured *in vitro* and those developing to morula or blastocysts were transferred into recipient cows. Nineteen calves were born of which 2 died at or shortly following birth, and another had to be euthanized at 10 months of age. Of the 16 remaining calves, transgene was detected in only 2 and in 1 of these calves the gene was only present in placental tissues and not in the live animal. In another more extensive study using a variety of different genes and promoters Hill et al.(1992) reported over 19,000 ova/embryos collected for producing transgenic cattle. Over 11,000 of these were injected; 1018 embryos were transferred; 193 calves were born of which 7 were transgenic but 3 died within one day of birth. While the majority of the ova collected for this study were from ovaries obtained from an abattoir, over one third (7651) were collected by surgically flushing oviducts from superovulated cows.

Clearly, millions of dollars have been spent to produce only a few transgenic cattle, and even more disheartening, because of our lack of knowledge concerning gene regulation and function, and the inability to control the number and location of gene copies incorporated into the genome, none of these transgenic calves have been of any real economic value. However, de-

spite all its inefficiencies and tremendous costs of production, the generation of only a single calf expressing the "right" transgene could produce great economic benefits to its producers. One needs to keep in mind that offspring from these animals will also be transgenic and propagation of transgenic animals after the first one is produced can be accomplished through simple breeding programs.

Progress has been made in recent years with producing transgenic cattle, but we still have a long way to go before the benefit of this technology will be fully realized. The ability to produce large numbers of embryos using ovaries obtained from local abattoirs has greatly reduced the cost of research and this technology will continue to be utilized for work involving the production of transgenic cattle. Reliable methods for screening embryos so to determine if they are transgenic prior to embryo transfer are desperately needed, and at least two reports this past year indicates progress in this area is also being made(Hyttinen *et al.*, 1994; Bowen *et al.*, 1990). By the combination of the ability to generate large numbers of embryos *in vitro* and the ability to screen the embryos prior to transfer the efficiency of generating transgenic cattle can be substantially increased. In addition, with the increased knowledge on gene regulation it should be possible in the near future to develop position-independent constructs that can overcome some of the regulatory drawbacks associated with random insertion.

The use of retroviral vectors is an alternative to pronuclear injection as they are natural genetic engineers with high efficiency in gene integration and single copy integration with few mistakes(Boselman *et al.*, 1989). However, these are derived from highly infectious and sometimes pathogenically dangerous viruses which has prevented their approval of use in situations where environmental release of the transgenic animals could occur, and their use has been limited to studies in chickens and rodents reared in secured environments(Kim *et al.*, 1993). Other disadvantages include the inability to control the site of integration of the exogenous DNA in the chromosome, problems with gene regulation, and technical problems in the construction of the retroviral vectors. The development of replication-defective retroviral vectors and subsequent use of these to produce bovine embryos expressing *E. coli* β-galactosidase gene under a β-actin promoter has recently been reported(Piedrahita *et al.*, 1992) and should stimulate more work in this area. However, due to the technical difficulties associated with the development and construction of replication defective retroviral vectors, the regulatory aspects of using retroviral vectors, and advances in the design of pronuclear injection vectors, it is unlikely that this method will gain widespread use for generation of transgenic cattle.

Conceptually, the most attractive method for producing transgenic cattle is that of gene targeting by homologous recombination in embryonic stem cells(Askew *et al.*, 1993; Evans *et al.*, 1990). While this technique is the most difficult and complex approach for producing transgenic animals, it has the advantage of allowing accurate control over the site in which exogenous genes are incorporated into the animal genome, which is not possible with pronuclear injection or the use of retroviral vectors. Moreover, unlike pronuclear injection and retroviral vectors, gene targeting can be used to inactivate and modify endogenous genes. Using this technology it is now possible to introduce single point mutations in selected areas of the genome(Evans *et al.*, 1990).

Unfortunately, due to the low efficiency of the procedure it can not be done directly on em-

bryos but requires the utilization of pluripotent embryonic stem cells or ES cells. ES cells can be cultured for prolonged periods of time without loss of pluripotentiality and as a result it is possible to genetically transform the cells in culture, identify the cell containing the desired modification, and reintroduce them into a host blastocyst. Due to its pluripotentiality, the ES cells are capable of interacting with the inner cells mass cells of the host blastocyst to form a chimera containing tissues derived from the transgenic ES cell. The problem with this technique is that to date, the isolation of ES cells from cattle has not been possible. While some progress has been made, and cell lines derived from the inner cell mass of bovine embryos have been established(Stice *et al.*, 1994; Strelchenko and Stice, 1994; Evans *et al.*, 1900), no chimeric animals have been produced by combining these cells with those from a normal embryo, and no transgenic livestock have been produced using this technology. The use of inner cell mass-derived cell lines to produce live calves following nuclear transfer as discussed above is an exciting breakthrough, however these experiments have yet to be repeated.

References

First NL. 1990. New animal breeding techniques and their application. J. Reprod. Fert. Suppl. 41:3-14.

Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF and Dressell MA. 1982. Normal development following *in vitro* fertilization in the cow. Biol. Reprod. 27:147-158.

Looney CR, Lindsey BR, Gonseth CL and Johnson DC. 1994. Commercial aspects of oocyte retrieval and *in vitro* fertilization for embryo production in problem cows. Therio. 41:67-72.

Whitesell TH, Hill KG, Miller DR, Jones AL and Wilson JM. 1992. *In vitro* embryo production from oocytes recovered from excised ovaries of terminally ill cows. Therio 37:332.

Matthew L. 1992. Specialty genetics consulting meeting. A.B.S.

Kajihara Y, Blakewood EG, Myers MW, Kometani N, Goto K and Godke RA. 1991. *In vitro* maturation and fertilization of follicular oocytes obtained from calves. Therio. 35: 220.

Armstrong DT, Holm P, Irvine B, Petersen BA, Stubbings RB, McLean D, Stevens GF and Seamark RF. 1991. Laparoscopic aspiration and *in vitro* maturation of ooyctes from calves. Therio. 35:182.

Armstrong, DT, Holm P, Irvine B, Petersen BA, Stubbings RB, McLean D, Stevens GF and Seamark RF. 1992. Pregnancies and live birth from *in vitro* fertilization of calf oocytes collected by laparoscopic follicular aspiration. Therio. 38:667-678.

Irvine B, Armstrong DT, Earl C, Holm P, McLean D and Seamark RF. 1993. Follicle development and oocyte recovery from calves with repeated gonadotropin stimulation and follicular aspiration. Therio. 39:237.

Bavister BD, Rose-Hellekart TA and Pinyopummintr T. 1992. Development of *in vitro* matured/*in vitro* fertilized bovine embryos into morulae and blastocyst in defined culture media. Therio. 37:127-146.

Voelkel SA and Hu TX. 1992. Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. Therio. 37:1117−1131.

Aoyagi Y, Fukui Y, Iwazumi Y, Urakawa M and Ono H. 1990. Effects of culture systems on development of *in vitro* fertilized bovine ova into blastocysts. Therio. 34:749-759.

Fukuda Y, Ichikawa M, Naito, K and Toyoda Y. 1990. Birth of normal calves resulting from bovine oocytes matured, fertilized, and cultured with cumulus cells *in vitro* up to the blastocyst stage. Biol. Reprod. 42:114-119.

Ellington JE, Carney EW, Farrell BP, Simkin ME and Foote RH, 1990. Bovine 1-2 cell embryo development using a simple medium in three oviduct epithelial cell coculture systems. Biol. Reprod. 43:97-104.

Kim CI, Ellington JE and Foote RH. 1990. Maturation, fertilization and development of bovine oocytes *in vitro* using TCM199 and a simple defined medium with co-culture. Therio. 33:433-440.

Brackett BG and Zuelke KA. 1993. Analysis of factors involved in the *in vitro* production of bovine embryos, Therio. 39:43-64.

Kim JH, Niwa K, Lim JM and Okuda K. 1993. Effects of phosphate, energy substrates, and amino acids on development of *in vitro*-matured, *in vitro*-fertilized bovine oocytes in a chemically defined, protein-free culture medium. Biol. Reprod. 48:1320-1325.

Gardner DK, Lane M, Spitzer A and Batt PA. 1994. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells; Amino acids, vitamins, and culturing embryos in groups stimulate development. Biol. Reprod. 50:390-400.

Rosenkrans CF and First NS. 1991. Culture of bovine zygotes to the blastocyst stage: Effects of amino acids and vitamins. Therio. 35:266.

Applewhite A and Westhusin ME. 1994. *In vitro* culture of bovine embryos in MBMOC and CR2. Therio. (In press).

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

Robl JM and Stice SL. 1989. Prospects for commercial cloning of animals by nuclear transplantation. Therio. 31:75-84.

Willadsen SM. 1986. Nuclear transplantation in sheep embryos. Nature 320:63-65.

Campbell KHS, Loi P, Cappai P and Wilmut I. 1994. Improved development to blastocyst of bovine nuclear transfer embryos reconstructed during the presumptive S-phase of enucleated activated oocytes. Biol. Reprod. 50:1385-1393.

Barnes FL, Collas P, Powell R, King WA, Westhusin M and Shepherd D. 1993. Influences of recipient oocyte cell cycle stage on DNA synthesis, nuclear envelope breakdown, chromosome constitution, and development in nuclear transplant bovine embryos. Mol. Reprod. Dev. 36:33-41.

Cheong HT, Takahashi Y and Kanagawa H. 1993. Birth of mice after transplantation of early cell-cycle stage embryonic nuclei into enucleated ooyctes. Biol. Reprod. 48:958-963.

Westhusin ME, Lavanduski MJ, Scarbroagh R, Looney CR and Bondioli DR. 1992. Viable embryos and normal calves following nuclear transfer into hoechst stained enucleated bovine demi-ooyctes. J. Reprod. Fert. 95:475-480.

Barnes F, Endebrock M, Looney C, Powell R, Westhusin M and Bondioli K. 1993. Embryo cloning in cattle: The use of *in vitro* matured oocytes. J. Reprod. Fert. 97:317-320.

Westhusin ME, Pryor JH and Bondioli KR. 1991. Nuclear transfer in the bovine embryo: A comparison of 5-day, 6-day, frozen-thawed, and nuclear transfer donor embryos. Mol. Reprod. Dev. 28:119-123.

Bondioli, KR. 1994. Commercial cloning of cattle

by nuclear transfer. In: Symposium on Cloning Mammals by Nuclear Transplantation; Seidel, G.E.(ed.), pp. 35-38.

Keefer CL, Stice SL, and Matthews DL. 1994. Bovine inner cell mass cells as donor nuclei in the production of nuclear transfer embryos and calves. Biol. Reprod. 50:935-939.

Bondioli KR, Westhusin ME and Looney CR. 1990. Production of identical bovine offspring by nuclear transfer. Therio. 33:165-174.

Wilson JM, Williams JD, Bondioli KR, Looney CR, Westhusin ME and McCalla DF. 1994. Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer(cloning), embryo transfer and natural mating. Animal Reprod. Dev. (In press)

Sims M and First NL. 1994. Production of calves by transfer of nuclei from cultured inner cell mass cells. Proc. Natl. Acad. Sci. 91:6143-6147.

Stice S, Strelchenko N, Betthauser J, Scott B, Jurgella G, Jackson J, David V, Keefer C and Matthews L. 1994. Bovine pluripotent embryonic cells contribute to nuclear transfer and chimeric fetuses. Therio. 41:301.

Strelchenko N and Stice S. 1994. Bovine embryonic pluripotent cell lines derived from morula stage embryos. Therio. 41:304.

Baker JF. 1990. Evaluation of maternal effects on birth and weaning characteristics. Texas A&M McGregory Field Day Report 1990, pp. 39.

McGrath J and Solter D. 1983. Nuclear transplantation in mouse embryos. J. Exp. Zool. 228:355-362.

DeChiara TM, Efstratiadis A and Robertson EJ. 1990. A growth deficiency phenotype in mice carrying an insulin-like growth factor II gene distrupted by targetting. Nature 345:78-80.

Watson AJ, Hogan A, Hanel A, Wiemer KE and Schultz GA. 1992. Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. Mol. Reprod. Dev. 31:87.

Eberwine J, Yeh H, Miyashiro K, Cao Y, Nair S, Finnell R, Zettel M and Coleman P. 1992. Analysis of gene expression in single live neurons. Proc. Natl. Acad. Sci. 89:3010-3014.

Paquin Platts DD, Bazer FW, Finnell RH and Piedrahita JA. 1994. Detection of gene expression in the preimplantation porcine uterus by reverse transcription-T7 RNA polymerase-dependent amplification(RT-TRDA). Therio. 41:269.

Jones, KL, Piedrahita JA, Finnell RH and Westhusin ME. 1994. Detection of gene expression in single preimplantation bovine embryos by reverse transcription-T7 RNA polymerase-dependent amplification (RT-TRDA). Therio. (In press)

Gordon JW and Ruddle FH. 1983. Gene transfer into mouse embryos: Production of transgenic mice to pronuclear injection. In:(Wu R, Grossman L, Moldare K, eds.) "Methods in Enzymol. Recombinant DNA", Part C. 101:411-433.

Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA and Ruddle FH. 1980. Genetic transplantation of mouse embryos by microinjection of purified DNA. Proc. Natl. Acad. Sci. USA 77:7380-7384.

Palmiter RD, Chen YY and Brinster RL. 1982. Differential regulation of metallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. Cell 29:701-710.

Palmiter RD, Wilkie TM, Chen HY and Brinster RL. 1983. Transmission distortaion and mos-

aicism in an unusual transgenic mouse pedigree. Cell 36:869-877.

First N, Kim T, McNamara G and Bleck G. 1991. Transgenic animals. 2nd ARRC International Symposium, p. 9-32.

Ebert KM and Schindler JES. 1993. Transgenic farm animals: Progress report. Therio. 39:121-135.

Krimpenfort P, Rademakers A, Eyestone W, van der Schans A, van den Broek S, Kooiman P, Kootwijk E, Platenburg G, Pieper F, Strijker R and de Boer H. 1991. Generation of transgenic dairy cattle using '*in vitro*' embryo production. Bio/Technology 9:844-847.

Hyttinen JM, Peura T, Tolvanen M, Aalto J, Alhonen L, Sinervirta R, Halmekyto M, Myohanen S and Janne J. 1994. Generation of transgenic dairy cattle from transgene-analyzed and sexed embryos produced *in vitro*. Bio/Technology 12:606-608.

Hill KG, Curry J and DeMayo FJ. 1992. Production of transgenic cattle by pronuclear injection. Therio. 37(1):222-225.

Bowen RA, Reed ML, Schnieke A, Seidel GE, Jr Stacey A, Thomas WK and Kajikawa O. Transgenic cattle resulting from biopsied embryos: Expression of c-ski in a transgenic calf. Biol. Reprod. 50:664-668.

Temin HM. 1987. Retrovirus vectors for gene transfer: Efficient integration into and expression of exogenous DNA in vertebrate cell genomes. In: "Gene Transfer" (Kuchelpati R, ed.). Plenum, New York, pp. 149-187.

Boselman RA, Hse RY, Boggs T, Hu S, Bruszewski J, Ou S, Kozar L, Martin F, Green C, Jacobsen F, Nicholson M, Schultz JA, Semon KM, Rishell W and Stewart RG. 1989. Germline transmission of exogenous genes in the chicken. Science 243:533-535.

Kim T, Leibfried-Rutledge ML and First NL. 1993. Gene transfer in bovine blastocysts using replication-defective retroviral vectors packaged with Gibbon ape leukemia virus envelopes. Mol. Reprod. Dev. 35:105-113.

Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM and Maeda N. 1992. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc. Natl. Acad. Sci. USA 89:4471-4475.

Askew GR, Doetschman T and Lingreil JB. 1993. Site-directed point mutations in embryonic stem cells: A gene-targeting tagand-exchange strategy. Mol. Cell. Biol. 13(7):4115-4124.

Evans MJ, Notarianni E, Laurie S and Moor RM. 1990. Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. Therio. 33:125.