# New Technologies for Manipulating Bovine Reproduction

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The reproductive process is a continuum of events that occur cyclically throughout the life of an animal, starting with fertilization, and continuing through embryonic and fetal development, parturition, the neonatal development period (including lactation), puberty, ovulation, and establishment of pregnancy once again. A number of malfunctions and technological interventions can be superimposed on this sequence, including post-partum anestrus, such technologies of selective breeding as artificial insemination and embryo transfer, and such management techniques as inducing twin pregnancies and decreasing intervals between calving. Many aspects of reproduction can be manipulated: induction of parturition, lactation, puberty, ovulation, twinning, abortion, etc. Detection of ovulation, diagnosis of pregnancy, and determining or manipulating the sex of offspring also are of obvious value. In addition, many things can go wrong in the reproductive process, including mastitis, dystocia, venereal disease, embryonic death, cystic ovaries, and dozens of other pathological conditions.

**Many approaches exist for manipulating reproduction and dealing with reproductive pathology, including more or less new technologies. I have chosen to concentrate on three items. The first is getting the cow pregnant; the second, in vitro oogenesis; and the third, adding genes to embryos.**

### Establishing Bovine Pregnancy

I am going to begin with a brief digression. It is embarrassing that greater than 95% of the beef cattle and over 30% of the dairy cattle in the United States become pregnant due to natural service. Although some billions of dollars have been invested in research on artificial insemination, control of estrus and ovulation, and technology of embryo transfer, we are doing no better than most Third World countries with our beef cattle reproduction programs. The Greeks and Romans did nearly as well.

I suspect that there is no single reason for this dismal performance, but rather a number of interacting causes:

1. Estrus and ovulation synchronization technologies are not really that good and are relatively expensive to purchase and apply.
2. Education and extension programs have not been good enough, particularly in providing sound demonstration projects.
3. Cow-calf operations have been unprofitable, and great efforts are made to minimize expenses.
4. Economics of beef cattle production have been obscured by land appreciation and depreciation, as well as tax laws that encourage tax avoidance strategies rather than efficient use of resources.
5. There is considerable cultural inertia. Raising beef cattle is more a way of life than a business, and there is reluctance to change.
6. Reproductive technologies are not needed or useful in many situations.
7. These factors interact in complex ways with other factors such as the huge supply of beef from cull beef cows and the dairy industry; import and export considerations; negative publicity regarding healthfulness of meat in diets, etc.

I have elaborated on these points to illustrate that technology requires more than efficacy for its adoption, and that better technology in most cases will be a minor factor in more profitable beef production. On the other hand, changes in beef cattle production over the next decade are likely to be phenomenal due to changes in tax laws, a national advertising program for beef, and the availability of bulls with statistically sound information concerning superiority of progeny. These factors plus improved technology may provide sufficient incentive for the use of artificial inseminations that within the next decade more than 20% of beef cattle pregnancies will result from this technique, a 500% increase over current practice. Similarly, use of artificial insemination will increase with dairy cattle, particularly with heifers. These increased percentages of use will be tempered by a marked decrease in total cattle numbers, perhaps approaching a 20% decrease by the end of the century.

*Technological Improvements in Synchronizing Ovulation.* Under most circumstances, current estrus synchronization strategies do not result in a practical method of using timed insemination effectively, which means that estrus detection is necessary. Some studies have incorporated use of estrogen or gonadotropin releasing hormone to induce ovulation. While this has been somewhat successful, it requires handling cattle an additional time. I suspect that using a delaying rather than an inducing approach to ovulation may be more successful. Possibly low dose release of a

progestin for 40-60 hours following luteolysis or progestin withdrawal, and then withdrawal of the low level progestin would synchronize ovulation precisely. Unfortunately, this probably would also require handling cattle an extra time.

*Estrus Detection.* In the absence of efficacious methods of synchronizing estrus and ovulation sufficiently for timed insemination, one might develop methods to circumvent or minimize the estrus detection effort. For example, cowside ELISA tests for several hormones, their metabolites, or other substances in milk, saliva, blood, or urine might be used to determine the interval to ovulation fairly precisely. Alternatively, some type of patch might be applied to the cows skin that is triggered by the luteinizing hormone surge to change color, much as a KaMaR is triggered. Monitoring this at milking time in dairy cattle would be easy.

Many different schemes have evolved to take advantage of statistical aspects of estrus synchronization. An example is breeding cows that come into estrus over a 4-day period and then giving the remainder prostaglandin in order to breed most cows in a short time and avoid double injection schemes. A scheme that may catch on fast with dairy cattle is to give prostaglandin to the eligible cohort of cows each Monday morning, and then check estrus on Wednesday, Thursday, and Fridays only. This has numerous advantages, including decreasing estrus detection time by more than half, increasing efficacy of detection by concentrating sexual activity, concentrating the work of artificial insemination including avoidance of weekends, forcing better reproductive management because of the required (though minimal) record keeping, and increasing fertility, as reported by MacMillan and Day (1982).

*Embryo Transfer.* Well into the future, as costs decline markedly, embryo transfer may replace artificial insemination. Acceptable pregnancy rates result when donor and recipient are in estrus within 36 hours of each other. Therefore, the timing of ovulation is much less critical than with insemination, and estrus detection in recipients may not be necessary for recipients. Other benefits of embryo transfer include selection of both dam and sire, higher pregnancy rates than with artificial insemination, more calves per dose of semen, and being able to sex the embryo. If these advantages can be maintained with frozen embryos, embryo transfer may replace artificial insemination in some circumstances. This seems unlikely before the end of the century.

Currently, about one out of every 400 dairy calves and one out of every 700 beef calves in the United States are from embryo transfer. In the short run, the decreased use of embryo transfer due to the new tax laws and poor economic conditions will have greater impact than increased use due to improved success rates and lower costs. I anticipate a moderate decrease in use of embryo transfer in the United States over the next few years, although specialty applications such as collection of embryos for export will increase transiently. In the longer run, use of embryo transfer will increase as economic conditions improve and technologies like sexing embryos become available. However, very fundamental changes will be required to increase use to more than 1% of calves born.

## In Vitro Oogenesis

At birth, each calf ovary contains hundreds of thousands of oocytes. Each year, in each cow, thousands of oocytes degenerate through normal atresia, and several are ovulated. Very old cows still have thousands of oocytes left in their ovaries. In this context, superovulation is a very inefficient method of harvesting oocytes.

There are two reasons to harvest oocytes. The first is to take advantage of the genetic value of a particular female, possibly preceded by progeny testing. The second is to provide a receptacle for genetic material from such other sources as sperm, cell lines kept in vitro, and somatic cells from other animals.

Thousands of oocytes can be obtained from each ovary rather easily by enzymatic digestion of slices of ovarian tissue. Two types of oocytes are obtained. One type, the minority, are surrounded by many nurse cells and enclosed in developing follicles. These oocytes are morphologically similar to ovulated oocytes, but from a molecular standpoint, are quite immature. The remainder, the majority, are very different from ovulated oocytes. They have no zona pellucida, have less than 10% of the volume of mature oocytes and must go through many biochemical steps in order to mature properly. Normally, this takes several months in vivo. Thousands of nurse cells are required for both types of oocytes to develop properly. Normally these develop by repeated division of nurse cells surrounding oocytes.

From this description, it is apparent that oocyte maturation is extremely complex. The intricacy of the molecular biology of this process is even more impressive. Nevertheless, great strides are being made in understanding how oocytes change from a state of suspended animation to a course of development that commits to either ovulation or degeneration. Perhaps the most impressive example is work with mouse ova (Eppig and Schroeder, 1986). They were able to grow immature oocytes in vitro for two weeks while they increased in size and underwent other appropriate changes. These oocytes then were fertilized in vitro, and the resulting embryos were transferred to recipients so that normal offspring were produced.

The main principle to evolve from this work was the necessity for growing the oocytes in the presence of a naturally occurring inhibitor of meiotic division, which is the final step that occurs before ovulation. Without this inhibitor, oocytes tend to begin meiotic division before they have matured properly in other respects. This extremely important finding may make similar studies with bovine oocyte maturation feasible. It will still take considerable work, but if successful, the method of choice for obtaining genetic offspring from a particular donor will be to remove

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one of her ovaries, obtain thousands of oocytes, and mature and fertilize them in vitro. Most would be cryopreserved, some before fertilization and others afterwards.

### Direct Manipulation of Bovine Genes

Over the past few years a remarkable technique has been developed by which DNA is injected into 1-cell embryos and becomes incorporated into the chromosomes of the resulting animal. The advantage of adding DNA at this stage of the life cycle is that every cell in the body gets the genetic material as the embryo goes from the 1-cell to the 2-, 4-, 8-cell stage, etc. This technology now is used in hundreds of laboratories to study basic biology of the mouse, and in dozens of laboratories to study farm animals. For technical and logistical reasons, success with this technology in farm animals has been rare thus far. Even if it were possible to add or modify genes routinely, there is so little known about the molecular biology of livestock that with a few exceptions, applications are years away. On the other hand, this technology can be used to obtain information about the molecular biology of livestock, and this information then can be applied in various ways.

*Problems with Current Methodology.* A fundamental problem with current methods is low success rates. Typically, the success rates with mice are about as follows: 90% survival of the ova after injection X 30% survival to term after embryo transfer X 80% survival to weaning X 20% having incorporated DNA X 40% expressing the gene (making the appropriate RNA and proteins) X 70% being fertile X 80% stably transmitting the new gene, or a 1% overall success rate. For some purposes, all of these steps are not necessary (e.g., if young are sacrificed at birth), but they would be necessary for breeding purposes. With mice, these low rates of success present no great difficulty. It is relatively inexpensive to obtain 1000 mouse embryos, and this number can easily be injected and transferred by one person in less than a month.

With livestock, considerable work is involved in obtaining, injecting, and transferring 1000 embryos. Also, it is difficult to inject genes into the pronuclei of 1-cell embryos of domestic animals because dense cytoplasm limits visualization. This may be dealt with by centrifuging the 1-cell embryos first to make the cytoplasm less dense. Another problem is that success rates are much lower than with mice, so the overall success rate seems to be about 1 per 1000 embryos injected with current methods. A number of transgenic pigs and sheep have been produced. With cattle, this is so difficult that to date not even one transgenic calf has been reported in the scientific literature, although several may be gestating.

There is an additional problem for transgenic animals of all species. The injected DNA is inserted randomly into the chromosomes in variable numbers of copies, and frequently in a backward direction. Thus, each transgenic animal made is different. Some of these problems can be overcome by using a defective retrovirus (e.g. Bovine Leukemia Virus) to insert the new genetic material, but frequently a new set of problems arises, such as lack of gene expression.

*Another Approach to Gene Insertion.* It is possible to remove cells from the inner cell mass of embryos and make cell lines from them in vitro. If they are cultured on a layer of appropriate feeder cells, they remain undifferentiated and can be placed back into embryos such that they frequently form parts of the developing fetus, including the germ cells. Cells kept in vitro in this way are called embryonic stem cells. If they are not kept on feeder cells, the embryonic stem cells are forced to differentiate to survive and then cannot form parts of a fetus.

The great thing about embryonic stem cells is that millions can be produced. DNA can be added to these in vitro in a variety of simple ways, and the resulting transgenic cells can be characterized, e.g. in number of copies of DNA, orientation, location on the chromosomes, potential for expression, etc. All of this is done in vitro with cells growing in an incubator. It by no means solves all problems in characterizing DNA insertion. For example, frequently there is a tissue-specific expression of genes, so this aspect could not be studied precisely in vitro.

On the other hand, the great majority of manipulators would be done in vitro without use of animals at all. Thus, costs would be minimal. When an appropriate gene insertion is characterized, a dozen of the cells would be injected into a blastocyst. Frequently, the cells will form parts of the fetus, e.g. one might have Holstein embryonic stem cells placed into a Hereford embryo. The resulting calf would be part Hereford, part Holstein. Up to 30% of resulting animals have the embryonic stem cells integrated into the germ line, so resulting calves would breed true to the genotype of the embryo used to make the embryonic stem cells. Nearly all of the work to date with embryonic stem cells has been done with mice. However, these techniques should work with other species.

With this approach, overall efficiency of making transgenic farm animals likely would improve by a factor of more than 10. It still would not be easy, but it seems to be the method of choice. With any of the methods, there is the huge problem of making animals homozygous for the new gene (only one of the two homologous chromosomes will have the original genetic change). Homozygozity is desirable so that the animals breed true rather than pass on the new gene to only half of their offspring. Also, there may be some deleterious effect of the homozygous condition, such as lower fertility or even embryonic lethality. This would be disastrous if the gene frequency became high in the population. Breeding to homozygosity to study these effects would entail some inbreeding, and would be a very long-term proposition.

*Applications of Transgenic Cattle.* Many seemingly obvious applications of gene injection are not really appropriate. For example, adding genes for growth to beef cattle likely would be economically disastrous because of the

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probable correlated response of larger mature size. One would get such large breeding females that overwintering costs would cancel any advantage of increased growth rates. On the other hand, one might be able to circumvent this problem by placing growth genes on the Y chromosome, so only males get large.

One obvious application of this technology is to delete genes functionally. This has occurred a number of times with transgenic mice. It provides an excellent method of studying physiological processes, particularly in animals homozygous for the effect. The most common approach is to insert some nonsence DNA into the gene of interest; this results in a nonfunctional gene. For example, this would be an excellent method of studying effects of follicle stimulating hormone. One simply deletes this gene and does various kinds of replacement therapy. It is much more physiological than removing the pituitary, which results in a very abnormal animal. Another approach would be to inactivate genes for receptors. Deletions could even have practical benefits. For example, a gene for a growth-hormone inhibiting factor might be inactivated resulting in more growth hormone, if that were desired. Similarly, inactivating the gene for inhibin would result in females that would ovulate multiple ova. This also would be disastrous in cattle for most production situations because of mortality with multiple births. On the other hand, it would be good for superovulation.

**I have presented only a small sample of potential applications of new technology to the cattle business. The first examples apply to routine management, and the latter examples to very specialized situations. Nevertheless, one can see that truly remarkable things are possible, although it is also clear that very few of these applications will have much impact in the near future.**

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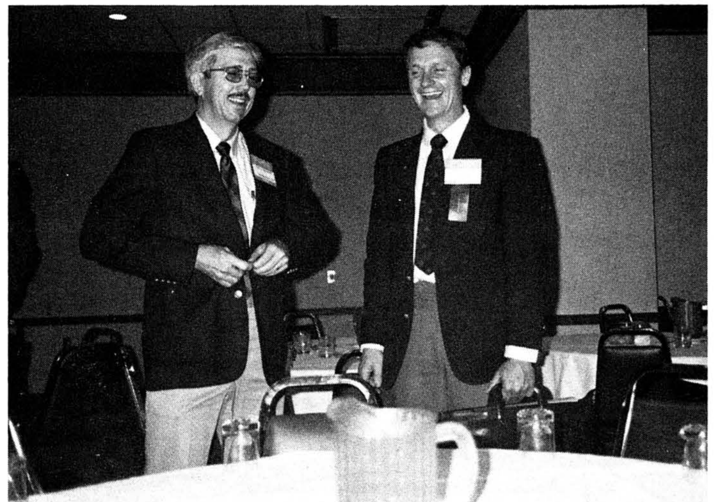

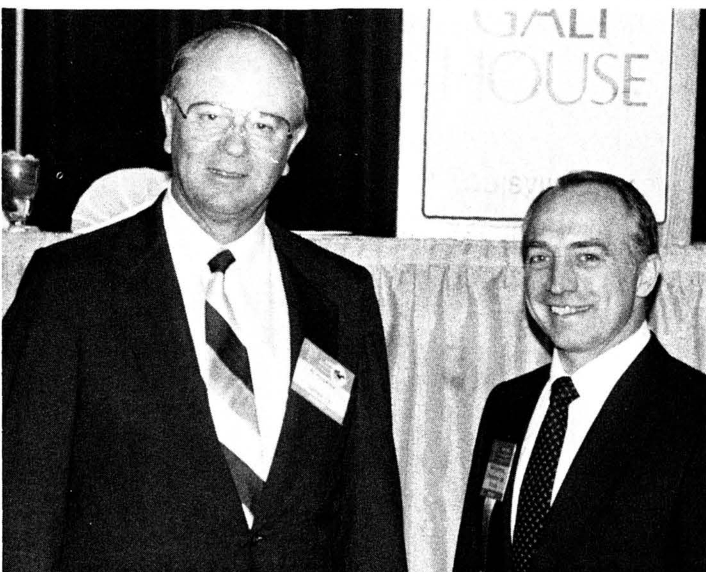

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