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PROSPECTS

# Making Transgenic Livestock: Genetic Engineering on a Large Scale

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**Abstract** The feasibility of introducing foreign genes into the genomes of cattle, goats, pigs, and sheep has only recently been demonstrated. Studies have thus far focused on improving growth efficiency or directing expression of pharmaceutical proteins to the mammary glands of these species. The general strategy for producing transgenic livestock and mice is similar. In addition to the obvious difference in scale between mice and livestock experiments, there are noteworthy obstacles that significantly reduce the efficiency of producing transgenic livestock. Low embryo viability, low transgene integration rates, and high animal costs contribute to project costs that can easily exceed hundreds of thousands of dollars. A better understanding of the mechanisms that govern transgene integration should lead to improved efficiencies. But, the full potential of the transgenic livestock system will not be fully realized until: 1) gene constructs can be designed that function in a reproducible, predictable manner; and 2) the genetic control of physiological processes are more clearly elucidated. Newly emerging approaches may resolve at least some of these issues within the next decade. Published 1992 Wiley-Liss, Inc.

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Since Jon Gordon first demonstrated the feasibility of introducing foreign DNA into the mammalian genome (Gordon et al., 1981), thousands of transgenic mice have been produced to aid in studies of gene function. The transgenic animal model is a tool and its primary utility will likely be to assist researchers in addressing fundamental scientific questions. There is little doubt that genetically engineered mice, rats, and rabbits will continue to serve basic science and the biomedical community for the next several decades. Notwithstanding these potential achievements, the topic of this review is not transgenic mice, but transgenic livestock. Some may envision giant pigs and sheep or cows producing rivers of milk. However, the agricultural community probably would not take kindly to the thought of rebuilding their infrastructure to accommodate larger animals, and dairies already have excess milk production capacity. So why would anyone want to produce transgenic livestock (used interchangeably with transgenic large animals and transgenic farm animals and restricted in this review to refer to cattle, pigs, sheep, and goats)?

Current goals of transgenic livestock producers fall into three general categories. Animal scientists would like to improve efficiency of producing livestock products; the pharmaceutical industry is considering the potential of genetically engineered animals to produce drugs; and the biomedical community would like to have large-animal models for study of genetic diseases.

To date, agricultural applications have focused almost exclusively on enhancing growth characteristics. If successful, livestock producers would benefit because the time required for animals to reach market weight (enhanced growth rate) would be reduced and less feed would be required to achieve market weight (increased feed efficiency). Consumers would benefit from leaner carcasses and reduced prices. The first transgenic farm animal experiments used a metallothionein growth hormone fusion gene (Hammer et al., 1985) to increase circulating growth hormone levels. Other experiments with similar goals have used a variety of fusion genes related to growth hormone (Brem et al.,

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1985; Ebert et al., 1988; Vize et al., 1988; Murray et al., 1989; Pursel et al., 1990; Rexroad et al., 1990a; Wieghart et al., 1990). Although no other agriculturally related approaches have progressed as far as the growth studies, experiments are being designed or are in early stages of implementation to increase wool production (Ward et al., 1988), enhance the healthfulness of offspring, and improve resistance to viral diseases.

The pharmaceutical industry is currently evaluating the potential of using large animals as "bioreactors" for production of drugs that are: 1) not effectively produced in cell or bacterial culture systems because of their complexity; 2) limited in supply because of limited availability of biological source material; 3) commonly extracted from tissues, such as human blood, that are now suspect because of concerns of contamination by known and unknown agents. Most projects aim to direct expression of transgenes to mammary glands, organs noteworthy for production of copious amounts of protein that can be harvested by non-invasive methods. The feasibility of this approach was reported in a landmark paper in which alpha-anti-trypsin was harvested from the milk of sheep (Simons et al., 1987). Others have subsequently shown that mammary gland gene expression can be altered in goats (Denman et al., 1991) and pigs (Wall et al., 1991).

A primary use of the transgenic mouse system has been to develop models for the study of human genetic diseases. However, now that transgenic rats have been produced (Hochi et al., 1990; Mullins and Ganten, 1990; Ganten et al., 1991), research may gradually shift to that species. A wealth of model data for human diseases has been derived from rat studies. The rat model has limitations as well, some of which could be overcome if a larger animal model were available. Pigs have been used extensively in nutritional, cardiovascular, and immunological research, and transgenic models designed to complement that work could be extremely valuable. However, as yet, no large-animal transgenic disease models have been reported. The reason for this may become clear later in this review.

## MAKING TRANSGENIC LIVESTOCK

The methods for producing transgenic livestock differ only in scale and minor detail from those used to produce transgenic mice. Because the basic mechanisms which govern transgenesis are not understood, practitioners of the art are forced to employ empirically derived approaches. It is not at all clear which parameters dictate the efficiency of producing transgenic animals. To be successful in this low-efficiency endeavor, one must have many eggs available to inject. Cattle, pigs, and sheep embryo donors are generally hormonally treated (superovulated) to maximize the yield of embryos per donor, and the embryos are flushed from their oviducts during surgery or at necropsy.

### Egg Harvest

Superovulation involves administering drugs at an appropriate stage of the estrus cycle to stimulate follicular development, followed by treatment with drugs to synchronize estrus and initiate ovulation. [Developmental biologists and gamete physiologists have a friendly on-going semantic debate regarding the proper terminology for single-cell female gametes. They are variously called eggs, ova, oocytes, zygotes, embryos, and preimplantation-stage embryos. In this review, the terms egg and embryo are used somewhat interchangeably. It is hoped that the context in which the terms are used will be sufficient for the reader to distinguish developmental states and fertilization status.]

Extensive research has been conducted to optimize superovulation regimes for cattle (Hasler et al., 1983; Lerner et al., 1986; Coleman et al., 1987). Protocol details may vary depending on age, breed, and climate, but in general follow the same basic format. Cow's behavior is monitored to determine reproductive status, and follicle-stimulating hormone (FSH) is administered during mid-luteal phase. Prostaglandin $F_2\alpha$ is administered to regress the corpus luteum, and cows are artificially inseminated after they show signs of estrus (Hawk et al., 1988). Cows are either slaughtered to recover 1-cell eggs from their oviducts or their oviducts are flushed by surgical intervention. Embryos were recovered in such a manner by Roschlau and colleagues, who reported one of the first successes in transgenic cattle production (Roschlau et al., 1988, 1989).

A little over 70% of treated cows yield eggs, of which about 60% are fertilized, resulting in approximately five fertilized eggs per donor cow (Hasler, 1983). At $650 per cow, bovine eggs can cost well over $120 a piece. To reduce costs, cows can be repeatedly superovulated, although embryo yield declines with successive attempts

(Hasler, 1983). Because of the high costs of this approach, it is largely being abandoned. Currently, the preferred source of cow embryos for microinjection is slaughterhouse ovaries.

In the past 5 years, methods have been developed to mature and fertilize immature eggs recovered from ovaries. The ovaries are collected from cows at slaughter (Fukui and Ono, 1988; Gordon and Lu, 1990) or on occasion taken by surgical means (Hill et al., 1992). The maturation and fertilization steps are efficient, typically approaching 80% in many laboratories (Gordon and Lu, 1990). Once the eggs have been microinjected into a pronucleus, they are generally cultured for 8 days before being transferred through the cervix into the uterus of foster mothers. On average, less than half the embryos survive the culture process (Gordon and Lu, 1990). Two laboratories have reported the successful production of transgenic cattle using the IVM/IVF/IVC protocol (Massey 1990; Krimpenfort et al., 1991; Hill et al., 1992).

In vitro methods are currently not very successful when applied to porcine gametes; consequently, eggs for microinjection must be harvested from superovulated gilts or sows. As with cows, the behavior of gilts and sows is monitored and treatment is initiated during the luteal phase of their reproductive cycle. Synchronization of estrus is usually achieved by feeding a progestogen for 5 to 9 days. Pregnant mare's serum gonadotropin (PMSG, which contains FSH-like activity) and human chorionic gonadotropin (hCG, which contains LH-like activity) are administered to stimulate follicle development and ovulation, respectively. Pigs are the big winner in the egg yield contest. About 90% of gilts or sows treated ovulate between 25 and 30 eggs on average, of which 90% are recovered and 90% are fertilized (Hunter, 1966; Webel et al., 1970; Christenson et al., 1973).

A variety of protocols is used to collect eggs from sheep, although no consensus has emerged on an optimum procedure. As with cows and pigs, the estrous cycle is synchronized prior to superovulation. However, unlike cows and pigs, sheep are seasonally anestrus (as are goats); therefore, the effectiveness of superovulation varies with season. Synchronization is accomplished by introducing a progestogen-impregnated vaginal pessary for 2 to 3 weeks. Superovulation is realized by administering a series of FSH injections (Rexroad and Powell, 1991) or injecting with PMSG and gonadotrophin-releasing hormone (GnRH) (Robinson et al., 1989). These and other superovulation regimes depress fertilization rate in ruminants, primarily because of their detrimental effect on sperm transport through the female reproductive tract (Hawk et al., 1987). To maximize fertilization, sperm are deposited directly into uterine horns, either with the aid of a laparoscope or by midventral laparotomy (Robinson et al., 1989; Rexroad and Powell, 1991). Under the best conditions, approximately 90% of ewes treated by one of these schemes ovulate, yielding about 10 eggs per responding donor. About 90% of the recovered eggs are fertilized.

### Microinjection

The equipment needed for microinjection of livestock eggs is, for the most part, identical with that used for the mouse. Eggs are positioned with an egg holder (fabricated from 1 mm glass tubing), which is attached to a micromanipulator (a mechanical device which translates hand movements into microscopic motions). Because the oocytes collected from farm animals are larger in diameter than mouse eggs (120 μm vs. 80 μm), it is advisable, although not imperative, to fabricate egg holders with an outside diameter of about 100 μm. Unlike mouse pronuclei that occupy a significant portion of the egg's cytoplasm, pronuclei of farm animal eggs are smaller with respect to the total egg volume. Thus, the injection needle must traverse more cytoplasm to contact a pronucleus. It is advisable to fabricate needles with a gradual taper to minimize the size of the hole created in the plasmalemma. Finally, as far as equipment is concerned, the microscope must be fitted with differential interference contrast optics (or an equivalent, such as Hoffman modulation optics). Phase contrast optics are not adequate.

Pronuclei in cow, pig, and sheep eggs are much more difficult to visualize than pronuclei in mouse or rabbit eggs. Both cow and pig embryos are optically opaque. Fortunately, the cytoplasmic material (thought to consist primarily of lipid droplets) that obscures the pronuclei can be displaced by centrifugation without compromising embryo viability (Wall et al., 1985; Wall and Hawk, 1988). Centrifugation stratifies the cytoplasm into two and sometimes three bands. The optically dense material is packed into one band, consuming about one-third of the egg

volume, and pronuclei are usually visible in the center of the egg.

Unlike cow and pig eggs, sheep eggs do not have dense droplets in their cytoplasm. Some do contain granular material that can be displaced by centrifugation (Nancarrow, 1985), but the granules are not usually an impediment to visualizing pronuclei. However, sheep pronuclei are the most difficult to see, even in perfectly translucent eggs. The refractive index of these pronuclei is apparently similar to that of the cytoplasm, resulting in little contrast between pronuclei and cytoplasm.

Pronuclei of cow eggs are more elastic and less firmly anchored in the cytoplasm than pronuclei of other species and consequently are the most difficult to inject. Cow pronuclei will either invaginate and/or move out of the way, unless the injection needle is very sharp. It is sometimes necessary to use the first densely packed band as a "backstop" to limit the movement of the pronucleus.

The most severely damaged mouse embryos lyse shortly after microinjection, providing a means of identifying non-viable embryos. Lysis is less common in microinjected livestock eggs, making it more difficult to identify damaged eggs. Although some have reported lysis of sheep embryos (Walton et al., 1987), others rarely observe this phenomenon (Rexroad et al., 1990b). Thus, there is no convenient means of eliminating damaged embryos before transfer to embryo recipients. Those that use 7-day in vitro culture systems for cattle embryos have a built-in selection process, albeit one that is so stringent that as many as two-thirds of the embryos die during culture. It is likely that a higher proportion of transgenic embryos die in culture than would die if there were a convenient means of returning them to the natural environment of a reproductive tract.

### Embryo Transfer

Ruminants (cows, sheep, and goats) bear triplets at most, and the health of the offspring and mother can be compromised if a cow gives birth to multiple offspring. Therefore, assuming a 30 to 50% embryo survival rate in the recipient dam, only two or three injected embryos are safely transferred into recipient ruminants. However, because pigs are polytocous (litter bearing), 30 or more injected embryos can be transferred into a single recipient sow, with the expectation that 8 to 10 of the embryos will develop to term. The litter-bearing characteristic of pigs significantly reduces the number of embryo recipients required when compared with the needs of monotocous species.

Pig, sheep, and goat embryos are transferred into oviducts by a surgical procedure similar to that used for mice. Cattle embryos also can be transferred surgically into oviducts, but success rates are usually low. Most commonly, cattle embryos are cultured to the morula or blastocyst stage of embryo development, 6 to 8 days after fertilization, and then deposited transcervically into the uterus by well-established techniques employed by the cattle embryo transfer industry (Hill et al., 1992).

## PRACTICAL CONSIDERATIONS

### Efficiencies and Costs

Monotocous species are at a clear disadvantage when considering number of eggs harvested per donor and number of embryo recipients required, but all species suffer from a low number of viable embryos (Table I). Almost 90% of injected embryos are lost between the time of transfer and parturition of embryo recipients. Studies with cattle and sheep embryos suggest that viability diminishes only 10% to 20% because of microinjection (Hawk et al., 1989; Rexroad and Wall, 1987), and centrifugation accounts for losses of a similar magnitude in pigs (Wall et al., 1985). Therefore, the major loss in embryo viability probably results from inadequate handling and culture conditions. Except for one curious exception, the efficiency parameters listed in Table II are similar for mice and farm animals. The exception is transgene integration frequency. Transgenes are detected in five times as many mouse pups as in offspring of farm animals. If the nature of this species differ-

**TABLE I. Comparison of Superovulation Response and Embryo Recovery**

| Item | Mouse | Cattle | Pigs | Sheep |
|---|---|---|---|---|
| Egg donors responsive to superovulation (%) | 80 | 70 | 90 | 80 |
| Eggs recovered per donor (No.) | 30 | 10 | 30 | 10 |
| Recovered eggs fertilized (%) | 80 | 60 | 90 | 90 |
| Injectable eggs per donor (No.) | 25 | 4 | 18 | 7 |

**TABLE II. Relative Efficiencies of Producing Transgenic Animals***

| Item | Mouse | Cattle | Pigs | Sheep |
|---|---|---|---|---|
| Eggs injected and transferred (No.) | 1,514 | 2,773 | 7,956 | 4,225 |
| Offspring per egg transferred (%) | 12 | 8 | 8 | 10 |
| Transgenics per offspring (%) | 10 | 10 | 8 | 6 |
| Transgenics per egg transferred (%) | 3.5 | 0.2 | 0.6 | 0.8 |
| Expressors per transgenic (%) | 60 | 50[a] | 62 | 33 |
| Expressing transgenics per egg transferred (%) | 2.1 | 0.1[a] | 0.3 | 0.3 |

*Compiled from Wall et al., 1990; Pursel, 1990; Rexroad et al., 1990a; and Massey, 1990.
[a]These values are purely speculative since no expressing transgenic cattle have yet to be reported.

**TABLE III. Comparison of Cost Estimates for Producing Transgenic Animals**

| Item | Mouse | Cow | Pigs | Sheep |
|---|---|---|---|---|
| Cost per animal ($) | 8 | 650 | 200 | 75 |
| Per diem cost per animal ($) | 0.25 | 15.00 | 8.50 | 2.50 |
| Number of animal days (No. animals × days on hand) | 204 | 30,135 | 2,412 | 20,400 |
| Cost of producing one expressing transgenic animal ($)* | 121.00 | 546K | 25K | 60K |

*K = $1,000.

ence was known, a strategy might be developed to overcome it.

The largest cost associated with producing transgenic livestock is the cost of the infrastructure required to house the animals, including land, fencing, and barns. Such costs can easily run in the millions of dollars, which may be the reason transgenic livestock generated to date have been produced exclusively by organizations that have pre-existing large-animal facilities. Even if the appropriate facilities are available, the purchase costs for animals and per diem costs for feed and care can easily exceed one or two orders of magnitude the costs associated with producing transgenic mice. Table III lists some rough cost estimates for a government research facility. No attempt was made to amortize the costs of the physical plant, nor were costs associated with maintaining the facilities (fence repair, tractor maintenance, etc.) included in these estimates. The parameter "Animal days" is the product of the number of animals required multiplied by the number of days the animals must be kept. The number of pig "Animal days" are small in comparison with other farm animals because more eggs can be harvested per donor sow, fewer embryo recipients are needed, and the gestation period is shorter (about 4 months for pigs vs. 9 months for cattle). The cost estimates for cattle are based on collecting embryos from superovulated donors, as are the estimates for the other species. However, if eggs were harvested from ovaries collected from slaughterhouses, the cost of producing an expressing transgenic calf might be reduced by 50% to 60%.

Transgenic large-animal experiments have been more costly than other types of large-animal studies. Typically, it is possible to design large-animal studies (nutrition, reproduction, breeding, etc.) so that a portion of experimental costs can be reclaimed by selling the animals after the experiments are completed. Recently, regulatory agencies have provided guidance for introduction of animals involved in transgenic research into the food chain (Federal Register, Vol. 56, No. 248, 56 FR 67054). This action by the regulatory agencies should be encouraging to those contemplating transgenic large-animal projects, since 90% or more of the animals born in these experiments (Table I) are not transgenic and are presumably normal in every respect.

## Major Limiting Factors

Clearly, the cost of producing transgenic livestock is the major factor limiting those interested in exploring the potential of this technology. The costs will be reduced when the efficiency of the processes is improved. Although the costs will never approach those for producing transgenic mice, they probably could be brought into line with costs normally associated with other livestock research.

The viability of embryos transferred after microinjection is only about 10%. However, this loss of 90% of the starting material cannot be

accounted for by the microinjection alone. When procedures are developed to increase viability of embryos subjected to in vitro manipulation, the efficiency of producing transgenic animals, including mice, could be increased substantially. This is currently a popular area of investigation by gamete physiologists and can be expected to yield incremental improvements soon.

Low frequency of transgene integration, as assessed by the proportion of transgenic offspring born, is another problem. Unfortunately, almost nothing is known about the mechanism of integration of transgenes, which makes interpreting the available empirical data difficult. For example, it is possible that every egg, properly injected, becomes transgenic, at least for a time. The transgenes may be excised at some point during development or they may integrate into cells that for one reason or another do not participate in forming the fetus. It is not likely that the efficiency of integration will be improved until some basic understanding of the underlying mechanism is elucidated. The primary impediment to gaining this understanding is a lack of interest by the scientific community in exploring this line of investigation, largely because integration in mice is reasonably high.

Approximately half of the costs of producing transgenic cattle, pigs, sheep, and goats is associated with the purchase, care, and feeding of embryo recipients, of which only about 10% carry transgenic fetuses. If a means were available to identify transgenic embryos prior to transferring them into foster mothers, up to 90% of embryo recipient costs of these species could be eliminated. Initial attempts to develop such an analytic procedure have been reported (King and Wall, 1988; Ninomiya and Yuki, 1989), but neither of these laboratories demonstrated that they could distinguish between integrated and unintegrated copies of transgenes in embryos.

Factors not directly related to either direct costs or efficient production also play a role in limiting the use of the transgenic livestock model system. These factors include identifying the appropriate genes to be introduced and overcoming the unpredictability of gene construct performance.

Superficially, the choice of the structural component of a transgene for bioreactor models can be based on need and cost of obtaining the desired drug by other methods. However, it remains to be determined whether or not the mammary gland can make the necessary post-transitional modifications for all categories of proteins. Any such limitations will necessarily limit drug production by this system.

The choice of transgenes designed to alter animal physiology is limited by what is known about the genetic control of the physiological trait and/or the ability to control expression of the transgene. The choice of the growth hormone gene, for the first transgenic livestock experiments, was probably a good one. The desired increase in growth rate, improved feed efficiency, and reduced carcass fat were observed (Hammer et al., 1985). However, undesirable side-effects, resulting from the inability to control the level of growth hormone gene expressed, doomed that approach. Other genes in the growth hormone cascade, including growth hormone releasing hormone and insulin like growth factor 1, also failed to produce the desired results because the genes did not elicit the desired physiological response (Pursel et al., 1990).

The growth hormone gene with a tightly controlled regulatory element, either inducible or developmentally controlled, may still be a viable approach. However, increasing circulating levels of hormones, such as growth hormone, that have a multitude of effects, may be less desirable than targeting specific functions, such as muscle development or lipid metabolism. It is likely that improvement in production traits will require coordinated interaction of several genes and will not be achieved by introduction of a single transgene.

## PROSPECTS

Finally, it is legitimate again to ask the question, "Why make transgenic livestock?", in light of the newly emerging stem cell technology. The current injection approach of introducing new genes provides a means of adding functions or altering function through physiological feedback mechanisms responsive to the added gene product. Although it is possible to ablate entire cell populations by introducing toxic genes (Wallace et al., 1991), that is a rather heavy-handed approach with which to abolish specific functions. Emerging embryonic stem cell technology may provide the solution. Embryonic stem cells can be genetically modified through homologous recombination techniques and those stem cells can then be used to modify the genetic composition of an embryo. Introducing point mutations,

replacing particular genes with mutant forms, or deleting genes will be a boon to genetic disease model builders.

The benefits of this elegant stem cell technology may be less obvious when applied to modifying production characteristics or developing bioreactor systems. Even though geneticists have searched, with only limited success for decades, for alleles that confer superior performance, the current emphasis on "the genome" may yield new pertinent information. Theoretically, "high performance" alleles could replace the endogenous form with stem cell technology, and bioreactor projects might benefit from modifying genetic control of the mammary gland, preventing or reducing expression of some constituents. To maximize the production of drugs, it may be necessary to reduce production of normal milk proteins to avoid saturating synthetic machinery of the mammary gland.

So, should the current transgenic livestock research be abandoned in favor of developing stem cell technology? Probably not. At this early stage in the development of stem cell technology, the current level of effort by the dozen or so laboratories engaged in that research is probably adequate. Development of a practical system is likely to be a decade or more away, whereas useful animals can probably be made with the current strategy. Furthermore, with only a moderate effort to develop livestock stem cell technology, it is likely, as with current transgenic technology, that the animal aspects of the technology will be in place before molecular genetic understanding is available to predictably manipulate animal physiology.

The transgenic animal model is a revolutionary tool that is providing the biomedical research community with an opportunity to ask questions that could not otherwise be addressed. It may still be a decade away, but the agricultural community (and eventually the consumer) will probably also be richly rewarded by applying this technology.

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