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CLONING BOVINE EMBRYOS: CURRENT STATUS AND FUTURE APPLICATIONS

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Introduction

Cloning animals entails transferring the nucleus of a cell obtained from the individual to be cloned into an unfertilized ovum that has had its metaphase chromosomes removed. If successful, the transferred nucleus is re-programmed so to direct development of a new embryo that is genetically identical to the animal from which the cell was obtained. This embryo can then be transferred into a surrogate mother for gestation to term and birth of a clone.

Although the history of animal cloning dates back to the early 1900’s (Spemann et al., 1938, McLaren, 2000), the first cloned mammals (sheep) were not reported until 1986 by Steen Willadsen, (Willadsen, 1986). In brief, Dr. Willadsen dissected a pre-implantation embryo into individual cells, and then utilized electrical pulses to fuse individual embryonic blastomeres with unfertilized ova in which he had removed the metaphase chromosomes. The resulting “cloned embryos” were then transferred into recipient females and developed into lambs which were genetically identical. This landmark accomplishment was the key event in history that spawned interest in the utilization of cloning to produce large numbers of genetically identical livestock. At that time, splitting embryos to produce identical twins was already becoming a popular method for increasing embryo production and thus the number of calves that could be derived from valuable embryo donor cows. However, splitting embryos was limited in its ability to produce only twins as pregnancy rates dropped dramatically when trying to split a single embryo more than one time. Cloning on the other hand offered the promise of producing literally thousands of genetically identical calves. This could be accomplished, “in theory”, by producing cloned embryos, then allowing them to divide several times in culture prior to using them for a second round of cloning to produce additional cloned embryos, and simply continuing to repeat the process, termed “multiple generation cloning”. With this approach, thousands of genetically identical embryos could be produced that when transferred into recipient females would result in thousands of genetically identical calves. The idea spawned visions of large herds of cloned bulls, cloned feedlot steers and cloned dairy cows. However, 20 years later, this idea has still not become reality. What happened? What went wrong? Is there still the possibility (what’s the probability) that this will someday occur? It’s a long sorted story that I certainly do not have time here to discuss in detail. I will however try to hit the highlights that bring us to the current status of cloning livestock, in particular, cattle.

Shortly after Dr. Willadsen's successful demonstration of producing cloned sheep he was recruited and hired by Granada Genetics, an entity of Granada Corporation based in Houston Texas. At that time Granada Genetics represented the largest commercial bovine embryo transfer company in the world and was interested in expanding their research program to include cloning and genetic engineering, then transferring these technologies to the commercial market. The challenge for Dr Willadsen was to adapt techniques he had utilized in sheep to produce cloned cattle. Success came quickly. In fact, by the time Willadsen's report involving cloned sheep was made public in *Nature*, pregnancies derived from cloned cattle embryos had already been obtained and the first cloned calves were born in 1986. Successful cloning of sheep and then cattle resulted in two other major companies investing time and resources to establish commercial cloning operations, Alta Genetics, Calgary Canada, and American Breeders Services, DeForrest Wisconsin. Over the next several years, all three of these companies had active cloning programs involving both research in addition to commercial applications.

Unfortunately, even though hundreds (if not thousands) of cloned cattle were produced during this time period and entered the market either as breeding stock, milk cows or for slaughter, the economics of producing cloned cattle using embryos as nuclei donors failed to support an ongoing viable business model, and by the early 1990s commercial activities involving the production of cloned cattle were at best, minimal. Multiple factors contributed to this scenario including the high cost of producing cloned offspring, low pregnancy rates resulting in low efficiency, enormous variability in the outcome (sometimes it worked and sometimes it didn't), and the infamous observation/documentation that a high proportion of the calves produced by nuclear transfer exhibited Large Offspring Syndrome (LOS) resulting in management problems as pertained to dystocia and death of cloned calves.

Although cloning bovine embryos never resulted in large-scale commercial application, interest in nuclear transfer and cloning livestock continued. New research focused on increasing the efficiency of the process and trying to understand problems associated with low pregnancy rates and LOS (Wilson et al., 1993). In addition, numerous studies were initiated to explore the utility of alternative cell types for use as nuclei donors. The most significant outcome of this research was the demonstration that cultured cell lines derived from either embryos or fetuses could be utilized to produce live cloned offspring (Campbell et al, 1996). While the efficiency of using cultured cells as donors for nuclear transfer was no more efficient than when using embryonic blastomeres (in fact it was most times much more inefficient), there were several advantages. First, cell culture allowed for the production of millions of cells with identical genotypes, using standard tissue culture techniques. As a result, millions of cells were readily available for use as nuclei donors thus bypassing the need for multigeneration cloning to produce large numbers of clones. More relevant, and the true driving force behind this work was the fact that cultured cell lines could be genetically engineered prior to utilization for cloning, resulting in a new more efficient approach for producing genetically engineered livestock (Schnieke et al., 1997) . In fact, it was this approach, with the goal of producing genetically engineered sheep that ultimately led to the utilization of somatic cells derived from adult animals to produce cloned livestock. In 1997, Wilmut et al reported the birth of a cloned sheep (Dolly), produced by nuclear transfer and using nuclei obtained from cultured mammary epithelial cells.

The birth of Dolly set into motion a new wave of interest in cloning. It also caused an enormous uproar in the bioethics community with concerns over adapting this technology for human cloning, a controversy that remains today. What made the birth of Dolly such an amazing feat was the fact that her birth went against more than 50 years of scientific dogma that suggested cloning mammals from differentiated cells collected from an adult mammal was biologically impossible. Obviously, this was not the case. It was simply a matter of trying new approaches and transferring enough embryos i.e. large enough numbers to finally obtain one that would develop to term. Since Dolly's birth, less than 10 years ago, an explosion in research efforts targeted at cloning mammals has occurred. Cloned animals resulting from somatic cell nuclear transplantation have now been reported in more than a dozen different species including all the major livestock species, cattle, goats, sheep, pigs, and horses (Wilmut et al., 1997; Cibelli et al., 1998; Hill et al., 2000; Keefer et al., 2000; Polejaeva et al., 2000). Work involving other species is currently ongoing, and information gathered to date suggests a wide variety of different animal species can be cloned by nuclear transplantation. These animals have all been cloned using cells obtained from adult animals. The most common cell used is normally a fibroblast, obtained by simply taking a sample of skin and applying tissue culture techniques to obtain millions of cells that are suitable for cloning. An obvious advantage of cloning from live animals is the ability to select individuals in which the phenotype is already known. Previous efforts involving the utilization of embryos and even fetal cells as nuclei donors could be used to produce genetically identical offspring, however besides the sex, there was no way to predict how this particular animal might develop as pertained to production characteristics. Cloning from adult cells now allowed one to select animals representing the very best of the best, and clone these e.g. the Grand Champion Bull or the milk cow that produced 45,000 # of milk per year. As such, title of this paper is really somewhat of a misnomer as the current status of cloning does not involve "embryo cloning" at all, rather cloning animals with superior phenotypes, using cells obtained from a small biopsy of skin.

Current Status

It is probably safe to say that world-wide there are more laboratory groups working on cattle cloning than in all other livestock species combined. It is also probably the case that more different cattle genotypes are represented by cloned offspring than genotypes of all other species combined. The ability of a number of different laboratory groups to successfully clone cattle is a result not only of numerous research programs focused on nuclear transfer in cattle, but the enormous base of knowledge that has been developed over the last 20 years involving the application of assisted reproductive techniques in cattle. Successful and repeatable procedures for *in vitro* oocyte maturation, *in vitro* fertilization, and *in vitro* embryo culture are now well established in cattle. Each of these represent a key step in the cloning process and in some cases are not as well established in other species. This is in part due to the ability to access large numbers of oocytes from abattoirs for use in research and at a relatively low cost. In terms of cloning a specific cow or bull, the ability to access large numbers of oocytes at a relatively low cost also provides the opportunity to carry out numerous attempts at cloning a specific animal. Therefore, even if the overall efficiency is low, chances are given enough trials and enough embryos transferred, a clone of most any cow or bull could be produced.

The efficiency of cloning cattle by nuclear transplantation is extremely variable. Due to the limited number of controlled experiments, it is difficult to determine the source of this variability and analyze potential interactions between different variables which include not only genotype, but the type of nuclei donor cell utilized and of course the laboratory group performing the work, just to name a few. The percentage of nuclear transfer embryos developing to the compact morula or blastocyst stage ranges from less than 5% to greater than 65%. Live births per embryo transferred are also extremely variable (0% - 83%). Of the calves born alive, a significant percentage die within one week of birth due to various health problems. Again, this varies, ranging from 0% to 100% of the calves failing to survive past one week of age (Kato et al., 1998: Hill et al., 2000). Looking at the overall averages when reviewing the scientific literature, the efficiency of producing cloned embryos that develop to the blastocyst stage in culture is similar to those produced by in vitro fertilization i.e. it is not uncommon for 45-50% development to the blastocyst stage. The major losses occur once the embryo is transferred into a recipient female and more specifically beyond 40-45 days of gestations. Initial pregnancy rates can average 40% -50%, again competitive with normal embryo transfer. However pregnancy loss can be extremely high after 50 days of gestation resulting in a calving rate of only 5-10%. Having said this, more recent data suggests these numbers are improving and as a result, large scale application of cloning may in fact be closer to reality than we think.

The Future

The high cost to produce small numbers of clones of individual donor cell lines will likely create self-imposed limits to commercial cloning in the purebred cattle industry. However, that's not the real enigma that besieges leaders in the industry and handcuffs them from using this potentially potent tool. By the time a bull or cow is determined to be worth cloning, i.e., has proven itself with sufficient numbers of superior offspring, it is most likely over five years of age. The breeder must ask himself if the money he spends today would be better spent on reproducing "old genetics", i.e., a clone, or on the next "super calf" with next generation EPDs/TPIs. The time lapse between the gestation of the clone, its birth, subsequent puberty, reproducing, and having its first calf crop on the ground, is normally about three years, and creates some serious head scratching considering that no genetic improvement is gained by cloning. Perhaps only a few proven herd sires and donor females from any breed will qualify as cloning prospects with the real potential to produce profits. None-the-less, these unique animals do indeed exist and several commercial companies currently offer cloning services to replicate the genotype of these individuals, for a fee. Cloning these superior individuals is certainly worth the cost and effort should unintentional death of the animal occur.

Ironically, the commercial sector of both the beef and dairy industries are likely the best candidates for cloning. Animals with exceptionally valuable phenotypes such as high producing dairy females or beef bulls that produce steers with “perfect” carcasses will be the candidates for genetic replication. However, the expense of cloning will have to be neutralized by high volume production of hundreds or even thousands of copies of each cell donor. In other words, as the volume of clones produced increases the cost will come down, on a per clone basis. Commercial companies are already offering volume discounts. As the cost for producing a cloned calf decreases when compared to other modes of reproduction, cloning large numbers of animals to increase herd size or provide replacement animals starts to make good business sense.

It’s important to note that clones of valuable beef bulls won’t be eaten, but their offspring will. Uniformity and predictability are two very important economic traits in the beef industry. Some, but certainly not all, of the criteria for selecting a beef bull to clone would be that he produces calves with low birth weight, high weaning and yearling weights, is disease resistant, an efficient feed converter, and has desirable carcass traits. It is rare that a single sire would pass on all or most of those traits to a high percentage of his offspring. A single animal exhibiting all these economically valuable traits in his genotype would be considered a genetic freak. Indeed he would be just that, and exactly the animal that should be cloned for commercial purposes. Imagine the uniformity of putting 5000 such male clones on 125,000 or so related females. What commercial buyer or packer wouldn’t want that set of calves? Retained ownership suddenly takes on a different meaning.

Cloning in the dairy industry has even greater ramifications. It’s not news that US dairies are getting bigger by the decade. However, expansion is expensive and, in some cases, superior genetics are difficult to acquire. Throw in a lack of biosecurity issues on purchased animals involving diseases such as Bovine Viral Diarrhea (BVD), Neospora, Bovine Leukemia Virus (BLV), Johne’s, and Leptospirosis and all of a sudden the task to grow seems very burdensome. Cloning on a massive scale seems a healthy solution. Reproducing or cloning several cell lines of ultra high producing females can generate in one gestation cycle hundreds or thousands of cows representing superior genetics and phenotypes that most dairy breeders spend their lifetime trying to raise just one. It’s no wonder considering the fact that most economically valuable traits like volume milk production, milk protein, fat values, good udder, sound feet and legs, highly fertile, plus longevity are all multigene traits. The odds of raising such a cow are mathematically very unlikely – she’s literally the one-in-a-million. Cloning her on a high volume basis, at least theoretically, makes sense. Use a biosecure recipient herd to nurture them and the dairyman now has control of his future. The real value of the clones wouldn’t be in their own production records, but more likely production of their offspring for generations to come. The biggest problem the dairyman would have is choosing a bull to breed to. Finding one that wouldn’t pull them down genetically could be a challenge.

A key word when considering cloning for commercial application is "theoretical". The history of cloning suggests that there are problems to overcome before cloning will become commercially feasible. Low conception rates of transferred embryos, early embryonic death, abortions, stillbirths, and perinatal deaths have all been reported. However, those problems are being defined and corrected. Current commercial cloning projects look promising with conception rates on nearly 600 NT embryo transfers at 40 days of gestation equal to or greater than *in vivo* produced control embryos from superovulated donors (59% vs. 54% respectively). By day 100 to 120 the cloned pregnancy rates are holding at 35%, but calving rates won't be available until after this manuscript goes to print (Brad Stroud, unpublished data).

For large commercial dairies looking to substantially expand their numbers cloning is certainly a consideration when compared economically to traditional embryo transfer (ET). For example, assume that a dairy has plans to expand by 1,000 females. A standard starting price for purchasing a frozen dairy breed embryo produced by traditional ET is about $300. The genetic value of that embryo would be at or below average for ET donor females in the dairy industry. Based on industry standards, a 50% calving rate could be expected from purchased frozen dairy embryos. Additionally, half of those calves would be unwanted males. Consequently, the dairyman would have to purchase 4,000 frozen embryos to produce 1,000 replacement females. At $300/embryo the initial investment in genetic material is $1,200,000 or $1200 per heifer calf.

Alternatively, the expansion project could be done utilizing nuclear transfer (cloning) technology. Assuming the dairyman would want to clone his best producing female there would be no initial investment in genetics. However, he could choose to buy a high producing female outside his herd, biopsy and freeze her tissue, then sell her to recover most, if not all, his initial investment. So, the economic model now shifts to the cost of cloning. Obviously, 100% of the offspring will be female, so that factor is removed. Assuming cloned embryos cost $250 each on a volume basis (ViaGen Inc., Austin, Texas), and the calving rates of transferred cloned embryos are 25%, the cost to produce 1,000 heifer calves would be [$250 x 4 x 1000 = $1,000,000. That's $1,000 per heifer calf produced by cloning as compared to $1200 per heifer calf produced by traditional ET.

The cost of transferring the embryos would be the same. Both scenarios, cloning and traditional ET, would require 4000 transfers to achieve 1000 heifers, so economically that's a washout. It could be argued that recipient wastage due to embryonic mortality and abortions is greater in the clone group since half of the pregnancies diagnosed at 40 days will be lost. However, data from ViaGen Inc. (unpublished) showed that eight of nine aborted recipients bred back to a bull within 90 days of the original 40 day pregnancy check. Although aborted clone recipients are an economic loss, so too are the recipients giving rise to unwanted bulls in the traditional ET group. At least the aborted clone recipients could be synchronized again for another clone whereas the recipients carrying unwanted male calves to term have wasted an entire gestational period.

When comparing all the economic factors listed above cloning stands pretty close to traditional embryo transfer. However, the model above considers a $300 embryo, which is the price for average quality genetics in the dairy industry. The dairy farmer who chooses to clone one of the most genetically elite females in the industry has an overwhelming advantage for a relatively similar investment. Who wouldn't choose to expand by producing 1000 copies of a lifelong healthy cow with good feet and legs that has had six or more lactations of close to 40,000 lbs plus high fat and protein as compared to 1000 heifers from mediocre donor females? The key of course to making this all work is directly tied to calving rate i.e. the efficiency of producing healthy calves by cloning.

Summary

In the early 1900s, Hans Spemann described what he called "The Fantastical Experiment". In essence, his vision involved the utilization of nuclear transplantation to someday be able to clone animals. Nearly a hundred years later, that vision has become reality. However, the large scale application of cloning cattle (or any livestock species for that matter) has yet to occur. To date, only those animals representing the elite of their breed have been selected for cloning. The efficiency of cloning cattle remains low, and the cost of producing a cloned calf high. However, as research continues, the efficiency of cloning cattle will increase. As seen in the examples provided here, just a slight increase in the efficiency could dramatically increase the utility and benefit of cloning and in turn the demand for cloned cattle. Some modern day visionaries obviously think we are already there.

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