

SOMATIC CELL CLONING IN THE BEEF INDUSTRY

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INTRODUCTION

Technically, cloning is the production of multiple genetically identical animals. Surprisingly, genetically identical animals have been produced for over 30 years using a technique of dividing embryos into two or more portions to produce multiple embryos. This technique was termed “splitting” and consequently did not generate as much public interest, or concern, as the current technique of nuclear transplantation which, for better or worse, is commonly referred to as “cloning”. Although splitting has been done commercially for many years its application is limited by the fact that only embryos can be divided and only two copies can be made. With each division of the embryo the resulting portions become smaller and less viable. Nuclear transplantation is an entirely different approach and has the potential of producing an unlimited number of genetically identical animals (Chan, 1999; Cibelli et al., 1998; Kato et al., 1998; Polejaeva and Campbell, 2000; Stice et al., 1998; Zakhartchenko et al., 1999a; Wilmut et al., 1997).

The theoretical basis for using nuclear transplantation for cloning is that all nuclei from an organism, with only a few exceptions, contain the exact same set of genes (Kato et al., 1998, 1999; Stice et al., 1998; Zakhartchenko et al., 1999a,b; Wakayama et al 1998, 1999; Wilmut et al., 1997). A skin cell contains the same set of genes as a liver cell, a kidney cell or any other cell in the body. Furthermore, small cell samples can be taken from an individual and greatly expanded in culture. Therefore, many genetic copies of an individual are technically available for making clones.

In each of these cells, however, a specific set of genes is used which results in each cell type having a unique function. We, therefore, need a method of turning off the genes that these cells are using in their original form and turning on the genes appropriate for each cell type in a complete, fully formed individual (Stice et al., 1998). The embryo does this naturally so the objective is to turn the cell into an embryo. The simple-minded approach that has been used is the removal of the genetic material from an egg and its replacement with the genetic material from one of the millions of somatic, or body cells, from the individual to be cloned. And, believe it or not, this actually works.

Techniques for the genetic modification of animals have also been available since the early 1980's (Chan, 1999; Piedrahita et al., 1999). As with cloning great interest was generated for application to farm animals; especially following a report of genetically modified mice that grew to 40% larger than nontransgenic littermates. The method of making genetic modifications in animals at this time was to microinject a few hundred to a few thousand copies of a gene into the pronucleus of a newly fertilized embryo (Chan, 1999; Piedrahita et al., 1999). As with cloning this method was used to produce a few transgenic cattle, sheep, goats and pigs. However, the method was limited, as for cloning, by the availability and expense of embryos and recipients. The nuclear transplantation method using cultured cells for nuclear donors has provided a much simpler and more efficient method for

genetically modifying agricultural animals (Chan, 1999; Cibelli et al., 1998; Piedrahita et al., 1999; Polejaeva and Campbell, 2000; Stice et al., 1998; Zakhartchenko et al., 1999a). Also, for the first time, it is now possible to insert a length of DNA into a predetermined site in the genome of agricultural animals (Chan, 1999; Cibelli et al., 1998; Piedrahita et al., 1999; Polejaeva and Campbell, 2000; Stice et al., 1998; Zakhartchenko et al., 1999a).

HISTORY OF MAMMALIAN CLONING RESEARCH

The method of nuclear transplantation was first developed and used successfully in amphibians in the 1960's (Wilson et al., 1995). Although several attempts were made to develop an efficient procedure for use in mammals over the years, it wasn't until 1983 that a technique was developed in mice that led to the first work in agricultural species (Wilson et al., 1995). This method is elegant and highly efficient.

The method first involves the removal of the genetic material from a recipient cell using a unique noninvasive approach. Although a sharpened micropipet is used for the procedure the cell membrane is so resilient that it is not penetrated (Robl et al., 1987). The pipet can be moved adjacent to the genetic material and with aspiration the genetic material is sucked into a membrane-bounded pocket, which is then pinched off from the cell. The membranes seal and the cell is perfectly intact.

Completion of the nuclear transplant process required the development of a method for inserting a nucleus into the enucleated recipient cytoplasm. To avoid disrupting the recipient cell membrane methods of cell fusion were attempted and proved to be successful (Robl et al., 1987). The resulting method for transplanting nuclei from one cell to another in mice was efficient and provided hope that similar methods could be practical for agricultural species.

Work began immediately to develop nuclear transplantation procedures for cows, pigs and sheep. This work was particularly difficult because efficient in vitro culture methods for oocytes and embryos had not yet been developed for these species. Ovulated oocytes and embryos, recovered from superovulated animals sent to slaughter were used. Resulting nuclear transplant embryos were transferred to the oviducts of sheep for several days before being recovered and transferred into recipient cows as blastocyst stage embryos (Robl et al., 1987).

Furthermore, methods developed for the mouse were not directly transferable to cows, sheep and pigs. The Sendai virus fusion system did not work at all. The cytoplasm of embryos in these species is much more opaque than the mouse making enucleation techniques more difficult and the size of the cells is different.

However, one of the greatest challenges in cloning embryos from agricultural species was not related to these technical difficulties. Interestingly, not long after the highly efficient procedure for nuclear transplantation in mice had been published a second paper was published by the same group of investigators reporting that the procedure would not work for cloning and, in fact, that "cloning by simple nuclear transplantation was biologically impossible". The mouse proved to be a very poor model for cloning and real success with this species was not realized until recently. Fortunately, work persisted on cloning

agricultural species and the first success was reported in the sheep in 1986, in the cow in 1987 and in the pig in 1989 (Robl et al., 1987).

Though successful, all cloning work at this time was based on a fallacy. Prior work in amphibians, and work that still stands today, indicated that cloning success decreased as a cell differentiation increased. Differentiation is the specialization that occurs in cells as the various organs and tissues form during embryo development. The thought was that differentiation was the result of a specific set of genes being turned on and the coincident inactivation of many other genes not needed in that specific cell type. It was thought that the inactivation of genes was an irreversible event, therefore, differentiation resulted in a cell having a specific function that could not be changed.

Consequently, cloning was done in agricultural animals, up until recently, using donor cells from early embryos (Robl et al., 1987). These cells were thought to be less differentiated than cells from either fetuses or adult animals. One of the disadvantages of using early embryos is that the number of nuclear donor cells is limited so the number of clones that can be produced is limited and efficiency is of utmost importance. For example, if a 32-cell embryo is used and the efficiency is 25% blastocyst production and 25% of these embryos surviving to term then the average number of clones produced is 2; the same as with embryo splitting.

During the decade following the first successes with cloning tremendous effort was placed on improving the efficiency of the embryo cell cloning procedure. One aspect of this effort was to develop and improve in vitro oocyte maturation and embryo culture systems in the cow and pig. Success was first realized in the cow, which greatly facilitated further research by making cow gametes and embryos readily available. A second aspect was to develop a better understanding of early development in agricultural species. Prior to this time nearly all work on mammalian development had been done in the mouse and very little was known about the normal process of embryo development in cows and pigs. This information was necessary for designing cloning manipulations that would simulate normal development as closely as possible. The third aspect of improving efficiency was developing novel approaches for manipulating embryos and refining these new methodologies. Ultimately, the embryo cell cloning procedure was proven not to be practical and interest in nuclear transplantation waned.

In 1997 the surprising and revolutionary discovery was made that differentiated cells could support development to term following fusion with an enucleated egg (Wilmut et al., 1997). This first work was done in sheep and was verified the following year in the cow. In the three years since this discovery was made interest in cloning research has exploded. To date somatic cell cloning has been successful in sheep, cow, goat, mouse and pig. Hundreds of cloned cattle and mice have been produced. Still work remains on understanding the process and improving efficiency.

NUCLEAR TRANSPLANTATION METHOD FOR CLONING CATTLE

The nuclear transplantation method for cloning cattle begins with recovery and preparation of the donor cells from the animal to be cloned. Tissue is recovered as a small disc of skin from the back of the ear. The ear is shaved and washed with a disinfectant solution. A

trochear is used to cut the disc of skin which is removed with a forceps, dipped in 70% ethanol placed in a tube containing a sterile saline solution (Standard Operating Procedures Of Cyagra). Good samples have been recovered from animals of any age and in some cases animals that have been dead for several hours (Cyagra of Kansas). The sample is then shipped to the laboratory by overnight express mail.

At the laboratory the skin sample is washed, cut into small pieces and digested with enzymes to free the fibroblast cells from the tissue. Interestingly, this process is not necessary and fibroblasts will actually creep out of the edges of the tissue, attach to the bottom of the culture dish and begin growing without any processing of the tissue at all. Generally, the cells are grown for several weeks, removed from the bottom of the dish with an enzyme treatment and then cryopreserved. Several days prior to nuclear transplantation a vial of the cells is thawed and grown until the cells cover the bottom of the dish (a confluent layer). The cells are then passed into multiple dishes to be used for several days of nuclear transplantation.

Oocytes are recovered from the ovaries of slaughtered animals. Ovaries are collected at the slaughterhouse then transported to the laboratory at room temperature. The oocytes are collected by aspirating antral follicles using a needle attached to a vacuum pump. The oocytes, in follicular fluid, are then sorted under a dissecting microscope and only oocytes with several layers of intact cumulus cells (the cells surrounding the egg) and a uniform cytoplasm are retained for use the following day as nuclear recipients.

Oocytes collected directly from ovarian follicles are not ready to be used in nuclear transplantation. They must undergo a process called maturation, which takes about 18 hours. After maturation the cumulus cells are removed from around the oocytes by shaking the cells vigorously in an enzyme solution. The mature oocyte is arrested, with condensed chromosomes, in the second metaphase of meiosis. It has extruded half of its DNA in a small cell called a polar body. The chromosomes are not readily visible in the oocyte and must be labeled with a fluorescent dye for removal.

Chromosome removal is done under a microscope at 400X magnification because the egg is only about 0.1 mm in diameter, about the size of the smallest speck of dust that can be seen. The microscope is set up with micromanipulators. The micromanipulators translate the very course movement of the hand into a very fine movement of a set of tiny glass micropipets. The micropipets are used to hold the egg and to remove the chromosomes. Chromosome removal involves fixing the egg in place with the holding pipet and inserting the enucleation pipet through the zona pellucida, the soft shell surrounding the egg. The chromosomes are located by a flash of UV light, which makes the fluorescent dye visible. The enucleation pipet is moved adjacent to the chromosomes and with aspiration are removed in a membrane enclosed bleb.

Donor cells are simply removed from the dishes using an enzyme treatment. They are then picked up individually and placed between the zona pellucida and the egg. These couplets are then fused together using a high voltage electrical shock. The electrical pulse creates a charge across the cell membranes pulling the nuclear donor cell tightly against the recipient cytoplasm. At some point the charge becomes sufficiently high that it ruptures the membranes creating many tiny holes between the two cells. When the holes reseal the two cells fuse together.

The next step in the process involves stimulating the nuclear transfer embryo to divide. Normally when the egg is fertilized the sperm imparts a signal that initiates cell division. Because we are omitting the sperm in nuclear transfer we have to use an artificial stimulus to initiate cell division. There are many approaches to doing this. The procedure we use consists of treating the nuclear transfer embryos with a chemical that elevates intracellular calcium following by a chemical that inhibits protein synthesis. Egg activation is a complex process and is currently an important area of further research.

The nuclear transplant embryos are then placed in culture for 7 to 9 days. During this time cell division takes place and some differentiation occurs so that the embryos have about 80 to 200 cells and cells that will form the placenta and fetus are clearly distinguishable. Then embryos are then transplanted into recipients for development to term.

RESULTS OF SOMATIC CELL CLONING IN CATTLE

To date, there are about 150 calves that have been produced using somatic cell nuclear transplantation in the world. About 30 different laboratories, with most being in Japan, have produced these calves. Overall development rate from nuclear transfer to healthy calf at term is about 1 to 3%.

A more detailed analysis provides information on when losses occur. The results can vary greatly depending on many factors; some known and many unknown. In general, development of cloned embryos in culture to the blastocyst stage at day 7 to 9 is about 15 to 20% but can vary from 5 to 50%. Pregnancy rates, transferring either one or two embryos per recipient, are generally high (40 to 60%) at day 35 but substantial losses occur by day 60 to 90 resulting in pregnancy rates of about 25% (Cyagra Data). Although some losses occur during the middle trimester of gestation they are generally low. In the last trimester substantial losses occur resulting in about 10 to 20% of the calves surviving to term (Hill et al., 1999). Unfortunately, perinatal and postnatal losses can be as high as 50% but with intensive care can be reduced to about 10 to 20%.

Prenatal, perinatal and postnatal losses have been documented in some detail. Prenatal losses generally occur as a result of fluid accumulation in the placenta (hydrops). The fluid accumulation gets sufficiently high that the cow aborts the calf. On gross examination the calf may look normal. Most calves have been taken by Caesarian section. Hydrops is noted in many of these pregnancies (Hill et al., 1999). In addition, the calves have an extraordinarily large umbilicus (Hill et al., 1999). They also typically have breathing difficulties and benefit from being placed on oxygen for a few hours (Hill et al., 1999). They also may have a variety of other problems such as fluid accumulation in various organs. By the first few days after birth most calves appear normal but may exhibit digestive problems. By sixty days of age the calves have generally outgrown any defects they might have had at birth.

The defects that are seen are remarkably consistent from lab to lab and appear to stem from gestational problems as opposed to genetic defects. Analysis of early conceptuses indicates that the placenta is likely the cause of most of the problems that have been observed. For unknown reasons placental attachment to the uterine lining does not occur properly in cloned embryos. This lack of adequate attachment may result in the fluid

accumulation, large umbilical cords and fluid accumulation in various organs that has been observed. The defect is sometimes observed in calves derived from in vitro produced embryos, which indicates that the defect may be the result of poor culture systems. However, it is unlikely that in vitro culture is the only cause of the problem (Cibelli et al., 1998).

CLONING IN THE CATTLE INDUSTRY

Somatic cell cloning has great promise but the limitations of low pregnancy rates and calf survival restrict its current use. At this time somatic cell cloning is still in the research phase. Considerable work needs to be done to improve survival rates and evaluate variations in results before cloning can be commercialized. The second phase will be small-scale commercialization of the technology to multiply animals of high value. As efficiency and the quality of embryos improves, cryopreservation will become feasible and large numbers of embryos will be sold in straws just as semen is today.

The ability to clone adult animals from easily cultured skin cells presents several opportunities to the beef industry. One opportunity is to preserve valued genotypes. To maintain the genetics of any animal in the herd indefinitely, a skin sample can be taken, processed and the cells frozen back for future use. Unlike semen, the cells contain a full copy of the genetic material rather than just half the chromosomes. Unlike embryos, the phenotype of the animal is known.

Cloning may also present a unique opportunity for marketing genetics. Evaluation of an animal by performance and progeny testing is costly and takes several years. Cloning may be useful in increasing the return from fully tested animals. These animals would be of great value to producers because of the predictability of the outcome.

Cloning could facilitate integration of segments of the beef industry for the production of specialty brand name products. Highly uniform brand name products would be helpful in increasing both value and consumption of beef products. Examples of possible products are high lean, low fat beef from double muscled animals or high quality beef from Japanese Waygu animals. Increased uniformity of beef products will improve consumer appeal and reduce the cost of production.

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