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Cloning: Adult vs. Embryonic Cells and Various Techniques Employed

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Abstract

The cloning of amphibians and mainly mammals is discussed focusing on somatic cell usage. Significant progress has been made in recent years and this is outlined with an emphasis on techniques and their differences. Special attention is given to the research producing Dolly the sheep and Millenium the cow. The advantages and disadvantages of cloning are reviewed with the exception of moral and ethical issues, which are omitted. The conclusion is drawn that much more study is needed before the economic and medical potential of cloning can be realized.

Introduction

The word “clone” is derived from the Greek word for twig, “klon,” which refers to the utilization of a piece of a plant to root an identical plant via asexual reproduction.¹ This type of cloning has been employed for centuries to propagate vegetation due to the fact that plant cells remain totipotent during cell differentiation. With animals, several lower invertebrates can be cloned by the segmentation process, i.e. earthworms regenerating as two animals when bisected. The only type of asexual reproduction occurring in vertebrates is identical twinning but it has been known since the 1940s that early mammalian embryo cells exhibit totipotency.² This was discovered through blastomere separation and culture, and embryo bisection. It is the loss of this totipotency with cell differentiation that is the main obstacle in the cloning of adult vertebrates.

Vertebrate cloning was first applied in amphibians. The nuclear transfer (NT) technique employed involved replacement of the maternal genetic component of an unfertilized egg with the nucleus of an undifferentiated embryonic cell.² This research revealed that NT success decreased with increasing differentiation of the nuclear donor cells suggesting that normal, full development could only occur with relatively undifferentiated nuclei. The first live offspring producing mammals resulted from NT with embryonic blastomeres from early cleavage stage embryos. This work performed in mice has since been utilized to produce rabbits, sheep, pigs, cattle, and rhesus macaques.² Subsequent experiments using cumulus cells, embryonic stem

cells, cultured embryonic cells, adult and fetal fibroblasts, cultured inner cell mass cells, leukocytes, adult mural granulosa cells, and fetal and adult cells have produced live offspring.²

Development of Nuclear Transplantation Technology

Amphibian cloning in the 1950s was accomplished using a microsurgical method of nuclear transplantation from early embryos to enucleated oocytes using a thin glass pipette.¹ Another method was oocyte nuclei disruption via UV irradiation. However, most of these cells showed no development. The success of these amphibian trials led to vertebrate experimentation. With this research, it was discovered that new microsurgical methods must be found because mammalian oocytes are approximately one thousand times smaller than those of amphibians. Cell fusion was looked at as an approach to donor nuclei introduction. This technique was modified and added to resulting in a highly efficient nuclear transfer method. Recipient pronuclei were removed without plasma membrane penetration by treating with cytochalasin B and colcemid, cytoskeletal inhibitors. These caused the plasma membrane to be rupture-resistant so that a glass pipette could be inserted through the zona pellucida adjacent to the pronuclei without membrane penetration. The pronuclei were removed via a membrane bound cytoplasm and the donor nucleus inserted by Sendai virus mediated cell fusion. This unearthed the idea that nuclei could be introduced with little effect on the manipulated embryo's viability.³

Further study with mouse pronuclei indicated that the timing of development followed that of the recipient cell. Other research was done with MII oocytes based on previous amphibian work. These results differed significantly from those of the pronuclei in that the transplanted nuclei changed completely in morphology with the nucleolus being very condensed like a pronucleus and the nucleus swelling greatly. This was good for creating a technique for

embryo development evaluation but problems still existed. One was that the zona pellucida of the unfertilized oocyte was not easy to pierce with the NT pipette. Another dealt with the fact that metaphase chromosomes within the oocyte are not visible in the cytoplasm and are therefore arduous to remove. Lastly, the cell had to be activated for onset of development. Because extensive work had been done with mice without any further progress, most research shifted to cattle.³

With bovines, one of the main objectives was to create a procedure using MII oocytes to produce live cloned offspring. To accomplish this goal, embryos and oocytes were collected from superovulated cows because IVM (in vitro maturation) and IVF (in vitro fertilization) were not yet established. These were used to deduce whether NT embryos could develop to full term using the previously described protocol. The prominent problems with this were that the superovulation-obtained embryos were expensive, rare, and relatively opaque due to high quantities of lipid granules present in the cytoplasm. This meant no chromosomes could be seen for removal. Using centrifugation, the genetic material could be isolated because the lipids floated to the top of the embryo.

Once the recipient pronuclei were removed and the donor nucleus in place between the zona pellucida and the inner membrane, another problem arose. The Sendai virus used to fuse the nucleus into the cytoplasm was ineffective in bovine oocytes. This was expected since the Sendai virus is of rodent origin but trials with bovine viruses also proved inadequate. This led to the use of electrical pulse, which resulted in an 80% fusion rate.³ This also solved the problem of activation because the initial electric shock seemed sufficient to initiate further development.

Obtaining Successful Development of Nuclear Transfer Embryos

Nuclear Reprogramming

Transferring a nucleus into an enucleated mature oocyte results in several morphological and biochemical changes in the cell. These changes occur within the nuclear components associated with reprogramming and include NEBD (nuclear envelope breakdown), nucleoli dispersion with premature chromosome condensation, pseudo-pronucleus formation, and donor nucleus edema after cytoplasmic activation.² Reprogramming should result in prompt inactivation of transcription in the transferred nucleus and initiation of zygotic gene expression as occurs in normal development. Yet cleavage, blastocyst formation, and the birth of viable offspring are the only indications of complete nuclear reprogramming in an NT embryo.

Normal cattle embryos and those made by NT display amazingly similar gene expression patterns with a 95% conservation of cDNA banding patterns by a problem occurs with certain genes.⁶ Among these are insulin-like growth factors (IGF) I and II. Inaccurate reprogramming like occurs with IGF I and IGF II could explain the inordinately high rate of embryonic, fetal, neonatal, and postnatal death in cloned animals.

Donor Cell Nucleus and Cytoplasm Synchrony

For accurate reprogramming, the stages of the cell cycle in the nucleus of the donor cell and in the cytoplasm of the recipient cell must be synchronized. The best choice for NT cytoplasm stage is the metaphase II arrested (MII) oocyte. This is due to the fact that at this stage, the cytoplasm contains high levels of MPF (maturation/meiosis/mitosis-promoting factor) that controls the initiation of mitosis and meiosis. MPF is composed of two proteins, cyclins and p34^{cdc2}. Protein kinase p34^{cdc2} activity is regulated by its association with cyclins and changes in its phosphorylation state. Activation of p34^{cdc2} during meiosis causes the cell to enter the M-

phase and results in NEBD and chromosome condensation. When fertilized, the oocyte response inactivates MPF to induce chromatin decondensation and pronuclei formation. A second rise of MPF after DNA replication at the S-phase induces mitosis yielding a 2-cell embryo. Transfer of a donor nucleus into a MII cytoplasm before activation causes the high levels of MPF to prompt NEBD and premature chromosome condensation. Activation of the cytoplasm leads to pronuclear formation and DNA replication.

If donor nuclei from the G_0/G_1 stage of mitosis/ meiosis are used, the right ploidy is preserved and the resulting daughter cells are diploid. The use of G_2 or M-phase nuclei yields tetraploid embryos because the cells have already replicated once and then replicate again. S-phase nuclei can produce aneuploid embryos due to rereplication of all or parts of the genome.

Most recent studies have utilized donor cells in a quiescent state dubbed G_0 . This state is achieved via serum deprivation or by using cells naturally arrested in G_0 *in vivo*. Cloned vertebrates have also been obtained from nonquiescent, active fetal fibroblasts and embryonic stem cells at G_2/M -phase. This indicates that cloning success is not solely dependent upon donor nuclei being in a quiet, G_0 state.

Embryonic and Fetal Cell Cloning

Embryonic and fetal cells were first used in cloning because of their totipotency, the ability to become any type of cell required. A problem with using embryonic blastomeres is that the cells are limited in numbers and additional nuclei must be generated via recloning of the embryos obtained from NT if large numbers of offspring are desired. However, the development of these recloned embryos declined significantly after one round of cloning.³ The main disadvantage of using embryonic and fetal cells to produce clones is that the genetic superiority or inferiority of the cloned cells is completely unknown. This presents a severe drawback if the

offspring are intended as agricultural economic commodities. This is the reason that adult somatic cells were first considered.

Adult Somatic Cell Cloning

Somatic cells of adult animals are differentiated. Many researchers believed that the inactivation of some genes during differentiation was irreversible. This idea has since been disproved. Wilmut and his coworkers at the Roslin Institute in Scotland used the mammary cell nuclei of a Finn Dorsett sheep and the recipient oocytes of Scottish Blackface ewes. The donor nuclei were cultured and serum deprived to obtain a quiescent state. Electrofusion and further culture produced embryos for transfer to sheep oviducts where the developing embryos incubated for seven days. After the incubation period, the embryos were moved to Scottish Blackface surrogate mothers. From this patented process, Dolly emerged.

It is important to note that of the 277 mammary cells used for NT, only 29 became developing embryos. Of these 29, only Dolly survived to parturition. She is the first mammal ever cloned from adult cells, proving that the differentiation process does not leave cells unable to be reprogrammed.

Since the announcement of Dolly's birth in 1996, other mammals have been cloned. Researchers from the University of Tokyo and the University of Hawaii created the first mice from adult cells using their own protocol dubbed the "Honolulu technique."⁴ Other animal scientists in Japan cloned calves, the first species of commercial value. From ten transferred embryos, eight calves were born. The cells were obtained from slaughterhouse oviducts and cumulus cells of a single cow. Of the eight offspring, four died at or soon after birth—one from pneumonia and the others from birthing complications.⁵

One of the most important breakthroughs in somatic cell cloning stems from work done at the University of Tennessee, Knoxville by Drs. J. Lannett Edwards and F. Neal Schrick. Millenium is the first cloned Jersey calf and among the first few cow clones in the United States. The nucleus of a Jersey cow and the enucleated egg of another cow were combined and transferred to an Angus cow. This recipient was chosen for immediate recognition at birth that the offspring was definitely not of the birth mother's genetic makeup. She is one of 95 initial embryos, a dramatic improvement over Dolly's success rate. *¹

The key difference in the creation of Millie and all previous somatic cell clones is that Millie was produced using standard cell culture techniques. This eliminates the need for a patented method like those used to create Dolly and the "Honolulu technique" mice. Another difference in this Jersey calf is that she will be registered as her nuclei donor's twin by the American Jersey Cattle Association and will be closely compared to her genetic identical in terms of production records to see if the cloning process affects factors like milk yield. This will aid in determining if the cloning process alters production in any significant manner.

Cloning Problems

Among the biggest concerns associated with cloning are high fetal wastage during pregnancy, high birth weight, above average perinatal and postnatal death, and virtual lack of adaptation to the post-parturition environment. These problems seem to amplify with somatic cell NT to rates higher than those seen with embryonic cloning. It is thought that a lack of or inappropriate gene expression at critical points of embryonic, fetal, and/or placental development may be the cause of the increased pregnancy loss. ² Research involving cattle found that the high NT embryo loss during the first trimester was due to abnormal placentation. The necessary nutrient-gas exchange via placentomes never occurred. Another problem stems from abnormal

* See end for schematic

surrogate mother response to pregnancy. Often inadequate mammary development and birth preparation by the mother are present at parturition leading to increased delivery by cesarean section. It is still under investigation as to the cause of this miscommunication between fetus and mother.

Telomere shortening

Telomeres are cellular structures that compose the termini of eukaryotic chromosomes. They consist of a repeating sequence (TTTAGGG) and regulate the replicative capacity of the cell that is used as an indicator of cellular senescence. Normal cells have a finite replicative capacity and once it is reached, the cell becomes incapable of further cellular division. Under ordinary somatic cell division, the telomeres shorten due to the primer requirement for DNA synthesis during replication. Dubbed the Hayflick phenomenon, this process results in loss of terminal repeats at the telomere and thus incomplete replication increasing with each cell division. Baseline measurements for telomere length have been obtained from somatic germ cells, which maintain a constant telomeric length.²

When Dolly's telomeres were analyzed, it was found that they were shorter than those of other sheep her age and were consistent with those of her 6-year-old donor nuclei. Restoration of telomere length did not occur because the germ cell line never became involved and NT protocol was not capable of telomere size restoration. In contrast, analysis of telomere length in calves derived from fetal fibroblasts indicated that their telomeres were significantly longer than age-matched controls. This could be due to NT protocol differences and donor cell types but these outcomes cannot be directly compared.

Mitochondrial DNA

In mammals, nuclear DNA is transmitted according to Mendelian genetics and mitochondrial DNA is transmitted maternally. When paternal mitochondria enter the oocyte during fertilization, they are rapidly eliminated in early preimplantation stages of embryo development.² Coevolution of nuclear and mitochondrial genes have resulted in nearly 100 critical interactions for proper ATP generation. Therefore a concern arises when transplanting a donor nuclei to an enucleated oocyte. Two different mitochondrial genomes could be present within one cell (heteroplasmy) thus disrupting the necessary nuclear-mitochondrial genome communications. Studies done with sheep show that mitochondrial DNA with NT embryos stem entirely from the recipient cell thus suggesting a deselection of donor cell mitochondrial DNA.² This does not mean, however, that the initial presence of two mitochondrial genomes does not negatively affect normal embryonic development.

Differences in Phenotype

Many skeptics are wary of clones that are not phenotypically identical to their nuclei donors. Phenotype is composed of not only genetics but also environment. Maternal uterine environment affects coat color due to melanoblast differences in migration and abundance. Maternal age, size, nutrition, immunity, and season of birth could also participate in phenotypic outcome. Therefore, genetically identical animals are not necessary phenotypically identical.

Cloning Benefits

Perhaps the biggest reason for cloning application is money. The economic value of genetically superior animals is reason enough to clone entire herds of highly productive offspring. Whole herds could be obtained in one generation rather than taking years if done by embryo splitting. The improved quality of products like milk and meat could be profitable for

many agricultural industries. Another major application is the production of animals resistant to economically devastating diseases. The possible saving of millions to billions of dollars a year by the livestock industry makes this one of the most attractive alternatives to producers.

The main application looked at by most animal scientists is the human implications of animal cloning. Already genetically modified sheep and pigs are producing products like insulin and clotting factors. Animals are being looked at as potential organ donors for human organ transplant patients. It is also being considered in the improvement of human infertility for parents whose age increases the risk of chromosomal abnormalities. A younger female's oocytes could be used with the desired nucleus transferred to produce a healthier baby than might otherwise be obtained.⁷

Conclusion

The potential for cloning is as of yet unrealized. The early stages of research are still in progress and while huge advancements have been made, many questions still lie unanswered. The possible benefits of cloning are phenomenal but the drawbacks are also considerable. These must be constructively evaluated and hopefully solved before cloning can become commonplace. Much more research must still be done before "pharming" can be viewed as a viable alternative to traditional farming methods.

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