
NOTES

Researchers desiring these animals should contact Dr. Carl Hansen of the NIH, Bethesda, Maryland.
This reference list is representative of the more than 100 citations on BHE rats in the literature. A complete list can be obtained from Dr. Carolyne D. Berdanier, Department of Foods and Nutrition, University of Georgia, Athens, GA 30602.

Nuclear Transplantation and Embryo Cloning in Mammals
Randall S. Prather, Ph.D.

INTRODUCTION

The ability to clone a potentially unlimited number of individuals would have innumerable applications. Identical individuals, or clones, would be perfect controls for experiments because all variation would be due to environmental factors such as treatments. Thus, fewer numbers of animals would be needed to make significant comparisons between treatments. In production agriculture, identical animals would require identical management systems. Clonal lines adapted to certain parts of the country, either naturally or by genetic manipulation, could be distributed to achieve maximum production. Given certain conditions (environmental stipulations such as rations and weather conditions) a performance guarantee could be provided with a set of clones at the time of purchase. Breeders could benefit from transferring a clonal line of frozen embryos to a clonal line of recipient females, because ease of giving birth and preweaning weight gains would be similar. Feedlots would benefit because a clonal line would perform as expected, thus guaranteeing a profit. Packing houses could purchase certain clonal lines that have identical meat characteristics, such as flavor, size, and marbling. Consumers would benefit from a more uniform product in their freezers. If this sounds like utopia for both science and agriculture, it is becoming fact. The technology necessary to achieve these goals is currently inefficient, but it does work and will be improved.

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CLONING

Potentially, the procedures to clone large numbers of individuals are numerous. Dedifferentiation of somatic cells is possible in theory but has yet to be demonstrated. Another possibility is to induce numerous rounds of mitosis in an early embryo without permitting any differentiation until enough cells have been produced to constitute numerous embryos. Again, this procedure has not been accomplished. A third prospect, first suggested by Spemann (1938), is to transfer nuclei from differentiated cells to a cell, such as an oocyte, that would induce dedifferentiation.

Developmental biologists are concerned with differentiation events. Cloning by nuclear transfer is actually a spin-off of a procedure designed to answer the more fundamental questions about differentiation because all of the nuclei of early embryos are presumed to be identical.

In 1938 Spemann proposed to evaluate the differentiation of nuclei during early development by conducting an experiment in which he transferred nuclei from cells at different stages of differentiation to enucleated, unfertilized oocytes. If all of the nuclei from a particular stage of development could direct development to term after nuclear transfer, they would be considered to be equivalent. The technical aspects of this experiment needed refinement and were not reported until 1952. Using Xenopus laevis, Briggs and King (1952) showed that nuclei from preblastula-stage embryos could direct early development, and they alluded to the fact that nuclei from beyond the blastula stage were less likely to result in similar development. The large amphibian eggs were ideal for these studies, and adults were subsequently produced as a result of nuclear transfer in both Xenopus laevis (Fischberg et al., 1958) and Rana pipiens (McKinnell, 1962).

In 1981 Illmensee and Hoppe reported successful nuclear transfer in the mouse. However, others were not able to repeat these results using the above procedure (McGrath and Solter, 1984; Robl et al., 1986). Undaunted by the discouraging results in mice, others more closely applied the successful amphibian procedures to other domestic animals, and in 1986 successful results were reported for both sheep (Willadsen, 1986) and cattle (Prather et al., 1986). Subsequent application to rabbits (Stice and Robl, 1988) and pigs (Prather et al., 1989a) has also resulted in offspring.

PROCEDURES AND THEORY OF NUCLEAR TRANSFER TECHNOLOGY

Nuclear Transfer

Initial studies in mammals involved simply transferring pronuclei between two zygotes. These experiments illustrated that pronuclear exchange procedures are compatible with development to term in mice (McGrath and Solter, 1983), cattle (Robl et al., 1987), and pigs (Prather et al., 1989a).

The basic procedures for cloning by nuclear transfer have been relatively unchanged since Briggs and King published them in 1952. The procedures for amphibians involve isolating cells from a donor embryo and aspirating a single cell into a micropipette. The internal diameter of the micropipette must be just small enough to cause the plasma membrane to rupture. The cytoplasmic contents are then deposited into the cytoplasm of an unfertilized oocyte. The unfertilized oocyte is activated either by the transfer procedure itself or by an additional stimulus (e.g., electrical stimulation), and the meiotic chromosomes of the oocyte are removed, leaving only the chromosomes of the transferred cell.

Current procedures in mammals are very similar. First the chromosomes are removed from an unfertilized oocyte, a procedure known as enucleation. This term is not technically correct because there is no nucleus present, but there is no better term available. Our laboratory uses techniques similar to those described by McGrath and Solter (1983), although other methods are also effective (Willadsen, 1986). For example, the unfertilized oocyte can be treated with cytochalasin, which imparts an elasticity to the plasma membranes. The oocyte is held adjacent to a holding micropipette with slight aspiration (Figure 1A). Next, a micropipette is inserted into the perivitelline space and the first polar body and underlying cytoplasm are removed. The metaphase chromo-

![FIGURE 1 Nuclear transfer procedures in cattle. A. An unfertilized oocyte arrested at meiotic metaphase II (arrow denotes the first polar body). A pipette is inserted through the zona pellucida and the first polar body and underlying cytoplasm are aspirated into the pipette and removed. B. An 8-cell stage embryo is held in place by aspiration to the holding pipette on the left and the transfer pipette is inserted through the zona pellucida and a single blastomere is aspirated into the pipette. C. Transfer of the 8-cell blastomere to the recipient enucleated oocyte (arrow denote the nucleus in the 8-cell stage blastomere). D. After nuclear transfer the recipient and donor cells are allowed to regain a spherical shape before electrofusion (arrow denotes transferred blastomere (400X magnification, diameter of the transfer pipette is 30 μm). (Reprinted with permission from Prather et al., 1987.)](https://academic.oup.com/ilarjournal/article-abstract/33/4/62/744299/27-February-2019)
somes can be indirectly visualized by using ultraviolet light and the DNA-specific stain bisbenzimide (Prather and First, 1990). After enucleation of the recipient oocyte, the donor embryo is held in place with the holding micropipette (Figure 1B). The transfer micropipette is inserted through the zona pellucida and the donor blastomere is aspirated into the micropipette and then deposited into the zona pellucida with the recipient oocyte (Figure 1C). Finally the nuclear transfer embryo is placed between two electrodes and electroporated with a mild electric current. Because of the thermodynamic instability of the small pores that form between the two cells, these pores congregate and coalesce to become a single channel. This causes fusion of the recipient oocyte and the transferred blastomere and also activation of the oocyte. Oocyte activation sets in motion the development and differentiation of the newly created embryo.

**Nuclear Remodeling and Reprogramming**

Presumably the association of unfertilized oocyte cytoplasm with the transferred nucleus results in a reprogramming of differentiation events. This is suggested by the development to term of unfertilized oocytes of sheep (Willadsen, 1986; Smith and Wilmut, 1989), cattle (Prather et al., 1987; Willadsen, 1989) (Figure 2), rabbits (Stice and Robl, 1988), and pigs (Prather et al., 1989a) but not of recipient pronuclear stage eggs in mice (McGrath and Solter, 1984), cattle (Robl et al., 1987), or rats (Kono et al., 1988).

In amphibians there is a dramatic exchange of proteins following nuclear transfer to an unfertilized oocyte. The transferred nucleus releases nuclear proteins and acquires cytoplasmic proteins (DiBerardino and Hoffner, 1975; Leonard et al., 1982). This protein exchange precedes an increase in nuclear volume following activation of the recipient oocyte (Merriam, 1969).

A specific antigen, and presumably protein, that follows this pattern of exchange in mammals is the J9 epitope on the A/C nuclear lamins. The nuclear lamins are intermediate filament-type proteins that reside on the inner nuclear membrane and polymerize and depolymerize with the cell cycle. In mammals there are generally three types of lamin proteins (designated A, B, and C) that are differentiated by molecular weight and isoelectric point. Lamin A is identical to lamin C except for an additional 98 amino acid tail (McKeon et al., 1987). Interestingly, in the mouse the A/C epitope is present in pronuclei and nuclei of two-cell stage embryos but absent in nuclei from the four-cell stage to implantation (Figure 3A,B). If a nucleus from an eight-cell stage embryo is transferred to an unfertilized oocyte and the oocyte is activated, then the A/C epitope is acquired in the transferred nucleus (Figure 3C,D). An identical result is observed in the pig after nuclear transfer to an enucleated activated oocyte (Prather et al., 1989b). However, the A/C epitope is not acquired when the nucleus is transferred to a pronuclear stage egg (Figure 3E,F) or when an enucleated pronuclear stage egg is used as a recipient (Prather et al., 1990).

Whether one interprets the data strictly as only a change in epitope exposure or loosely as the changes observed by the antibody as a result of changes in the proteins present, the conclusion reached is the same. Nuclei transferred to a pronuclear stage egg are not remodeled to resemble a pronucleus, while a nucleus transferred to a freshly activated unfertilized oocyte is remodeled to resemble a pronucleus. This remodeling presumably results in a reprogramming of developmental events. No specific examples of molecular reprogramming have been reported in mammals, but many have been reported in detail in amphibians (Gurdon, 1986; DiBerardino, 1987). Two examples of highly specific molecular reprogramming will be described below.

Differentiating myotome cells first begin producing muscle specific actin at the gastrula stage of the developing amphibian embryo. If a myotome cell nucleus is transferred to an enucleated activated oocyte, muscle-specific actin RNA synthesis ceases. When the resulting embryo develops to the gastrula stage and the myotome cells begin to differentiate, muscle-specific actin is produced only in the differentiating myotome cells (Gurdon et al., 1984). Another example of specific gene regulation is the 5S ooc gene, which is transcribed for a short period of time as the embryo passes through the late blastula stage. Nuclei from neurulae-stage embryos can

**FIGURE 2** Calves that have resulted from the transfer of 9- to 16-cell stage nuclei to unfertilized eggs. The calves are derived from different donor embryos and are therefore not genetically identical. The calves were named Copy and Fusion. Left to right, Dr. R.S. Prather, Dr. F.L. Barnes and Dr. N.L. First. Photograph by Meg Theno.
FIGURE 3 Nuclear lamin antigens before and after nuclear transfer to an unfertilized oocyte. A. Lamin A/C immunofluorescence (antibody J9) in a pronuclear stage and a 16-cell stage egg. B. DNA localization in the eggs pictured in panel A. Note that the pronuclei have a dimmer localization. This is due to their being haploid as well as dispersed over a larger area. All embryonic nuclei evaluated to date have a positive staining for B type lamins. C-D. Lamin A/C and lamin B, respectively, immunofluorescence localization in a mouse oocyte to which was transferred a 16-cell stage nucleus coincident with activation of the oocyte, followed by 4 hours of culture (arrow denotes transferred nucleus). E-F. Lamin A/C and lamin B, respectively, immunofluorescence localization in a mouse zygote to which was transferred a 16-cell stage nucleus followed by 4 hours of culture, small arrow indicated endogenous pronuclei (one of the pronuclei is out of the focal plane and is located directly below the endogenous pronucleus). Note that although the transferred nucleus has a positive reaction for the lamin B epitope, the A/C epitope is absent in the nucleus transferred to the zygote (C-D). This is in contrast to the nucleus transferred to the oocyte which was activated coincident with the nuclear transfer (E-F). (Prather et al., 1990.)

Attempts to clone mice have been discouraging. No one has been able to reproduce the work of Illmensee and Hoppe (1981) who first reported cloning of mice by nuclear transfer. McGrath and Solter (1984) published a report showing that nuclear transfer techniques similar to those used by Illemnsee and Hoppe did not result in the development of mice to term. Robl et al. (1986) hypothesized that the nuclear transfers did not go to term because the maternal to zygotic transition occurs at the two-cell stage and that enucleated zygotes are incapable of reprogramming this dramatic change in transcription. They suggested that nuclear transfer between the late two-cell and early eight-cell stage is more likely to result in development to term because there are relatively few differentiation events (both morphological and biochemical) within this developmental phase. Using this procedure, Robl et al. (1986) were able to show that development could proceed to midterm, and others showed that offspring could be produced (Tsunoda et al., 1987). However, complete reprogramming of the transferred nucleus was not observed (Barnes et al., 1987). More recently, the procedures for amphibian nuclear transfer have been more closely applied to the mouse, that is, the transfer of nuclei to enucleated, activated, meiotic metaphase-II-stage oocytes; however, offspring have yet to be born (Modlinski et al., 1990). In conclusion, development after nuclear transfer appears to be different in the mouse than in most other mammals. Although cloning by nuclear transfer has been successful in cattle, sheep, pigs, and rabbits, repeatable results in the mouse have yet to be reported.
that have the capacity to contribute to the somatic and germ cells of a chimera when injected into the blastocoel cavity of a blastocyst-stage embryo (reviewed by Prather et al., 1989c). ES cells are relatively undifferentiated cells and, therefore, are potentially excellent donor cells for nuclear transfer. Because they can be transformed and cultured in vitro, it is possible that ES cells could be used in creating a clonal transgenic line of animals. These clonal lines would all contain the gene of interest in the same location within the genome. Unfortunately similar ES cell lines have yet to be established in any of the domestic species (Evans et al., 1990).

Cloning by Splitting or Nuclear Transfer

Another method of producing identical individuals is by splitting the embryo into two or more parts. In the cattle industry this is routinely accomplished between the morula and blastocyst stages. If embryos are transferred fresh (as opposed to frozen-thawed), a calving rate of up to 105 percent can result (Leibo and Rall, 1987). The main difference between this method of producing identical individuals and the nuclear transfer method is the absence of reprogramming after splitting. For example, if a 32-cell-stage bovine embryo is split, after 1 day the two halves will develop into blastocysts. Because a minimum number of cells is required to form a competent blastocyst, the maximum number of animals that can result from splitting is about four (Robl and First, 1985). In contrast, if a 32-cell-stage blastomere is transferred to an enucleated, activated, meiotic metaphase-II-oocyte, after 1 day it should be a 2-cell-stage embryo, i.e. immediately after nuclear transfer the nucleus should be reprogrammed to behave as a zygote. This method of cloning has the potential to produce an unlimited number of identical individuals.

Genetic Equivalence and Identical Individuals

Although in theory animals produced from nuclear transfer embryos all have identical nuclear genomes, DNA rearrangements, diminutions, mutations, translocations, and gene amplifications can occur. One or more of these DNA alterations could occur during the first round of the cloning procedure or in subsequent serial nuclear transfers, causing divergence within a clonal line. Thus, it is very important to keep accurate records so that if a DNA alteration occurs, retrospective analysis can determine when it occurred and which subclonal lines are affected. From a broad perspective; however, these changes are likely to have little influence on the genetic similarity of a clonal line.

Since economics regulate that the source of recipient oocytes in domestic animals will likely be from slaught-
APPLICATIONS OF CLONAL TECHNOLOGY

The production of identical individuals would be useful to at least two groups of people: researchers and food producers. Researchers are always interested in cutting the cost of experiments. Since clones theoretically have no genetic variation, differences in responses to an experimental protocol in animals from a single clonal line should be caused by either environmental factors or treatments. With a clonal line, as with naturally identical twins, there also should be less variation within treatments (Biggers, 1986), and therefore, fewer numbers of animals should be needed for statistically valid comparisons (Biggers, 1986; Erb, 1990). Comparing results between clonal lines would give an insight into genetic differences in experimental responses.

Commercial agriculture could also benefit from clonal lines of livestock expressly suited for specific environmental conditions, such as animals able to digest certain kinds of roughage or animals resistant to heat stress or certain diseases. Management decisions will have similar effects on all animals. Clonal lines of animals may even be developed that have specific meat characteristics that fulfill consumer demands. When new demands or more efficient clonal lines are defined, these new genetics could be rapidly disseminated (reviewed by Robl and Stice, 1989).

In conclusion, nuclear transfer and cloning technology has immense potential for answering scientific questions, as well as for providing a source of food to meet the needs of the next century.

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REFERENCES


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Issues for Institutional Animal Care and Use Committees (IACUCs)

The Public Health Service Responds to Commonly Asked Questions

The National Institutes of Health’s (NIH’s) Office for Protection from Research Risks (OPRR) is responsible for developing, implementing, and overseeing compliance with the Public Health Service (PHS) Policy on the Humane Care and Use of Laboratory Animals (Policy). The PHS Policy, along with the U.S. Department of Agriculture’s (USDA’s) Animal Welfare Regulations, are the two principal federal documents setting forth requirements for animal care and use by institutions using animals in research, testing, and education. A primary function of OPRR in assisting institutions in implementing PHS Policy is to respond to policy-related questions. The following represent a few of the most commonly asked questions from institutions and the OPRR responses:

1. Are avian embryos covered by the PHS Policy, i.e., must their proposed use be reviewed and approved by institutional animal care and use committees (IACUCs)?

PHS Policy is applicable to proposed activities that involve live vertebrate animals. While embryonal stages of avian species develop vertebrae at a stage in their development prior to hatching, OPRR has interpreted “live vertebrate animal” to apply to avians (e.g., chick embryos) only after hatching.

This report was prepared by the staff of the Division of Animal Welfare, Office for Protection from Research Risks, National Institute of Health, in Bethesda, Maryland.