

CLONING HUMAN BEINGS

The Science of Animal Cloning

Commissioned Paper

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WHAT IS CLONING?

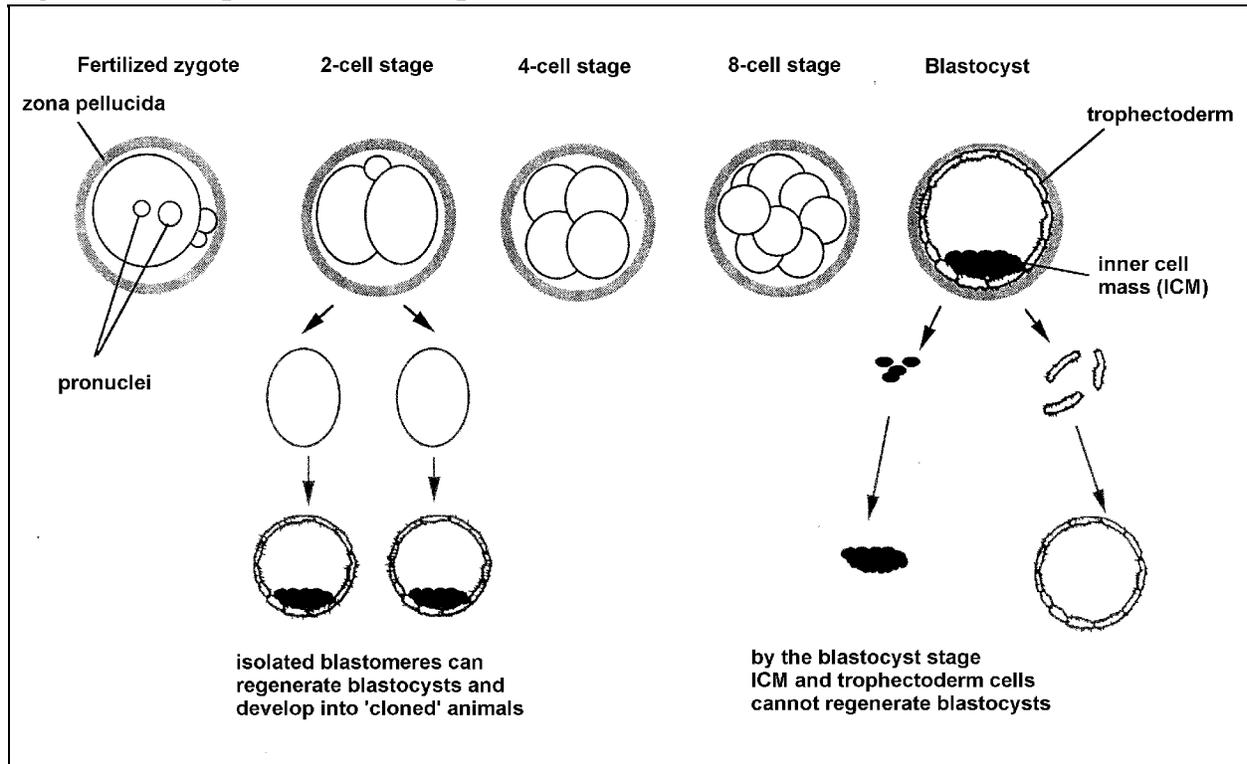
The word “clone” is used in many different contexts in biological research, but in simple terms it means a set of genetically identical individuals. Scientists talk about cloning DNA—the process of making and propagating a set of identical copies of a particular piece of genetic material, or about cloning cells—taking a single cell in culture and allowing it to multiply into a cell line. These techniques are some of the basic tools of the trade of modern biomedical research and are not currently a source of much public concern. But when we talk about cloning whole animals, especially mammals, the public rightly wants to know what is going on and why.

Genetically identical copies of whole organisms—clones—are commonplace in the plant breeding world. Many valuable horticultural or agricultural strains are maintained solely by vegetative propagation from an original plant, and never by sexual reproduction. This reflects the ease with which it is possible to regenerate a complete plant from a small cutting. The ability to propagate a valuable animal strain in the same way would revolutionize the agricultural business. However, in the animal kingdom, development is much less flexible than in plants. Many simpler invertebrate species have the ability to regenerate a whole organism from a small piece, although this is not necessarily their usual mode of reproduction. Vertebrates have lost this ability entirely, although regeneration of missing limbs, organs, or tissues can occur to varying degrees.

Although an adult vertebrate cannot make another adult, natural cloning does occur, in a limited way, with the formation of identical twins. These arise in humans and other mammals by chance separation of a single embryo into halves at an early stage of development. The resulting offspring will be genetically identical, deriving from one zygote—the result of the fusion of one egg and one sperm. A clone of two is not very remarkable, but it is a clone nonetheless. Experimental separation of cells from the early embryos of several mammalian species has shown that it is possible in some cases to get larger clones from one egg (Figure 1). In mice, only separated two-cell blastomeres are capable of generating entire mice (Rossant 1976), but in some domestic species, like sheep, it is possible to get separated eight-cell blastomeres to develop into viable offspring (Willadsen 1981).

At best, efficiency of this technique is never 100 percent, so the number of clones is small. However, the experiments are scientifically important, because they demonstrate that the cells of the early embryo are totipotent; that is, they retain the full potential to form an entire animal. As development proceeds, cells begin to differentiate into specialized cell types and can no longer revert back to the beginning of development (Figure 1). If this stability of the differentiated state could be reverted in some way, then producing animals from later differentiated cells or even adult cells would become feasible. Nuclear transfer experiments, first performed in amphibians in the 1960s, demonstrated how this could be done.

Figure 1: Preimplantation development in mammals



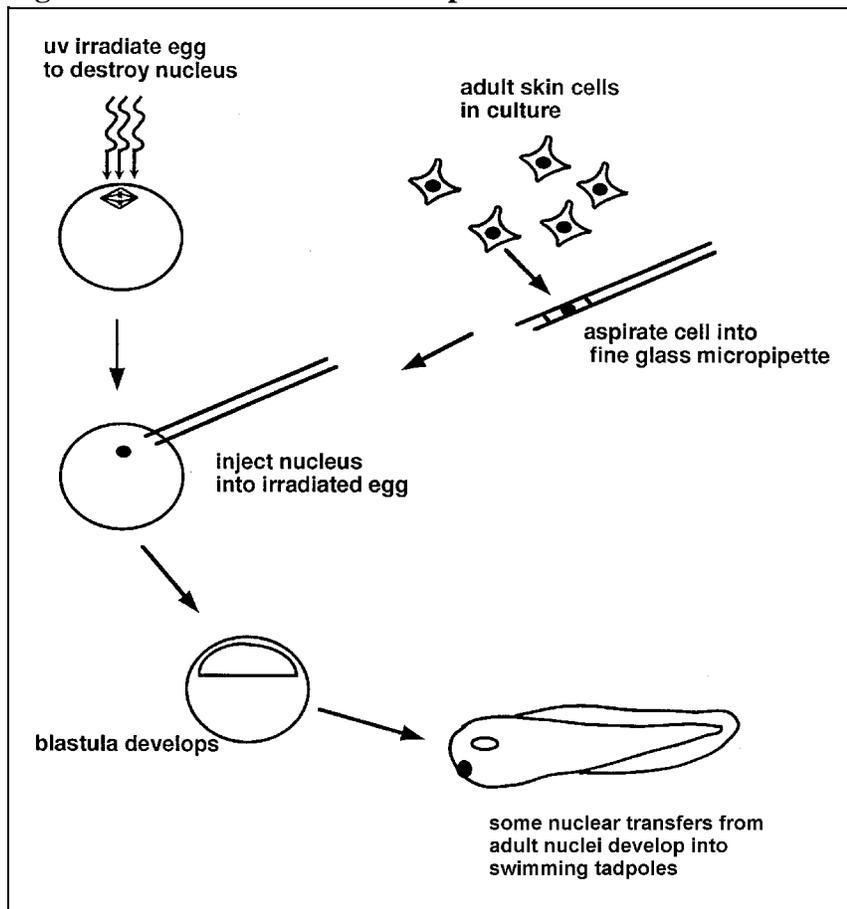
THE SCIENTIFIC QUESTION: DOES DIFFERENTIATION INVOLVE IRREVERSIBLE CHANGES IN GENETIC CONTENT?

The information for all the proteins produced in all the specialized cell types of the body is encoded in the DNA of the zygote and must be passed on intact into the next generation via the germ cells that form the eggs and sperm. However, there is no absolute requirement that the somatic cells—those cells not destined to give rise to the germ cells—retain the full genetic content in an unmodified form. A differentiated cell like a neuron must keep a set of neural-specific genes active and silence those genes specifically required to make muscle, liver, and other tissues. How does it do this? Is it an active process, in which genes are still present but repressed in some way, or is the DNA for the inactive genes lost or irreversibly inactivated in some way? In the early 1960s our understanding of the mechanisms of gene regulation was still rudimentary and this general question was extremely important.

Elegant experiments in *Xenopus laevis* by John Gurdon, following earlier experiments in *Rana temporaria* by Briggs and King (1952), provided strong evidence that the genetic content of differentiated somatic cells is essentially unchanged from that of the early embryo. Nuclei from donor differentiated cells were injected into recipient eggs from which the nucleus, containing the DNA, had been inactivated (Figure 2). If the donor nucleus could direct normal development of the recipient egg, this would strongly suggest that differentiation cannot involve permanent

changes in the genetic material. The first series of experiments used intestinal epithelial cells from swimming tadpoles (Gurdon 1962), and adult frogs were produced, albeit at a very low efficiency. The intestinal cells used were highly specialized brush-border gut cells, but were not derived from the final adult frog and so might not be terminally differentiated. Gurdon and colleagues (1975) performed another carefully controlled series of experiments in which they used nuclei from adult skin cells for transfer. Over 99 percent of the cultured cells expressed keratin, a differentiated marker of skin cells, and 4 percent of the nuclei transferred eventually gave rise to fully developed tadpoles. These experiments provided the strongest evidence to date that the nuclei of terminal differentiated cells could be reactivated by the cytoplasm of the egg and redirect normal development.

Figure 2: Nuclear transfer in amphibians



However, no viable adult frog was ever produced from an adult differentiated nucleus and there was a strong decline in the rate of recovery of feeding tadpoles with progressive age of nuclei transferred. This left open the theoretical possibility that complete reactivation of the adult

nucleus was prevented by some kind of irreversible change in the genetic material, and that there was, indeed, a progressive decline in nuclear potential with age. However, careful analysis at the time suggested that the major reason for developmental failure of transplant embryos was chromosomal abnormalities acquired as a consequence of the process of nuclear transplantation itself. The cell cycle of adult cells is much slower than the rapid pace of cell division in the early frog embryo. Expecting a transplanted nucleus to reprogram its gene expression, replicate its DNA, and enter normal embryonic cell cycles within an hour of nuclear transfer is unrealistic. The remarkable thing is that some nuclei manage to do so, rather than that so many do not.

The general conclusion from the amphibian nuclear transfer experiments of Gurdon and others was that the differentiated state did not involve major irreversible changes in the DNA. This conclusion was reached in the 1960s and early 1970s and has not been challenged in the intervening years (Gurdon 1974).

THE STABILITY OF THE DIFFERENTIATED STATE: OUR UNDERSTANDING TODAY

As our understanding of the regulation of gene expression has grown, we have learned that most patterns of differentiated gene expression are maintained by active control mechanisms (Blau 1992), in which combinations of regulatory proteins bind to control sequences adjacent to genes and turn them on or off. The particular differentiated state of a cell depends on its particular combination of regulatory proteins. This is not the only mechanism of gene control. There are some cases in which actual rearrangements and deletions of DNA occur, as in the expression of the immunoglobulin and T-cell receptor genes in lymphocytes. Heritable modification of the DNA by methylation can also affect gene expression. However, the overwhelming evidence suggests that given the right environment, it should be possible to activate or inactivate almost any gene in a cell.

This environment need not be the cytoplasm of the egg. Cell fusion experiments, in which different cell types are fused into one multinucleate cell called a heterokaryon, have demonstrated that extensive reprogramming of differentiated nuclei can occur. For example, when muscle cells are fused with non-muscle cells of various sorts, muscle-specific genes are activated in the non-muscle cells (Blau et al. 1985). Similarly, globin genes can be activated in many cell types after fusion with erythroid cells (Baron and Maniati 1986). These and other kinds of experiments have led to the isolation of specific protein factors that regulate cell differentiation, such as the myogenic factors that regulate the formation of the muscle cell lineages (Weintraub 1993).

All of this information has shown that the stability of the differentiated state is not absolute and, therefore, it should be theoretically possible to reprogram adult cells to reinitiate earlier programs of differentiation. Nuclear transfer experiments pointed the way and molecular biology is continuing to define the components of the regulation of cellular differentiation.

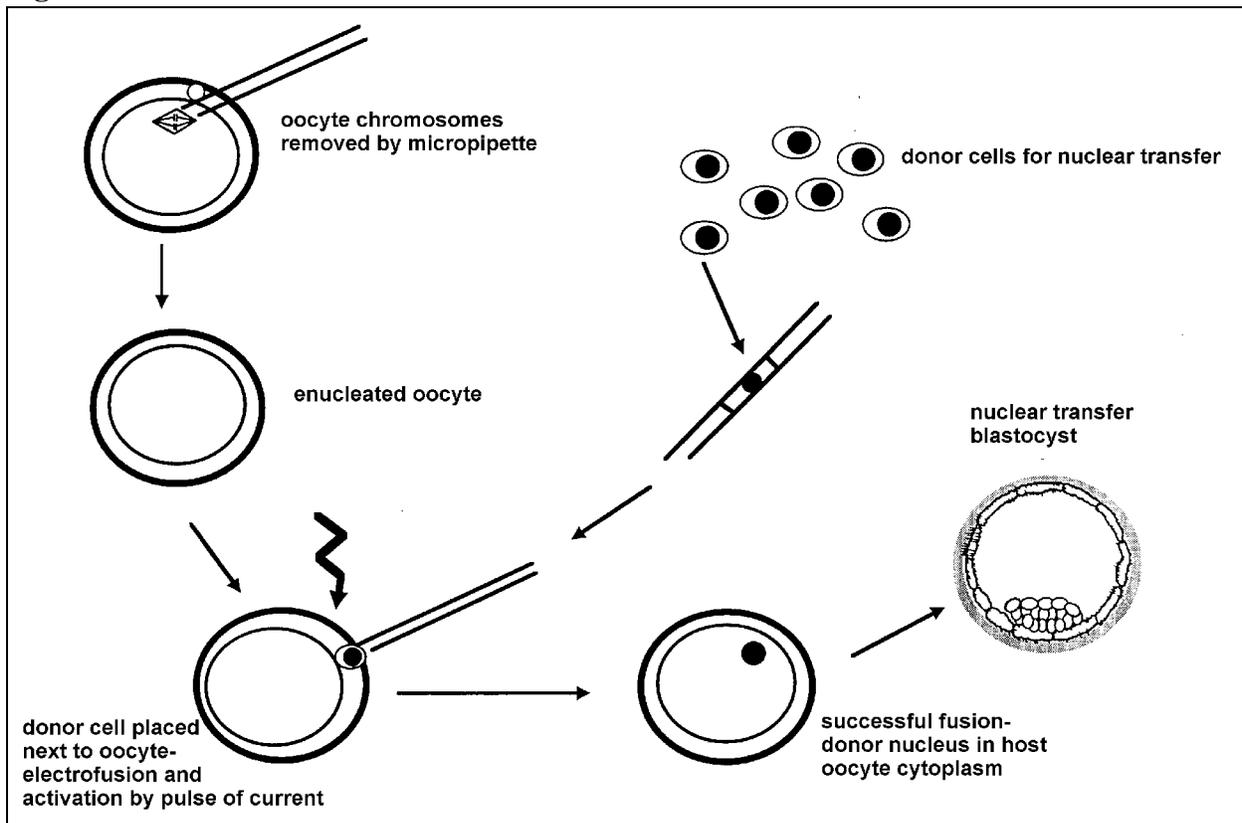
NUCLEAR TRANSFER IN MAMMALS: THE EARLY EXPERIMENTS

Following success in nuclear transfer experiments in frogs, there were some attempts in the 1970s to repeat the experiments in mice, the mammal of choice for experimental manipulation. It was known that early development occurs at a considerably slower rate in mammals than in amphibians, giving hope that reprogramming of the donor nucleus would occur more efficiently. In mice it takes about a day from fertilization to the first cleavage, giving ample time, it was thought, for the reprogramming of gene expression and adjustment of the cell cycle. This proved not to be the case. Early experiments showed that nuclei of adult cells fused with fertilized eggs did not undergo nuclear swelling or nuclear division (Graham 1969).

A careful series of experiments by McGrath and Solter in the mid 1980s showed that nuclei could be successfully exchanged between zygotes, with 90 percent reaching the blastocyst stage and beyond (McGrath and Solter 1984). Nuclei from 2-cell embryos could direct development to the blastocyst stage, but nuclei from later cleavage stages could not successfully recapitulate development after nuclear transfer. In fact, in mice, nuclei show less totipotency than whole cells—many experiments have shown that blastomeres from as late as the early blastocyst stage are still totipotent when combined with other embryonic cells (Rossant and Pedersen, 1986). This means that the failure of nuclear reprogramming has to be the result of something other than irreversible changes to the genetic material of the cells. In 1986, Willadsen showed that, unlike the situation in mice, enucleated unfertilized eggs from sheep could be fused with 8-cell stage blastomeres and viable offspring produced (Willadsen 1986).

Most recent experiments have used nuclear transfer into enucleated unfertilized oocytes, a procedure that prolongs the period of possible reprogramming before the donor nucleus has to undergo the first cleavage division. Oocytes arrested at metaphase II of meiosis, prior to fertilization, are enucleated by aspiration of the metaphase chromosomes into a fine glass micropipette (Figure 3). The nuclear donor cell is introduced under the egg membrane, or zona pellucida, and fused to the enucleated oocyte. The major technical advance in the last few years has been the use of electrofusion for both fusion of cells and activation of the oocyte. When the enucleated oocyte and the nuclear donor cell are subject to short electrical pulses in culture, membrane breakage and fusion occurs between the two cells and the electrical pulse also begins the processes of egg activation that would normally be triggered by fertilization. Using this approach, viable offspring have been obtained after nuclear transfer from 8-cell blastomeres in the mouse (Cheong et al. 1993) and from later stages of development in several other species, as will be discussed below.

Figure 3: Nuclear transfer in mammals



REPROGRAMMING IN THE OOCYTE ENVIRONMENT

There has been some study of the events that occur once an embryonic nucleus is exposed to the oocyte cytoplasm, and some, but not all, of the parameters that affect success of nuclear transfer are known (Fulka et al. 1996). Oocytes used for fusion are arrested in metaphase II of meiosis and only proceed to complete division, with extrusion of the second polar body, after fertilization or activation by some artificial signal, such as electrical current. In this arrested state, levels of maturation-promoting factor (MPF) are high. This cell-cycle regulatory complex promotes mitosis. When transplanted nuclei are introduced into the high MPF-containing oocyte environment, they usually undergo DNA replication, nuclear envelope breakdown, and premature chromosome condensation. Activation of the oocyte causes a decline in MPF activity and the nuclear envelope is reformed around the donor nucleus. The nucleus now takes on the appearance of the pronucleus of the egg, which is large and swollen. It is assumed that this process begins the reprogramming of the transferred nucleus, by exposing the chromatin to the oocyte cytoplasm and beginning the exchange of donor nuclear proteins for oocyte-derived proteins (Prather and First 1990).

Whether exposure to MPF and/or nuclear swelling is an absolute prerequisite for later development seems to be still unclear. Experiments in a number of species have shown that when nuclei are fused with oocytes that have been activated some hours prior to fusion, no DNA replication, chromosome condensation, or nuclear swelling occurs, but normal development can occur (Campbell et al. 1993, Campbell et al. 1994, Stice et al. 1994). Prefusion of blastomere and enucleated oocyte, with activation being induced after a few hours in culture, has also been attempted with success (Campbell et al. 1996). In all cases, the numbers of surviving progeny are too small to determine whether the differences in the success rates of the various treatments are statistically significant.

What is clear, however, is that the cell cycle stage of the donor nucleus does affect success. In rabbits, cows, sheep, and mice (Campbell et al. 1993, Cheong et al. 1993, Collas et al. 1992), experiments have shown that nuclei from cells in the early phases of the cell cycle do better than cells in S-phase or beyond. In the first phase of the cell cycle, G1, cells are diploid and relatively quiescent. They then enter a period of DNA replication, called S-phase, followed by another rest phase, called G2, where they have twice the diploid amount of DNA in preparation for mitosis. Because DNA replication is induced after nuclear transfer in the usual protocol, where fusion and activation are simultaneous, any nucleus that has more than the diploid DNA value upon transfer will end up with too much DNA, which will likely result in chromosome anomalies. Thus, the need to transfer G1 nuclei is paramount if chromosome damage is to be avoided. It seems likely that the failure to use carefully synchronized donor nuclei underlies some of the difficulties that have been reported in achieving successful nuclear transfer development in different species.

NUCLEAR TRANSFER IN MAMMALS: THE CURRENT STATE OF PLAY

Over the past ten years or so, there have been several reports of successful nuclear transfer experiments in mammals, nearly all of them using cells taken directly from early embryos. Surveying the literature on embryonic nuclear transfer, we find that the oldest embryonic nucleus that can successfully support development differs among species. In mice, no nucleus older than the 8-cell stage has been used successfully (Cheong et al. 1993). Four-cell blastomere nuclei have been successfully used in pigs (Prather et al. 1989), while in rabbits, 32- to 64-cell morulae can be used as nuclear donors (Yang et al. 1992). In cows and sheep, inner cell mass (ICM) cells from the 120-cell blastocyst stage have been used successfully (Collas and Barnes 1994, Smith and Wilmut 1989). Indeed, in both cows and sheep, cell lines have been made from ICMs and nuclei from these cells have been able to reprogram development after nuclear transfer. In the first experiments of this sort by Sims and First (1994), bovine ICM cells were grown in low-density cell suspensions for up to 28 days and then used as nuclear donors, without any attempt at synchronization of the cell cycle of the donor cells. Of those successfully fused, 24 percent developed to the blastocyst stage, and 4 out of 34 (12 percent) blastocysts transferred to recipient cows developed into normal calves. This success rate compares favorably with those using earlier

blastomeres, and suggests that it might be possible to achieve nuclear transfer success from permanent cell lines established from early embryos.

Wilmut and colleagues established permanent epithelial cell lines from sheep embryos and used these as nuclear donors after as many as 13 passages in culture (Campbell et al. 1996). In an attempt to avoid the problems of nuclear transfer of non-G1 nuclei into activated oocytes, they subjected their donor cell line to serum starvation prior to nuclear transfer. Under these conditions, where the cells are starved of essential nutrients, the cells exit the cell cycle and enter the so-called G0 state. Fusion of G0 nuclei to oocyte cytoplasm means that all nuclei can be activated to reenter the cell cycle together and problems of cell cycle asynchrony between donor and host are avoided. It was also suggested that the G0 state might actually be beneficial in terms of increasing the capacity of the nucleus to be reprogrammed by the oocyte cytoplasm. However, there is currently no direct evidence to support this, nor to conclude that nuclei synchronized in G0 are any better than nuclei synchronized in G1. Approximately 14 percent of fusions resulted in development of blastocysts, and 4 out of 34 (12 percent) embryos transferred developed into live lambs. The success rate in sheep and bovine experiments was almost identical, and suggested that long-term passage of early embryo cells need not inhibit their ability to be reprogrammed by the oocyte environment. Would the same be true of adult cells?

AND THEN CAME DOLLY

All of this background work led up to the famous Dolly, the first mammal to develop from the nucleus of an adult somatic cell (Wilmut et al. 1997). Wilmut and colleagues took fetal fibroblast cells and cells derived from the mammary gland of an adult sheep and applied the same approach of synchronizing cells in G0 prior to nuclear transfer. They reported successful production of live offspring from both cell types. Twenty-nine out of 247 (12 percent) of successful fusions between adult mammary gland nuclei and enucleated oocytes developed to the blastocyst stage, and 1 out of 29 (3 percent) blastocysts transferred developed into a live lamb—Dolly. This experiment was, in fact, the first time any adult animal had been derived from nuclear transfer of an adult nucleus, since the frog experiments generated only swimming tadpoles. However, the amount of new information about the stability of the differentiated state derived from this experiment was small, since no attempt was made to use only fully differentiated cells expressing specialized mammary gland proteins for the transfer, as was done for the skin cell experiments in frogs. The successful nuclear transfer animal could have derived from a less-differentiated, stem-cell-like cell in the population. The excitement generated by Dolly was more related to the realization that there may be no theoretical barrier to nuclear transfer into the oocyte from any cell of the body in any mammalian species. Hence, the science fiction scenario of copying or “cloning” an adult mammal, including humans, became science fact.

Several important questions remain unanswered about how feasible cloning from adult cells really will be, especially since only one successful adult nuclear transfer animal has been produced to date.

1. ***Are there true species differences in the ability to achieve successful nuclear transfer?***
We have seen that the published data suggest that nuclear transfer in mice is much less successful than in larger domestic animals. Part of this difference may reflect the intensity of research in this area in the last ten years, where agricultural interests have meant that more nuclear transfer work has been performed in domestic animals than in mice. But part of it may be real and reflect another critical component for the successful reprogramming of the donor nucleus—namely, the time between nuclear transfer and the activation of the embryonic genome. In order for a differentiated nucleus to redirect development in the environment of the egg, its particular constellation of regulatory proteins must be replaced by those of the egg in time for the embryo to be able to use the genome of the donor nucleus to transcribe the genes it needs for normal development. In mammals, unlike many other species, the early embryo rapidly needs to use the embryonic genome and cannot survive on the proteins and messenger RNA inherited from the mother in the egg. The time at which embryonic gene activation occurs varies among species—the late 2-cell stage in mice (Schultz 1993), the 4- to 8-cell stage in humans (Braude et al. 1988) and the 8- to 16-cell stage in sheep. The later onset of embryonic gene transcription in sheep provides an additional round or two of cell divisions in which nuclear reprogramming can occur, unlike the rapid genome activation in the mouse. Donor nuclei do turn on some of the 2-cell stage-specific genes after nuclear transfer in the mouse, but protein synthesis patterns are not identical between nuclear transfer and normal embryos (Latham et al. 1994). Further cross-species comparisons are needed to assess the importance of this difference in the time of genome activation for the success of nuclear transfer experiments.

2. ***Will imprinting affect the ability of nuclei from later stages to reprogram development?*** In mammals, the phenomenon of genomic imprinting means that the paternally and maternally inherited genomes are not equivalent (Solter 1988). Some heritable imprint is established on the chromosomes during gametogenesis, such that certain genes are expressed from only one of either the maternally or paternally inherited copies later in development. Imprinting explains why parthenogenetic embryos, with only maternally inherited genes, and androgenetic embryos, with only paternally inherited genes, fail to complete development (Fundele and Surani 1994). Nuclei transferred from a diploid organism, whether from the embryo or the adult, should contain both maternal and paternal copies of the genome and so not suffer the problems of parthenogenesis. However, an adult nucleus, if it is to be successful in reprogramming development, should retain intact the chromosomal imprints that normally determine whether maternal or paternal gene copies will be active. The successful generation of an adult sheep from an adult cell nucleus suggests that the imprint can be stable, but it is possible that some instability of the imprint, particularly in cells in culture, could limit the efficiency of nuclear transfer from adult cells. It is interesting that nuclear transfer embryos produced from established bovine embryonic cell lines died in mid-gestation, with specific deficiencies in placental development (Stice et al. 1996). Placental development has been found to be particularly sensitive to imprinting effects in mice (Moore and Haig 1991).

3. ***Will processes of cellular aging affect the ability of adult nuclei to program development?*** As somatic cells divide, they progressively age and there is normally a defined number of cell divisions that they can undergo before senescence. Part of this aging process involves the progressive shortening of the ends of the chromosomes, the telomeres. Germ cells and cancer cells evade this chromosome aging by possessing a telomerase activity that can keep telomeres full length (Chiu and Harley 1997). It seems likely that returning an adult mammalian nucleus to the oocyte will expose it to sufficient telomerase activity to reset telomere length, since oocytes have been found to be potent sources of telomerase activity (Mantell and Greider 1994).

4. ***Will the mutation load accumulated by adult cells affect nuclear transfer efficiency and predispose to cancer and other diseases?*** As cells divide and organisms age, mistakes and alterations in the DNA will inevitably occur and will accumulate with age. If these mistakes occur in germ cells, a heritable mutation occurs, but mutations in somatic cells are not necessarily harmless. Sporadic somatic mutations in a variety of genes can predispose a cell to become tumorigenic. Transfer of a nucleus from a somatic cell carrying such a mutation into an egg would transform a sporadic somatic mutation into a germline-equivalent mutation in all cells of the body, with presumably severe consequences on the likelihood of that mutation leading to malignant transformation. The risks of such events occurring following nuclear transfer is difficult to estimate.

WHY PURSUE ANIMAL CLONING RESEARCH?

The continued pursuit of nuclear transfer as a means of producing genetically identical copies of embryonic or adult organisms largely has been driven by technological needs rather than by the pursuit of basic knowledge of cellular differentiation. The goals are:

1. to generate groups of genetically identical individuals for research purposes,
2. to rapidly propagate “elite” animal stocks,
3. to improve the efficiency of generation and propagation of transgenic livestock, and
4. to generate targeted genetic alterations in domestic animals.

1. Making Clones for Research Purposes

Inbred strains of mice have been a major mainstay of biological research for years. These mice have been bred by brother-sister mating for many generations until they are essentially all genetically identical and homozygous (i.e., they carry two identical copies of all genes). Experimental analysis is then simplified, because variations in response to experimental treatment due to variations in genetic background can be eliminated. Clearly, generating homozygous inbred lines in larger animals with long generation times and small numbers of offspring is not readily achieved. The concept of generating small groups of identical animals by nuclear transfer has been proposed as an alternative strategy and apparently underlies the recent report from Oregon on successful nuclear transfer from early embryonic nuclei in monkeys. Repeated cycles of nuclear

transfer can expand the number of individuals derived from one donor nucleus, a trick first used in *Xenopus* experiments. Thus, the first nuclear transfer embryo is allowed to divide to early blastomere stages, and then those cells are used as donor nuclei for another series of transfers. This process can be carried on indefinitely, in theory, although practice suggests that fusion rates decline with each cycle of transfers. One experiment in cows, for example, produced a clone of 54 early embryos from nuclear transfer of a single blastomere nucleus from one parent embryo after three cycles of transfer (Stice and Keefer 1993). Viable calves were produced from all three cycles.

This approach is likely to be limited in its usefulness as a research tool, however. It must be remembered that a clone of animals derived from nuclear transfer from one individual is self-limited. Cloned animals do not breed true unless they are derived from an inbred stock, and each clone will differ genetically from a clone derived from another individual. Thus each member of a clone has to be made by the difficult procedure of nuclear transfer, and generation of large enough clones to be useful as experimental groups is likely to be prohibitively expensive in most animals.

2. Propagating Desirable Stocks

In animal breeding strategies, rapid spread of desirable traits within stocks of domestic animals is of obvious commercial importance. Artificial insemination and embryo transfer can increase the effective reproductive output of individual elite male and female animals, respectively, and are widely used in the livestock business. Nuclear transfer cloning, especially from adult nuclei, could provide an additional means of increasing the average “genetic merit” of a given generation of animals. The ability to make identical copies of adult prize cows, sheep, and pigs is a feature unique to nuclear transfer technologies and may well be used in livestock production if the efficiencies of adult nuclear transfer can be improved. The net effect of multiplying genetically favorable individuals by cloning will be to reduce the overall genetic diversity in a given livestock line, with possible adverse long-term consequences. Efforts will have to be made to ensure maintenance of a pool of genetic diversity for the future.

3. Improved Generation and Propagation of Transgenic Livestock

There is considerable interest in being able to genetically alter farm animals by introduction and expression of foreign DNA sequences in their genome. So-called transgenic animals were first made in mice by microinjection of DNA into the pronucleus of the egg. In a proportion of cases, the injected DNA integrates into a host chromosome and is then passed into all cells of the mouse and into the next generation as though it were a host gene. With the right DNA sequences attached, the foreign gene can be expressed and function in the transgenic environment. This ability to add genes to the genome has been a major research tool for understanding gene regulation and for making mouse models of certain human diseases. It has also been applied to other species, including livestock species. Proposed applications of this technology to livestock improvement include the possible introduction of growth-enhancing genes, genes that affect milk

quality or wool fibers, and disease-resistance genes (Ward and Nancarrow 1995). Progress has been slow. Initial results of attempting to manipulate meat production by overexpression of growth hormone in pigs led to undesirable side effects (Pursel et al. 1989). In light of the likely resistance of consumers to genetically manipulated meat, it seems probable that the use of transgenesis for livestock improvement will be limited.

The major activity in livestock transgenesis is focused on pharmaceutical and medical applications. The milk of livestock animals—sheep, goats, and cows—can be modified to contain large amounts of pharmaceutically important proteins by expression of human genes under the control of mammary gland-specific sequences (Houdebine 1994). In sheep, greater than 50 percent of the proteins in milk can be the product of a human transgene (Colman 1996). Even the milk of transgenic mice can yield milligram quantities of recombinant proteins. Since many such proteins are pharmaceutically active at very low concentrations, it is estimated that production of human drugs from transgenic animals could be a multimillion-dollar industry in the coming years. Regulatory approval for drugs prepared from milk is not yet in place.

The other major area of commercial interest is the use of transgenic animals for human organ transplantation. Pig organs in many cases are similar enough to human organs to be potentially useful in organ transplants if problems of rejection of the so-called xenograft could be overcome. Prevention of acute phase rejection of the xenografts has already been achieved by expression of human complement regulatory proteins in transgenic pigs. Further transgenic manipulation may lead to improved graft survival. Several companies are exploring the possibility of pig organ transplants despite possible risks of cross-species transfer of pathogenic viruses and the likely public resistance to xenografts.

How does nuclear transfer come into all this transgenic animal work? Transgenesis by zygote injection is inefficient. Not all injected eggs will develop into transgenic animals, and then not all transgenic animals will express the transgene in the desired manner. Characterizing a transgenic line of livestock is a slow and expensive business. Nuclear transfer would speed up the expansion of a successful transgenic line, but, perhaps more important, it would allow more efficient generation of transgenic animals in the first place. Foreign DNA could be introduced into cell lines in culture, and cells containing the transgene in the right configuration could be grown up and used as a source of nuclei for transfer, ensuring that all offspring are transgenic.

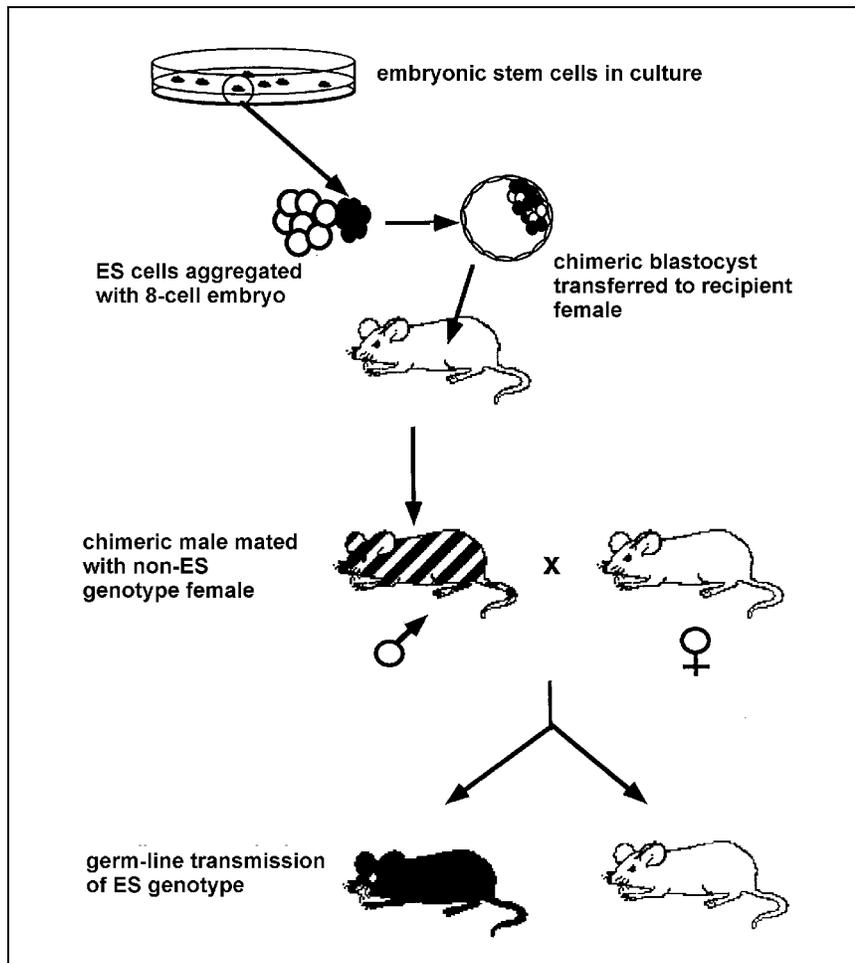
4. Generating Targeted Gene Alterations

The most powerful technology for genetic manipulation in mammals—gene targeting—was developed in mice, and depends on the ability of mammalian DNA, when added to cells in culture, to recombine homologously with identical DNA sequences in the genome and replace them. Thus, mutations or other desired alterations can be introduced into the genome in a directed and controlled manner and their effects studied (Capecchi 1989). This technology would have been of limited use, however, without some means of taking those changes generated in culture and reintroducing them into animals. In mice, this can be achieved by the use of embryonic stem (ES)

cells. These are cell lines derived from the ICM of the blastocyst, which can be cultured indefinitely in the undifferentiated state but which retain the potential to form all cells of the animal, including the germ cells, when returned to the environment of the early embryo (Figure 4). These “chimeric” animals can then be bred to transmit the ES genotype into the germ line. Thus, any genetic alteration made in the ES cells in culture can be introduced back into mice (Robertson 1986).

The combination of homologous recombination and ES cell technology has been responsible for the explosion of knock out mice, in which specific genes have been deleted from the genome. These mice enhance understanding of normal gene function and allow generation of accurate models of human genetic disease. Gene targeting approaches can also be used to ensure correct tissue-specific expression of foreign transgenes and to misexpress genes in inappropriate tissues. If applied to domestic animals, this technology could increase the efficiency of transgene expression by targeting transgenes to appropriate regions of the genome for expression. It could also be used to mutate endogenous genes so as to influence animal health and productivity or to

Figure 4: Generation of germline chimeras from embryonic stem cells

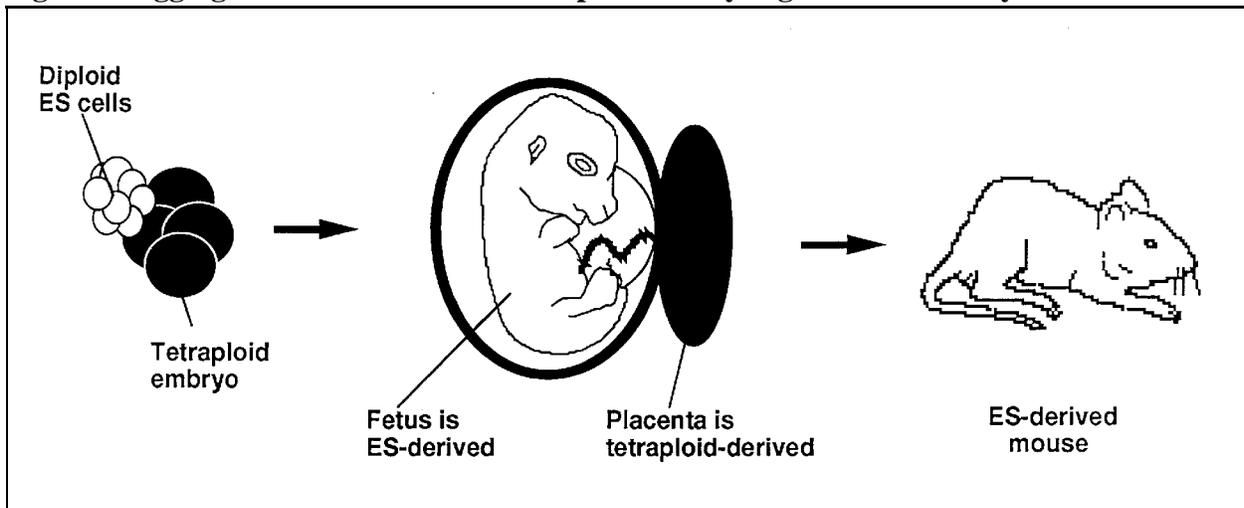


help prevent rejection of xenografts. However, to date, there are no fully validated ES cell lines in domestic animals. Nuclear transfer from non-pluripotent cell lines, as reported by Wilmut et al. 1997, provides a possible alternative to the ES cell route for introduction of targeted gene alterations into the germ line.

At this point it is unclear whether homologous recombination can occur efficiently in the kinds of cell lines used for sheep nuclear transfer. Experiments in mice have indicated that the ES cell environment is particularly conducive to homologous recombination, and efficiencies of targeted mutation tend to be much lower in non-ES lines. Attempts to generate ES cells from other species are continuing—primate (Thomson et al. 1995), rat (Iannaccone et al. 1994), and pig (Wheeler 1994) lines have been reported, and this may still be the best route to achieve precise gene alterations in domestic animals.

Apart from the fact that ES lines do not exist, the other argument for using nuclear transfer to introduce germ-line genetic alterations in farm animals is that it avoids one generation of breeding from chimeras, an important factor in farm animals with long generation times and small litter size. In fact, ES cells can also be used directly to generate cloned animals carrying the gene alteration of interest without the intermediate chimeric step. Clonal ES cell lines have been shown to be capable of forming entire mice when combined with developmentally compromised host embryos (Figure 5) (Nagy et al. 1993). Although this procedure is not yet very efficient, it illustrates the remarkable properties of these cells and suggests that similar approaches could be applied in other species.

Figure 5: Aggregation of ES cells with tetraploid embryos generates entirely ES-derived mice



HOW CAN WE USE INFORMATION FROM NUCLEAR TRANSFER EXPERIMENTS FOR HUMAN BENEFIT?

The biotechnology applications of nuclear transfer cloning in mammals are clear, but the underlying science does offer some opportunities for further understanding of the reversibility of the differentiation process. The demonstration that in mammals as in frogs, the nucleus of an adult cell can be reprogrammed by the environment of the early embryo, provides further impetus to studies on how to reactivate embryonic programs of development in adult cells—with exciting prospects for regeneration and repair of diseased or damaged human tissues and organs. Information on the mechanisms of reprogramming of the adult nucleus in the egg cytoplasm may provide clues as to how to reprogram adult differentiated cells directly without the need for oocyte fusion.

It may not be necessary to reprogram terminally differentiated cells, but rather to stimulate proliferation and differentiation of the quiescent stem cells, which are known to exist in many adult tissues—including the nervous system (Gage et al. 1995). Experiments in this area are likely to focus more on the conditions required for direct stimulation of the stem cells in specific tissues than on the actual use of nuclear transfer to activate novel developmental programs. These approaches to cellular repair using adult stem cells will be greatly aided by an understanding of how stem cells are established during embryogenesis. ES cells provide an interesting model for such studies, since they represent the precursors of all cell lineages in the body. They can be stimulated to differentiate in vitro into precursors of the hematopoietic, endothelial, neuronal, and muscle cell lineages, among others (Weiss and Orkin 1995), and they thus provide a potential source of stem cells for regeneration of all tissues of the body.

Once we have learned more about how to control the differentiation of mouse ES cells, one could envisage the generation of human ES-type cells as essentially endless sources of stem cells for tissue regeneration. Such cell lines could be generated from “spare” in vitro fertilized embryos or from fetal germ cells, as has proved possible in mice (Matsui et al. 1992). One could even envisage using nuclear transfer from an adult cell to generate an early embryo and therefore an ES line for each individual human, which would be ideally tissue-matched for later transplant purposes. This seems a rather expensive and far-fetched scenario; a more likely scenario would involve the generation of a few widely used and well-characterized human ES cell lines that had been genetically altered to prevent graft rejection in all possible recipients.

ETHICAL CONCERNS

As we move into the realms of direct human embryo manipulation, the ethical implications of this research become more apparent. It is outside the scope of this paper to discuss the various scenarios in which human nuclear transfer might be considered. Work with embryonic stem cells and genetic manipulation of early embryos in different species (including nuclear transfer) is already providing unparalleled insights into fundamental biological processes and promises to provide great practical benefit in terms of improved livestock, improved means of producing

pharmaceutical proteins, and prospects for regeneration and repair of human tissues. Great care should be taken in crafting any ethical or legal guidelines on human cloning to avoid inhibiting legitimate research in animals or humans that has the potential to provide immense benefits for the future.

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