

Chapter Two

THE SCIENCE AND APPLICATION OF CLONING⁴

The report in February 1997 that scientists in Scotland had cloned a sheep, Dolly, led to much public discussion of "cloning" of animals and speculation about the possibility of cloning humans. The term "cloning" is used by scientists to describe many different processes that involve making duplicates of biological material. In most cases isolated genes or cells are duplicated for scientific study, and no new animal results. This type of cloning, using genes and cells, has led to many medical advances such as providing insulin to treat diabetes and therapies for hemophilia. The sheep experiment was different; it used a cloning technique called "somatic cell nuclear transfer" and resulted in an animal that was a genetic twin—although delayed in time—of an adult sheep. This technique of transferring a nucleus from a somatic cell into an egg that produced Dolly was an extension of experiments that had been ongoing for over 40 years. These experiments were aimed at understanding how development of an animal from a single fertilized egg is carried out. In recent years the agricultural industry has been trying to improve nuclear transplantation cloning to facilitate the breeding of desirable livestock and some biotechnology companies are exploring ways to use nuclear transfer cloning to improve the production of therapeutic drugs. In addition to drug production, understanding the details of nuclear transplantation cloning might lead to new therapies to treat human disease. For instance it might be possible to grow human cells and tissues for transplantation and grafts that would not be rejected after transfer, as they often are today. These kinds of benefits are currently only hypothetical and much additional research will be needed in animal systems. Although the birth of Dolly was lauded as an amazing success, in fact the procedure is not perfected. Only one sheep was produced from over two hundred nuclear transfers. In addition, it is not yet clear whether Dolly is normal or whether she could have subtle problems that might lead to serious diseases. Using this technique to produce a human child might result in, for example, malformations or disease due to problems inherent in the technique. Thus, while using animals to understand the biological process that produced Dolly holds great promise for future medical advances, there is no current scientific justification for attempting to produce a human child at this time with this technique.

What is Cloning?

The word clone is used in many different contexts in biological research but in its most simple and strict sense, it refers to a precise genetic copy of a molecule, cell, plant, animal, or human being. In some of these contexts, cloning refers to established technologies that have been part of

⁴ Much of this chapter is derived from the material contained in two commissioned papers provided by Janet Rossant, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto; and by Stuart Orkin, Children's Hospital and Dana Farber Cancer Institute, Boston.

agricultural practice for a very long time and currently form an important part of the foundations of modern biological research.

Indeed, genetically identical copies of whole organisms are commonplace in the plant breeding world and are commonly referred to as “varieties” rather than clones. Many valuable horticultural or agricultural strains are maintained solely by vegetative propagation from an original plant, reflecting the ease with which it is possible to regenerate a complete plant from a small cutting. The developmental process in animals does not usually permit cloning as easily as in plants. Many simpler invertebrate species, however, such as certain kinds of worms, are capable of regenerating a whole organism from a small piece, even though this is not necessarily their usual mode of reproduction. Vertebrates have lost this ability entirely, although regeneration of certain limbs, organs, or tissues can occur to varying degrees in some animals.

Although a single adult vertebrate cannot generate another whole organism, cloning of vertebrates does occur in nature, in a limited way, through multiple births, primarily with the formation of identical twins. However, twins occur by chance in humans and other mammals with the separation of a single embryo into halves at an early stage of development. The resulting offspring are genetically identical, having been derived from one zygote, which resulted from the fertilization of one egg by one sperm.

At the molecular and cellular level, scientists have been cloning human and animal cells and genes for several decades. The scientific justification for such cloning is that it provides greater quantities of identical cells or genes for study; each cell or molecule is identical to the others.

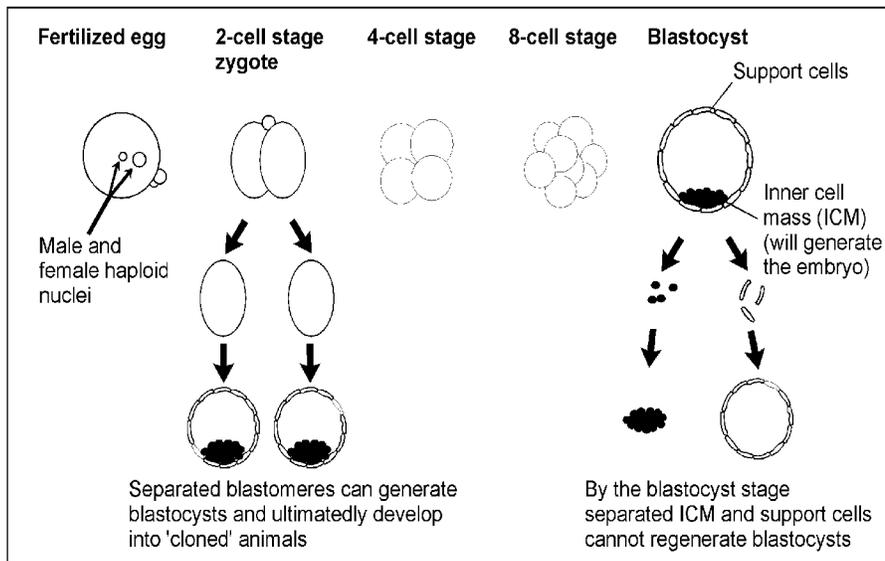
At the simplest level, molecular biologists routinely make clones of deoxyribonucleic acid (DNA), the molecular basis of genes. DNA fragments containing genes are copied and amplified in a host cell, usually a bacterium. The availability of large quantities of identical DNA makes possible many scientific experiments. This process, often called *molecular cloning*, is the mainstay of recombinant DNA technology and has led to the production of such important medicines as insulin to treat diabetes, tissue plasminogen activator (tPA) to dissolve clots after a heart attack, and erythropoietin (EPO) to treat anemia associated with dialysis for kidney disease.

Another type of cloning is conducted at the cellular level. *Incellular cloning* copies are made of cells derived from the soma, or body, by growing these cells in culture in a laboratory. The genetic makeup of the resulting cloned cells, called a cell line, is identical to that of the original cell. This, too, is a highly reliable procedure, which is also used to test and sometimes to produce new medicines such as those listed above. Since molecular and cellular cloning of this sort does not involve germ cells (eggs or sperm), the cloned cells are not capable of developing into a baby.

The third type of cloning aims to reproduce genetically identical animals. Cloning of animals can typically be divided into two distinct processes, *blastomere separation* and *nuclear transplantation cloning*.

In blastomere separation, the developing embryo is split very soon after fertilization when it is composed of two to eight cells (see figure 1). Each cell, called a blastomere, is able to produce a new individual organism. These blastomeres are considered to be totipotent, that is they possess the total potential to make an entire new organism. This totipotency allows scientists to split animal embryos into several cells to produce multiple organisms that are genetically identical. This capability has tremendous relevance to breeding cattle and other livestock.

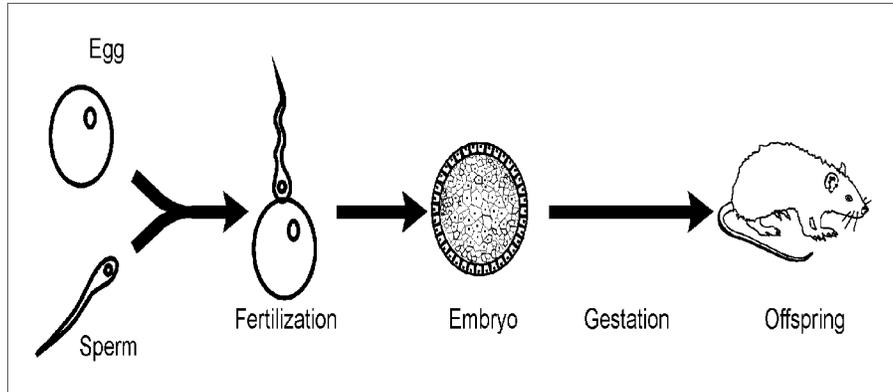
Figure 1. Preimplantation embryo development in mammals



In the early 1980s, a more sophisticated form of cloning animals was developed, known as nuclear transplantation cloning. The nucleus of somatic cells is diploid—that is, it contains two sets of genes, one from the mother and one from the father. Germ cells, however, contain a haploid nucleus, with only the maternal or paternal genes. In nuclear transplantation cloning, the nucleus is removed from an egg and replaced with the diploid nucleus of a somatic cell. In such nuclear transplantation cloning there is a single genetic "parent," unlike sexual reproduction where a new organism is formed when the genetic material of the egg and sperm fuse (see figure 2). The first experiments of this type were successful only when the donor cell was derived from an early embryo. In theory, large numbers of genetically identical animals could be produced through such nuclear transplantation cloning. In practice, the nuclei from embryos which have

developed beyond a certain number of cells seem to lose their totipotency, limiting the number of animals that can be produced in a given period of time from a single, originating embryo.

Figure 2. Sexual reproduction



The new development in the experiments that Wilmut and colleagues carried out to produce Dolly was the use of much more developed somatic cells isolated from adult sheep as the source of the donor nuclei. This achievement of gestation and live birth of a sheep using an adult cell donor nucleus was stunning evidence that cell differentiation and specialization are reversible. Given the fact that cells develop and divide after fertilization and differentiate into specific tissue (e.g., muscle, bone, neurons), the development of a viable adult sheep from a differentiated adult cell nucleus provided surprising evidence that the pattern of gene expression can be reprogrammed. Until this experiment many biologists believed that reactivation of the genetic material of mammalian somatic cells would not be complete enough to allow for the production of a viable adult mammal from nuclear transfer cloning.

The Science That Led to Dolly

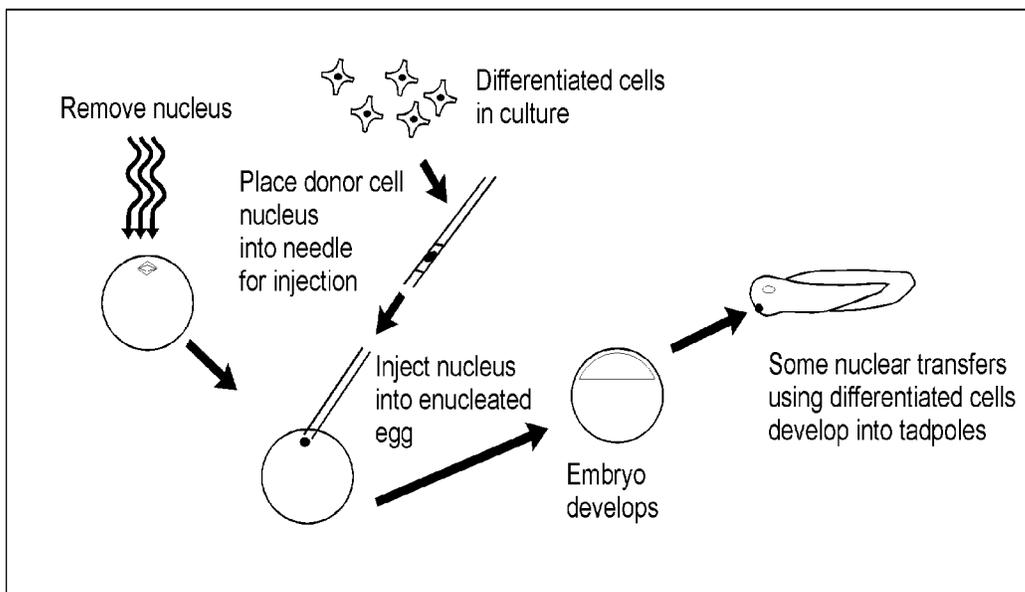
Until the birth of Dolly, developmental and molecular biologists focused their efforts on understanding the processes of cellular differentiation, the regulation of genes during this process, the factors that stimulate differentiation, and the reversibility of this process. Biologists have investigated whether, once cellular differentiation occurs, the process is reversible. These questions have by no means been fully answered by the appearance of Dolly. If anything, the existence of Dolly stimulates even more speculation and inquiry. This section describes the background of the science that led to the birth of the cloned sheep, including early studies of differentiation and development, research on regulation of gene expression, experiments using nuclear transfer in animals, and studies of cell programming and division.

Early Studies of Differentiation and Development

Nearly every cell contains a spheroid organelle called the nucleus which houses nearly all the genes of the organism. Genes are composed of DNA, which serve as a set of instructions to the cell to produce particular proteins. Although all somatic cells contain the same genes in the nucleus, the particular genes that are activated vary by the type of cell. For example, a differentiated somatic cell, such as a neuron, must keep a set of neural-specific genes active and silence those genes specific to the development and functioning of other types of cells such as muscle or liver cells.

Investigations which began over 40 years ago sought to determine whether a differentiated somatic cell still contained all genes, even those it did not express. Early experiments in frogs and toads by Gurdon (1962) and by Briggs and King (1952) provided strong evidence that the expression potential of the genes in differentiated cells is essentially unchanged from that of the early embryo. Nuclei from donor differentiated cells were injected into recipient eggs in which the nucleus had been inactivated (figure 3). The first series of experiments used cells from tadpoles as the source of donor nuclei (Gurdon, 1962) and adult frogs were produced, albeit at a very low efficiency. Although the cells used were highly specialized, they were not derived from the adult frog, so the cells might not have been fully differentiated.

Figure 3. Nuclear transfer carried out in frogs



In these experiments, because isolated nuclei were used, other cellular components were not transferred to the recipient egg. Among those other cellular components is an organelle called the mitochondrion, the energy-producing component of the cell. Although most of the genes specifying this cellular component reside in the nucleus, the mitochondrion itself houses some of its own genes. Thus, in somatic cell nuclear transfer, mitochondrial genes are not transferred to the enucleated egg along with the nuclear genes. Because there are some serious diseases associated with mitochondrial genes, nuclear transplantation could allow an embryo to develop with new, healthy mitochondria from a donor.

Gurdon and colleagues performed another carefully controlled series of experiments in which they used nuclei from adult frog skin cells for transfer to an enucleated egg (Gurdon, et al., 1975). Four percent of the nuclei transferred eventually gave rise to fully developed tadpoles. These experiments provided evidence that the genes contained in the nuclei of differentiated cells could be reactivated by the cytoplasm of the egg and thus direct normal development, but only up to a certain stage. No viable adult frog ever developed from these tadpoles and there was a decrease in the number of tadpoles born as the age of the transferred nucleus increased. This left open the possibility that complete reactivation of the adult nucleus was prevented by some irreversible change in the genetic material, and that there was a progressive decline in nuclear potential with age.

Careful analysis, however, suggested that the major reason for developmental failure of the transplanted embryos appeared to be chromosomal abnormalities that occurred during the process of nuclear transplantation itself. The rate of cell division of adult cells is much slower than that of the cells of the early frog embryo. Thus, in reality, for this technique to work it would be necessary that the transplanted adult nucleus reprogram its gene expression, replicate its DNA, and enter the normal embryonic cell division cycle within an hour of nuclear transfer. It is remarkable, given the mechanics and timing of the process, that any nuclei from adult somatic cells were successful in generating an embryo. Although they did not produce normal adult animals, the amphibian nuclear transfer experiments of Gurdon and others succeeded in demonstrating that the differentiated state of adult somatic cells do not involve major irreversible changes in their DNA.

Regulation of Gene Expression

In recent years, it has been determined that most patterns of differentiated gene expression are maintained by active control mechanisms, in which particular genes are turned on or off by regulatory proteins (Blau, 1992). Further studies suggested that it might be possible to reprogram the gene expression of somatic cells so that they perform a different task. The role of a particular cell type (e.g., muscle, liver, or skin) depends on the combination of regulatory proteins it expresses. While in certain specialized cells, such as white blood cells, actual rearrangements and deletions of DNA occur, for the most part, however, gene expression is not regulated by the loss of DNA but by the turning off of specific genes. Thus, it should be possible to activate or inactivate almost any gene in a cell, given the right cellular environment containing the appropriate regulatory molecules.

To reprogram the gene expression of a somatic cell it is not essential to fuse it with an egg; in some cases re-programming can occur through fusion of two adult cells. Cell fusion experiments, in which different somatic cell types are fused, 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), and, similarly, genes that code for hemoglobin can be activated in many cell types after fusion with red blood cells (Baron and Maniatis, 1986). These and other kinds of experiments have led to the isolation of specific factors that regulate cell differentiation, such as the gene that regulates the formation of muscle cells (Weintraub, 1993).

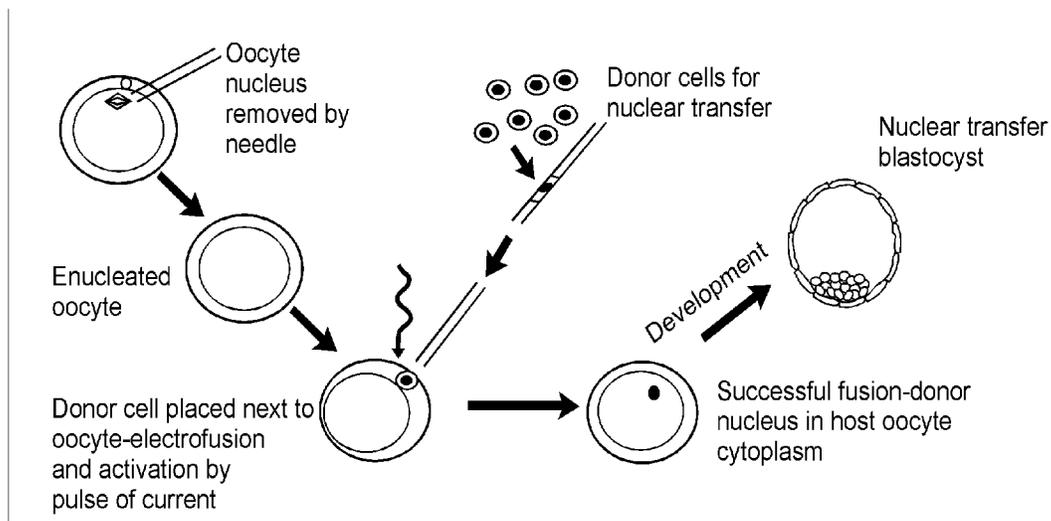
These studies have further demonstrated that the stability of the differentiated state is not absolute. Thus, given the appropriate regulatory molecules and enough time to reprogram an adult nucleus, somatic cells can re-initiate earlier programs of differentiation.

Nuclear Transfer in Mammals

Experiments in mammals have also suggested that it is possible to reprogram adult somatic cells. Following success in the nuclear transfer experiments in frogs, scientists attempted to repeat the experiments in mice. It was known that early development occurs at a considerably slower rate in mammals than amphibians, giving hope that reprogramming of the donor nucleus would occur more efficiently. For example, the first cell division in mice occurs about a day after fertilization, giving ample time, it was thought, for the reprogramming of gene expression and adjustment of the cell division cycle. This proved not to be the case. Early experiments showed that nuclei from somatic cells fused with fertilized eggs did not undergo nuclear division (Graham, 1969).

However, a series of experiments in mice in the mid 1980s showed that nuclei could be successfully exchanged between fertilized eggs, with 90 percent reaching the blastocyst stage of embryonic development and beyond (McGrath and Solter, 1984). Nuclei recovered and transplanted from embryos at the two-cell stage could direct development to the blastocyst stage. Nuclei transferred from embryos at later stages, however, could not successfully recapitulate development. In fact, in mice, nuclei show less totipotency than whole cells. Many experiments have shown that blastomeres up to 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 reported experiments with sheep. Unlike the situation in mice, enucleated eggs from sheep could be fused with blastomeres taken from embryos at the eight-cell stage to provide donor nuclei and viable offspring were produced (Willadsen, 1986).

Figure 4. Nuclear transfer via electrofusion in mammals



Recent experiments have used nuclear transfer into enucleated unfertilized eggs (figure 4). Using these very early stage eggs prolongs the period of possible reprogramming before the donor nucleus has to undergo the first division. And the advent in the last few years of electrofusion for both fusion of cells and activation of the egg has been another major advance, because activation and fusion occur simultaneously. Because these experiments use fusion of two cells and not simple injection of an isolated nucleus, all of the cellular components are transferred. Thus, the mitochondria, which contain some genes of their own, are transferred along with the nucleus. Because an enucleated egg also contains mitochondria, the result of a fusion experiment is a cell with a mixture of mitochondria from both the donor and the recipient. Since the mitochondrial genes represent an extremely small proportion of the total number of mammalian genes, mixing of mitochondria per se is not expected to have any major effects on the cell. However, if the nucleus donor suffers from a mitochondrial disease, and the egg donor does not, then mixture of the mitochondria may significantly alleviate the disease.

Over the past ten years or so there have been numerous reports of successful nuclear transfer experiments in mammals, nearly all of them using cells taken directly from early embryos. The oldest embryonic nucleus that can successfully support development differs among species. Four-cell blastomere nuclei have been successfully used in pigs (Prather, et al., 1989). In mice, no nucleus older than the eight-cell stage has been used successfully (Cheong, et al., 1993). In rabbits, 32- to 64-cell early embryos can be used as nuclear donors (Yang, et al., 1992). In cows and sheep, cells from what is called the inner cell mass (ICM) of the 120-cell blastocyst stage (see figure 1) have been used successfully (Collas and Barnes, 1994; Smith and Wilmut, 1989). Indeed, in both cows and sheep, cell lines have been made from these ICM cells and nuclei from these cells have been used to reprogram development after transfer into enucleated unfertilized eggs.

In the first experiments of this sort by Sims and First (1994), cow cells derived from embryos were grown in the laboratory for up to 28 days, and then used as nuclear donors, without any attempt at synchronization of the cell division cycle of the donor cells. Of those successfully fused with eggs, 24 percent developed to the blastocyst stage, and 4/34 (12 percent) of the blastocysts transferred to recipient cows developed into normal calves. This success rate compares favorably with those seen using earlier blastomeres and suggests that it might be possible to achieve successful nuclear transfer from permanent cell lines established from early embryos.

Reprogramming of Nuclei and Synchronization of the Cell Division Cycle

There has been some study of the events that occur once a transferred nucleus is exposed to the cytoplasm of the egg and some, but not all, of the parameters that affect success of nuclear transfer are known (Fulka, et al., 1996). Enucleated eggs used for fusion only proceed to division after activation by some artificial signal, such as the electrical current used in the electrofusion technique. When donor nuclei are introduced into the enucleated egg, they usually undergo DNA replication, nuclear envelope breakdown, and chromosome condensation. After activation of the egg the nuclear envelope is reformed around the donor chromosomes. The nucleus now takes on the appearance of a typical egg nucleus at this stage, which is large and swollen. It is assumed that this process begins the reprogramming of the transferred donor nucleus by exposing the chromosomes to the egg cytoplasm and beginning the exchange of egg-derived proteins for the donor nucleus' own proteins (Prather and First, 1990).

It is not clear whether exposure to proteins found in the earliest stages of development and/or nuclear swelling is a prerequisite for reprogramming for later development. Experiments in a number of species have shown that, when nuclei are fused with eggs that have been activated some hours prior to fusion, no DNA replication, chromosome condensation, or nuclear swelling occurs, but normal development can transpire (Campbell, et al., 1994; Stice, et al., 1994).

Once again, it is not obvious which of the processes described above are required for normal development. In rabbits, cows, sheep, and mice (Cheong, et al., 1993; Collas, et al., 1992) experiments have shown that nuclei from cells in the early phases of the cell division cycle do better than cells in later stages. In the first phase of the cell cycle, termed G1 (for Gap phase 1), cells contain only one complete set of chromosomes and are relatively quiescent. They then enter a period of DNA synthesis or replication, called S-phase, followed by a rest phase, called G2 (Gap phase 2), at which time they each have a duplicate copy of each chromosome. This doubling of the chromosomes is in preparation for cell division where an equal number will be divided between the two daughter cells. Because DNA replication is induced *after* nuclear transfer, any nucleus that has initiated replication *before* transfer will end up with too much DNA, which will likely result in chromosome anomalies. Thus, the need to transfer nuclei in the G1 phase before replication is initiated, is likely to be important to avoid chromosome damage that will prevent development of the embryo into a viable offspring.

Changes in Technique that Allowed for the Birth of Dolly

In background work that preceded the birth of Dolly, Wilmut and colleagues established cell lines from sheep early embryos, or blastocysts, and used these cells as nuclear donors (Campbell, et al., 1996). In an attempt to avoid the problems of nuclear transfer of non-G1 nuclei into activated eggs, they starved the donor cell line by removing all nutrients from the medium prior to nuclear transfer. Under these starvation conditions, the cells exit the cell cycle and enter the so-called "G0" state (Gap phase 0), similar to the G1 phase in which chromosomes have not replicated. Fusion of G0 nuclei with eggs ensures that the donor chromosomes have not initiated replication prior to fusion. It was also suggested that the G0 state might actually increase the capacity of the nucleus to be reprogrammed by the egg cytoplasm. However, there is currently no direct evidence to support this, nor to conclude that nuclei synchronized in the G0 stage are any better than nuclei synchronized in G1. For Wilmut and colleagues, approximately 14 percent of fusions resulted in development of blastocysts, and 4/34 (12 percent) embryos transferred developed into live lambs. Two died shortly after birth. The success rate in sheep and cow experiments was almost identical, and suggests that division of cells in culture for many days does not inhibit the ability of their nuclei to be reprogrammed by the egg environment. Could the same be true of nuclei from fully differentiated somatic cells?

All of this background work led up to Dolly (Wilmut, et al., 1997). Wilmut and colleagues took late embryo, fetal cell cultures, and cell cultures derived from the mammary gland of an adult sheep and applied the same approach of synchronizing the cells in the G0 stage prior to nuclear transfer. They reported successful production of live offspring from all three cell types, although only 29 of 277 (11 percent) of successful fusions between adult mammary gland nuclei and enucleated oocytes developed to the blastocyst stage, and only 1 of 29 (3 percent) blastocysts transferred developed into a live lamb. This experiment was, in fact, the first time any fully developed animal had been born following transfer of a somatic cell nucleus, since the earlier frog experiments only generated tadpoles.

It should be noted, however, that the amount of new information regarding the stability of the differentiated state derived from this experiment is small, as no attempt was made to document that the donor cells were fully differentiated cells, the genes of which expressed specialized mammary gland proteins. In the earlier experiments with frogs, the fact that the donor cells were fully differentiated was documented in such a manner. In the present case, Dolly could have been derived from a less-differentiated cell in the population, such as a mammary stem cell.

Remaining Scientific Uncertainties

Several important questions remain unanswered about the feasibility in mammals of nuclear transfer cloning using adult cells as the source of nuclei:

First, can the procedure that produced Dolly be carried out successfully in other cases? Only one animal has been produced to date. Thus, it is not clear that this technique is reproducible even in sheep.

Second, are there true species differences in the ability to achieve successful nuclear transfer? It has been shown 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; agricultural interests have meant that more nuclear transfer work has been performed in domestic animals than in mice. But part of the species differences may be real and not simply reflect the greater recent effort in livestock. For example, in order for a differentiated nucleus to redirect development in the environment of the egg, its constellation of regulatory proteins must be replaced by those of the egg in time for the embryo to use the donor nucleus to direct normal development of the embryo. The species difference may be the result of the different times of embryonic gene activation.

In mammals, unlike many other species, the early embryo rapidly activates its genes and cannot survive on the components stored in the egg. The time at which embryonic gene activation occurs varies between species—the late 2-cell stage in mice (Schultz, 1993), the 4-8 cell stage in humans (Braude, et al., 1988) and the 8-16 cell stage in sheep. The later onset of embryonic gene activation and transcription in sheep provides an additional round or two of cell divisions during which nuclear reprogramming can occur, unlike the rapid genome activation in the mouse. 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. In humans, for example, the time period before gene activation is very short, which might not permit the proper reprogramming of genes after nuclear transfer to allow for subsequent normal development.

Third, will the phenomenon of genetic imprinting affect the ability of nuclei from later stages to reprogram development? In mammals imprinting refers to the fact that the genes inherited on the chromosomes from the father (paternal genes) and those from the mother (maternal genes) are not equivalent in their effects on the developing embryo (Solter, 1988). Some heritable imprint is established on the chromosomes during the development of the egg and the sperm such that certain genes are expressed only when inherited from the father or mother. 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 diploid cells, whether embryonic or adult, should contain maternal *and* paternal copies of the genome, and thus not have an imbalance between the maternally and paternally derived genes.

The successful generation of an adult sheep from a somatic 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 somatic cells. It is known that disturbances in imprinting lead to growth abnormalities in mice and are associated with cancer and rare genetic conditions in children.

Fourth, will cellular aging affect the ability of somatic cell nuclei to program normal 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, and other genetic changes. Germ cells (eggs and sperm) evade telomere shortening by expressing an enzyme, telomerase, that can keep telomeres full length. It seems likely that returning an adult mammalian nucleus to the egg environment 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).

Fifth, will the mutations that accumulate in somatic cells affect nuclear transfer efficiency and lead to cancer and other diseases in the offspring? As cells divide and organisms age, mistakes and alterations (mutations) in the DNA will inevitably occur and will accumulate with time. If these mistakes occur in the sperm or the egg, the mutation will be inherited in the offspring. Normally mutations that occur in somatic cells affect only that cell and its descendants which are ultimately dispensable. Nevertheless, such mutations are not necessarily harmless. Sporadic somatic mutations in a variety of genes can predispose a cell to become cancerous. Transfer of a nucleus from a somatic cell carrying such a mutation into an egg would transform a sporadic somatic mutation into a germline mutation that is transmitted to all of the cells of the body. If this mutation were present in all cells may lead to a genetic disease or cancer. The risks of such events occurring following nuclear transfer are difficult to estimate.

Why Pursue Animal Cloning Research?

Research on nuclear transfer cloning in animals may provide information that will be useful in biotechnology, medicine, and basic science. Some of the immediate goals of this research are:

- to generate groups of genetically identical animals for research purposes
- to rapidly propagate desirable animal stocks
- to improve the efficiency of generating and propagating transgenic livestock
- to produce targeted genetic alterations in domestic animals
- to pursue basic knowledge about cell differentiation

Cloning Animals for Research Purposes

Inbred strains of mice have been a mainstay of biological research for years because they are essentially genetically identical and homozygous (i.e., both copies of each gene inherited from the mother and father are identical). Experimental analysis is simplified because differences in genetic background that often lead to experimental variation are eliminated. Generating such homozygous inbred lines in larger animals is difficult and time consuming because of the long gestation times and small numbers of offspring. The concept of generating small groups of identical animals by nuclear transfer has been proposed as an alternative strategy to obtaining a genetically identical group of animals, and apparently underlies a recent report from Oregon on successful nuclear transfer from early embryonic nuclei in rhesus macaque monkeys (Weiss and Schwartz, 1997).

Repeated cycles of nuclear transfer can expand the number of individual animals derived from one donor nucleus, allowing more identical animals to be generated. 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 successful fusion rates decline with each cycle of transfer. One experiment in cows, for example, produced 54 early embryos after three cycles of transfer from a single blastomere nucleus from one initial embryo (Stice and Keefer, 1993). Viable calves were produced from all three cycles of nuclear transfer.

This approach is likely to be limited in its usefulness, however. A group of cloned animals derived from nuclear transfer from an individual animal is self-limited. Unless they are derived from an inbred stock initially, each clone derived from one individual will differ genetically from a clone derived from another individual. Once a cloned animal is mated to produce offspring, the offspring will no longer be identical due to the natural processes which shuffle or recombine genes during development of eggs and sperm. Thus each member of a clone has to be made for each experiment by nuclear transfer, and generation of a large enough number of cloned animals to be useful as experimental groups is likely to be prohibitively expensive in most animals.

Advantages of Nuclear Transfer Cloning for Breeding Livestock

In animal breeding, the rapid spread of certain traits within stocks of domestic animals is of obvious commercial importance and has very long historical standing. Artificial insemination and embryo transfer can increase the effective reproductive output of individual elite male and female animals and are widely used in the livestock industry. Nuclear transfer cloning, especially from somatic cell nuclei, could provide an additional means of expanding the number of chosen livestock. 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 chosen animals by cloning will be to reduce the overall genetic diversity in a given livestock line, likely with severe adverse long-term consequences. If this technique became widespread, efforts would have to be made to ensure a pool of genetically diverse animals for future livestock maintenance.

Improved Generation and Propagation of Transgenic Livestock

There is considerable interest in being able to genetically alter farm animals by introduction and expression of genes from other species, such as humans. So-called "transgenic animals" were first developed using mice, by microinjection of DNA into the nucleus of the egg. This ability to add genes to an organism has been a major research tool for understanding gene regulation and for using the mouse as a model in studies of certain human diseases. It has also been applied to other species including livestock. Proposed applications of this technology to livestock improvement include the possible introduction of growth-enhancing genes, genes that affect milk quality or wool fibers, or disease-resistance genes (Ward and Nancarrow, 1995). But progress has been slow. Initial results of the manipulation of meat production by expression of excess growth hormone in pigs led to undesirable side effects (Pursel, et al., 1989).

Currently, the major activity in livestock transgenesis is focused on pharmaceutical and medical applications. The milk of livestock animals can be modified to contain large amounts of pharmaceutically important proteins such as insulin or factor VIII for treatment of human disease by expressing human genes in the mammary gland (Houdebine, 1994). In sheep greater than 50 percent of the proteins in milk can be the product of a human gene (Colman, 1996). The milk of even transgenic mice can yield large (milligram) quantities of recombinant proteins. Since many such proteins are active at very low concentrations, it is estimated that production of human drugs from transgenic animals could be considerably more cost-effective than current methods.

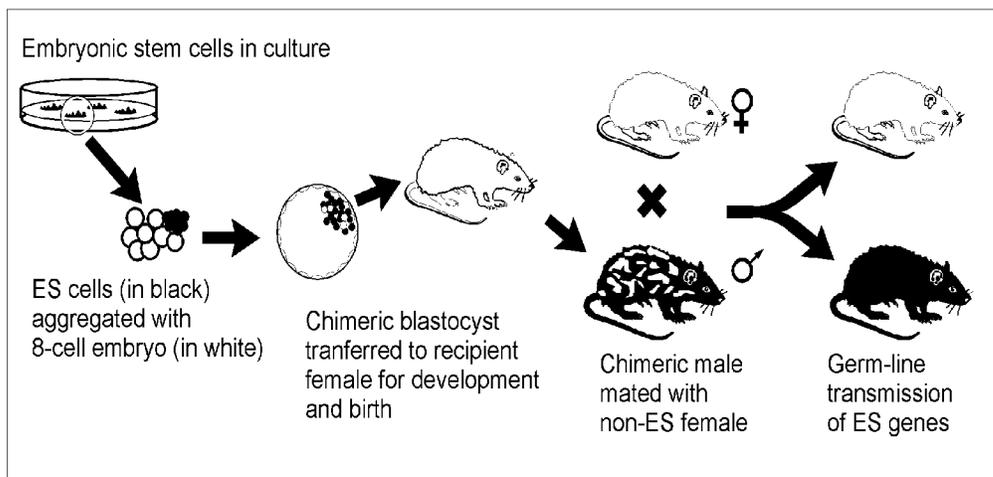
Another major area of interest is the use of transgenic animals for organ transplantation into humans. Pig organs, in many cases, are similar enough to humans to be potentially useful in organ transplants, if problems of rejection could be overcome. Rejection can already be partly overcome by the expression of human complement (a component of the immune system) regulatory proteins in transgenic pigs. Further transgenic manipulation such as the expression of human antigens in pigs could alleviate organ shortages by minimizing or eliminating the rejection of pig organs transplanted into humans, although other barriers, such as the possible transmission of viruses from pigs to humans, must be overcome.

However the current method of directly injecting genes into fertilized eggs is inefficient. Not all injected eggs will develop into transgenic animals, and then not all transgenic animals will express the added gene in the desired manner. The production of transgenic livestock is slow and expensive. Nuclear transfer would speed up the expansion of a successful transgenic line, but, perhaps more importantly, it would allow more efficient generation of transgenic animals in the first place. Foreign DNA, such as a human gene, could be introduced into cell lines in culture and cells expressing the transgene could be characterized and used as a source of donor nuclei for cloning, and all offspring would likely express the human gene. This, in fact, was the motivation behind the experiments that led to the production of Dolly. If a human gene such as that for insulin could be expressed in the mammary gland, the milk of the sheep would be an excellent source of insulin to treat diabetes.

Generating Targeted Gene Alterations

The most powerful technology for gene replacement in mammals was developed in mice. This technique adds manipulated or foreign DNA to cells in culture to replace the DNA present in the genome of the cells. Thus mutations or other alterations that would be useful in medical research can be introduced into an animal in a directed and controlled manner and their effects studied, a process called *gene targeting* (Capecchi, 1989). This technology would be of limited use, however, without some means of taking the changes generated in cultured cells and reintroducing them into animals. In mice, this can be achieved by the use of embryonic stem (ES) cells that are capable of being cultured indefinitely in the undifferentiated state. ES cells retain the potential to form all cells of the animal, including the germ cells, when returned to the environment of the early embryo (figure 5). As the technique is currently used in mice, the first generation of animals generated from the ES cells are "chimeric," that is they are made up of a mixture of cells from two different animals. These mice must then be bred one more time to transmit altered genes to the next generation. Using this technique, any genetic alteration made in the embryonic stem cells in culture can be introduced back into mice (Robertson, 1986).

Figure 5. Generation of mice from embryonic stem cells

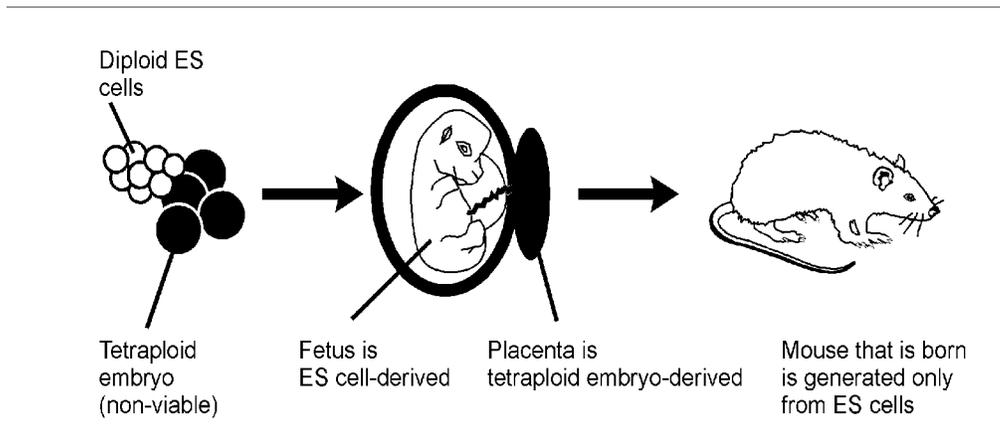


This use of gene replacement and embryonic stem cell technology has been responsible for the explosion in the generation of "knock-out" mice, in which specific genes have been deleted from the genome. These mice have been invaluable in current studies to understand normal gene function and to allow the generation of accurate models of human genetic disease. Gene targeting approaches can also be used to ensure correct tissue-specific expression of foreign genes and to suppress the expression of genes in inappropriate tissues. If applied to domestic animals, this technology could increase the efficiency of the expression of foreign genes by targeting the introduced genes to appropriate regions of the chromosome. It could also be used to directly alter the normal genes of the organism, which could influence animal health and productivity or to help

develop transgenic organs that are less likely to be rejected upon transplantation. However, to date, there are no fully validated embryonic stem cell lines in domestic animals. Nuclear transfer from somatic cell lines into an egg, as reported by Wilmut and colleagues, provides a possible alternative to the embryonic stem cell route for introduction of targeted gene alterations into the germ line of animals.

Apart from the fact that embryonic stem cell lines have not yet been produced from farm animals, the other argument for using nuclear transfer to introduce germ line genetic alterations in farm animals is that it eliminates one generation of breeding from the initial chimeric animals. This is an important time and cost saving factor in farm animals with long generation times and small litter size. However this factor might not be as important as once thought. In mice, it turns out, embryonic stem cells can also be used to generate cloned animals carrying gene alterations directly without the initial generation of chimeric animals. When 'tetraploid' embryos that are not themselves capable of developing normally are used as the host cells, the entire mouse fetus can be derived directly from the normal diploid ES cells (figure 6)(Nagy, et al., 1993). Although this procedure is not yet very efficient, it illustrates the remarkable properties of ES cells and suggests that similar approaches could be applied in other species such as farm animals.

Figure 6. Mice can be generated directly from ES cells without first generating a chimera



Basic Research on Cell Differentiation

The basic cellular processes that allowed the birth of Dolly by nuclear transfer using the nucleus from an adult somatic donor cell are not well understood. If indeed the donor cell was a fully differentiated cell and not a rare, less differentiated stem cell that resulted in this cloned sheep, there will be many questions to ask about how this process occurred. How the specialized cell from the mammary gland was reprogrammed to allow the expression of a complete developmental program will be a fascinating area of study. Developmental biologists will want to know which genes are reprogrammed, when they are expressed, and in what order. This might

shed light on the still poorly understood process of sequential specialization that must occur during development of all organisms.

Molecular biologists will also likely learn much from studying how reprogramming and reactivation occurred. What regulatory proteins in the host egg participated in the reprogramming? How did these proteins interact with each other and the DNA so that inactive genes from the mammary gland cells might be activated again? Answers to these kinds of questions will contribute to our overall understanding of how cells grow, divide, and become specialized.

Basic research into these fundamental processes may also lead to the development of new therapies to treat human disease. It is not possible to predict from where the essential new discoveries will come. However, already the birth of Dolly has sparked ideas about potential benefits that might be realized. To explore the possibility of these new therapies, extensive basic research is needed.

Much of this basic research will likely be done in the mouse as this animal is widely used by developmental biologists, and thus a great deal is already known about its development. However, as described above, the use of cloning in other animals—such as cows, pigs and sheep—by agricultural and biotechnology companies will also contribute to understanding of the basic processes involved. The study of nuclear transplantation cloning in a wide variety of animals will be very useful. Although many of the basic cellular mechanisms underlying animal development are the same in all mammals, there are subtle developmental variations that often lead to major technical differences in working with a particular species. Because a technique is often perfected in one species before being applied to another, knowing which parts of the techniques are widely applicable and which might need to be perfected for the given species will be of great value. This body of research into animal systems will answer many questions about the feasibility of various new therapeutic applications being proposed for human cells. New innovations in treating human disease can be tested in animal systems to determine if the basic foundation of the idea is sound before experiments using human cells would be required. Thus the path to testing the potential therapies to treat human disease, described below, should initially go through testing in animal models before progressing to human cell research.

Potential Therapeutic Applications of Nuclear Transfer Cloning

The demonstration that, in mammals as in frogs, the nucleus of a somatic cell can be reprogrammed by the egg environment provides further impetus to studies on how to reactivate embryonic programs of development in adult cells. These studies have exciting prospects for regeneration and repair of diseased or damaged human tissues and organs, and may provide clues as to how to reprogram adult differentiated cells directly without the need for oocyte fusion. In addition, the use of nuclear transfer has potential application in the field of assisted reproduction.

Potential Applications in Organ and Tissue Transplantation

Many human diseases, when they are severe enough, are treated effectively by organ or tissue transplantation, including some leukemias, liver failure, heart and kidney disease. In some instances the organ required is non-vital, that is, it can be taken from the donor without great risk (e.g., bone marrow, blood, kidney). In other cases, the organ is obviously vital and required for the survival of the individual, such as the heart. All transplantation is imperfect, with the exception of that which occurs between identical twins, because transplantation of organs between individuals requires genetic compatibility.

In principle, the application of nuclear transfer cloning to humans could provide a potential source of organs or tissues of a predetermined genetic background. The notion of using human cloning to produce individuals for use solely as organ donors is repugnant, almost unimaginable, and morally unacceptable. A morally more acceptable and potentially feasible approach is to direct differentiation along a specific path to produce specific tissues (e.g., muscle or nerve) for therapeutic transplantation rather than to produce an entire individual. Given current uncertainties about the feasibility of this, however, much research would be needed in animal systems before it would be scientifically sound, and therefore potentially morally acceptable, to go forward with this approach.

Potential Applications in Cell-based Therapies

Another possibility raised by cloning is transplantation of cells or tissues not from an individual donor but from an early embryo or embryonic stem cells; the primitive, undifferentiated cells from the embryo that are still totipotent. This potential application would not require the generation and birth of a cloned individual. Embryonic stem cells provide an interesting model for such studies, since they represent the precursors of all cell lineages in the body. Mouse embryonic stem cells can be stimulated to differentiate *in vitro* into precursors of the blood, 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.

It might be possible to take a cell from an early blastomere and treat it in such a manner as to direct its differentiation along a specific path. By this procedure it might be possible to generate in the laboratory sufficient numbers of specialized cells, for example bone marrow stem cells, liver cells, or pancreatic beta-cells (which produce insulin) for transplantation. If even a single tissue type could be generated from early embryonic cells by these methods and used clinically, it would constitute a major advance in transplantation medicine by providing cells that are genetically identical to the recipient.

One could imagine the prospect of nuclear transfer from a somatic cell to generate an early embryo and from it an embryonic stem cell line for each individual human, which would be ideally tissue-matched for later transplant purposes. This might be a rather expensive and far-fetched

scenario. An alternative scenario would involve the generation of a few, widely used and well characterized human embryonic stem cell lines, genetically altered to prevent graft rejection in all possible recipients.

The preceding scenarios depend on using cells of early human embryos, generated either by *in vitro* fertilization or nuclear transfer into an egg. Because of ethical and moral concerns raised by the use of embryos for research purposes it would be far more desirable to explore the direct use of human cells of adult origin to produce specialized cells or tissues for transplantation into patients. 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 even 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 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.

Another strategy for cell-based therapies would be to identify methods by which somatic cells could be “de-differentiated” and then “re-differentiated” along a particular path. This would eliminate the need to use cells obtained from embryos. Such an approach would permit the growth of specialized cells compatible with a specific individual person for transplantation. Although at the current time this strategy is highly speculative, ongoing research in animal systems may identify new approaches or new molecular targets that might make this approach feasible.

It will be of great importance to understand through experiments in animals how the environment of the egg reprograms a somatic cell nucleus. What cellular mechanisms can be elucidated? What components are involved in these processes? Can we direct cells along particular developmental pathways in the laboratory and use these cells for therapy? The capacity to grow human cells of different lineages in culture would also dramatically improve prospects for effective somatic gene therapy.

Assisted Reproduction

Another area of medicine where the knowledge gained from animal work has potential application is in the area of assisted reproduction. Assisted reproduction technologies are already widely used and encompass a variety of parental and biological situations, that is, donor and recipient relationships. In most cases, an infertile couple seeks remedy through either artificial insemination or *in vitro* fertilization using sperm from either the male or an anonymous donor, an egg from the woman or a donor, and in some cases surrogacy. In those instances where both individuals of a couple are infertile or the prospective father has non-functional sperm, one might envision using cloning of one member of the couple’s nuclei to produce a child.

Although this constitutes an extension of current clinical practice, aside from the serious, moral, and ethical issues surrounding this approach, there are significant technical and medical causes for caution, some of which were described in the research questions enumerated above.

In most situations of assisted reproduction, other than the intentional union of the gametes by *in vitro* techniques, the fertilized egg and initial cells of the early embryo are not otherwise manipulated. In some rare cases, such as preimplantation genetic diagnosis, the embryo is manipulated by the removal of one of the identical cells of the blastomere to test its genetic status. In contrast, if nuclear transfer were to be used as a reproductive option, it would entail substantially more invasive manipulation. Thus far, the animal cloning of Dolly is a singular success, one seemingly normal animal produced from 277 nuclear transfers. Until the experiment is replicated the efficiency, and even the validity, of the procedure cannot be fully determined. It is likely that the mere act of manipulating a nucleus and transferring it into an egg could decrease the percentage of eggs that go on to develop and implant normally, as well as increase the rate of birth defects.

Cloning and Genetic Determinism

The announcement of Dolly sparked widespread speculation about a human child being created using somatic cell nuclear transfer. Much of the perceived fear that greeted this announcement centered around the misperception that a child or many children could be produced who would be identical to an already existing person.

This fear reflects an erroneous belief that a person's genes bear a simple relationship to the physical and psychological traits that compose that individual. This belief, that genes alone determine all aspects of an individual, is called "genetic determinism." Although genes play an essential role in the formation of physical and behavioral characteristics, each individual is, in fact, the result of a complex interaction between his or her genes and the environment within which they develop, beginning at the time of fertilization and continuing throughout life. As social and biological beings we are creatures of our biological, physical, social, political, historical, and psychological environments. Indeed, the great lesson of modern molecular genetics is the profound complexity of both gene-gene interactions and gene-environment interactions in the determination of whether a specific trait or characteristic is expressed. In other words, there will never be another you.

While the concept of complete genetic determinism is wrong and overly simplistic, genes do play a major role in determining biological characteristics including a predisposition to certain diseases. Moreover, the existence of families in which many members are affected by these diseases suggest that there is a single gene that is passed down with each generation that causes the disease. When such a disease gene is identified, scientists often say they have "cloned the gene for" breast cancer, for instance, implying a direct cause and effect of gene and disease. Indeed, the recent efforts of the Human Genome Project have led to the isolation of a large number of genes

that are mutated in specific diseases, such as Duchenne Muscular Dystrophy, and certain types of breast and colon cancer.

However, recent scientific findings have revealed that a “one-gene, one-disease” approach is far too simplistic. Even in the relatively small list of genes currently associated with a specific disease, knowing the complete DNA sequence of the gene does not allow a scientist to predict if a given person will get the disease. For example, in breast cancer there can be many different changes in the DNA, and for some specific mutations there is a calculated risk of developing the disease, while for other changes the risk is unknown. Even when a specific genetic change is identified that "causes" the disease in some people, others may be found who have the same change but do not get the disease. This is because other factors, either genetic or environmental, are altered that mask or compensate for "the" disease gene. Thus even with the most sophisticated understanding of genes, one cannot determine with certainty what will happen to a given person with a single change in a single gene.

Once again, the reason rigid genetic determinism is false is that genes interact with each other and with the environment in extremely complex ways. For example, the likelihood of developing colon cancer, a disease with a strong hereditary component and for which researchers have identified a single "causative" gene, is also strongly influenced by diet. When one considers a human trait that is determined by multiple genes, the situation becomes even more complex. The number of interactions between genes and environment increases dramatically. In fact, the ability to predict what a person will be like knowing only their genes becomes virtually impossible because it is not possible to know how the environment and chance factors will influence the outcome.

Thus the idea that one could make through somatic cell nuclear transfer a team of Michael Jordans, a physics department of Albert Einsteins, or an opera chorus of Pavarottis, is simply false. Knowing the complete genetic makeup of an individual would not tell you what kind of person that individual would become. Even identical twins who grow up together and thus share the same genes and a similar home environment have different likes and dislikes, and can have very different talents. The increasingly sophisticated studies coming out of human genetics research are showing that the better we understand gene function, the less likely it is we will ever be able to produce at will a person with any given complex trait.

Conclusions

The term “clone” has many meanings but in its simplest and most scientific sense it means the making of identical copies of molecules, cells, tissues, and even entire animals. The latest news about cloning Dolly the sheep involved somatic cell nuclear transplant cloning. In this process the nucleus from an adult somatic cell is transplanted into an enucleated ovum to produce a developing animal that is a “delayed” genetic twin of the adult.

There are many applications that nuclear transfer cloning might have for biotechnology, livestock production, and new medical approaches. Work with embryonic stem cells and genetic manipulation of early embryos in animal 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.

However, the possibility of using human cloning for the purposes of creating a new individual entails significant scientific uncertainty and medical risk at this time. Potential risks include those known to be associated with the manipulation of nuclei and eggs and those yet unknown, such as the effects of aging, somatic mutation, and improper imprinting. These effects could result in high rates of failed attempts at pregnancy as well as the increased likelihood of developmentally and genetically abnormal embryos.

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