The future of cloning

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The future of cloning

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It is now possible to make clones, or exact genetic copies, of sheep, cows, goats, mice and, probably, humans. This opens the way towards the production of replacement body parts from adult cells.

Cloning techniques have been in use for centuries. The practice of taking cuttings is universal among gardeners, and large companies now propagate desirable plant strains in their millions. Lower invertebrates can also be cloned — cut an earthworm or flatworm in half, for example, and the missing halves will regenerate to create two genetically identical individuals. Although vertebrates cannot be cloned by these routes, identical twins are naturally occurring genetic clones. Moreover, the method of nuclear transplantation, first developed about 40 years ago in frogs, has been successfully used to make clones of sheep, mice, cows and goats, and it could probably be applied to people too. By taking a few non-reproductive cells from adult mammals, identical replicas can be created without damage (or even inconvenience) to the donors.

But what is the way forward for cloning techniques in the next century? Combined with other cell-biological procedures, they could open the way towards a type of tissue-replacement therapy that avoids the problems of immune rejection. What can we achieve using this methodology, and what are the potential benefits for humans?

How is it done?

In vertebrates, fertilization begins with the union of the sperm and the egg. The unfertilized egg is stopped at a certain stage of the cell-division cycle, and the sperm provides an activation stimulus that triggers the resumption and completion of cell division. The egg and sperm 'pronuclei' then swell, their chromosomes unravel from the tightly packed, 'condensed' state in which they are stored, and DNA replication can proceed. The chromosomes then recondense, the nuclear membrane dissolves, and the fertilized egg divides into two identical daughter cells.

Nuclear transfer subverts fertilization by replacing the female genetic material of an unfertilized egg with the nucleus from a different cell. This was first done successfully on frogs in the 1950s, in the United States and Britain. A non-reproductive (somatic) cell, such as an intestinal epithelial cell, was ruptured by suction into a glass microneedle. Its nucleus, surrounded by a layer of cytoplasm, was then injected into an unfertilized egg from which the female genetic material had been removed or destroyed by ultraviolet irradiation. Some of these nuclear-transplant embryos developed normally to swimming tadpole or adult stages, and genetic markers were used to show that only the transplanted nucleus — and not the egg pronucleus — had contributed the genetic material of the resulting embryo[1].

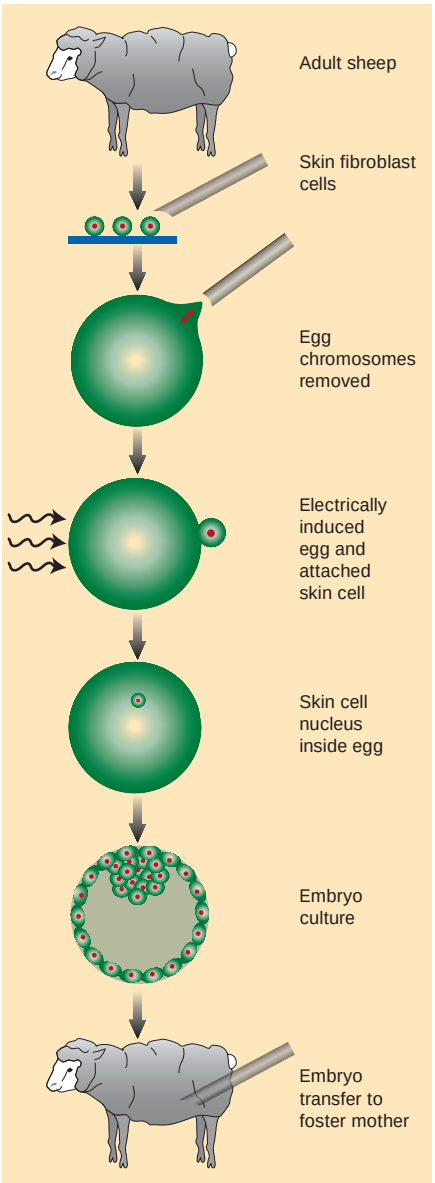

Figure 1 **Nuclear-transplantation techniques in mammals. The genetic material is removed from the recipient cell (an egg), then replaced by a nucleus from a donor cell. The resulting embryo is then transferred to a surrogate mother. The clones are genetically identical to the donor.**

Mechanical disruption of donor cells was also tried in mammals. Until recently, however, it was an unsuccessful or unreproducible method of nuclear transfer. Consistent success in producing live, full-term animals was achieved in livestock by fusing a donor cell from an early embryo with either unfertilized or mock-fertilized eggs as recipients[2] (Fig. 1). The female genetic material is sucked out, then the recipient cell is fused with the donor nucleus using electrical or chemical methods. In most experiments, the donor nucleus comes from a dividing cell, and it should be able to divide in harmony with the recipient. Nuclear transfer in livestock, using somatic cells as donors and unfertilized eggs as recipients, has been successful (the most famous example being Dolly the sheep), but only in a small percentage of cases[3]. Live mouse births were initially achieved only by using donor nuclei from very early embryos, although a piezo-electrically controlled microinjection device has facilitated success with the nuclei of adult cells[4].

How can we tell that mammals obtained by nuclear transfer are genetically identical to the donor cell? Confirmation has come from microsatellite[5] and DNA fingerprinting[6] analyses. However, only genetic material in the nucleus will be identical to the nuclear donor. A cell's energy-producing factories, the mitochondria, also contain their own genome. Because mitochondria are located in cytoplasm, they are inherited through the cytoplasm-packed recipient (the egg) rather than through the donor[7]. The mitochondrial DNA in mammalian clones is therefore entirely maternal in origin.

Conservation of the genome

For successful cloning, it is probably essential for donor cells to contain a full complement of genes. Germline cells (the eggs and sperm) have a complete set of genes; that is, those needed for all cell types such as skin cells, intestine and so on. But for nuclear transfer to work, an adult cell that has already been programmed as, say, a skin cell, needs to be somehow reprogrammed so that it regains genetic totipotency — the ability to guide the formation of all the different cell types that make up an animal.

Early experiments with the frog *Rana pipiens* showed that nuclear totipotency is lost very early in development, at a stage known as gastrulation[8]. However, in another species of frog, *Xenopus laevis*, a transplanted nucleus has been shown to retain genetic totipotency. Indeed, the first clones of adult males and females were obtained in this species from embryonic nuclei (Fig. 2). Although the proportion of successful nuclear transfers decreased as increasingly differentiated donor cells were used (that is, cells more specialized for a particular function), at least a few normal tadpoles (1.5% of the total nuclei transplanted) were obtained from the most advanced donor cells[9]. These experiments culminated in the derivation of genetically marked, fertile male and female adult frogs by transplanting nuclei from intestinal epithelial cells of feeding tadpoles[10]. Later experiments used other kinds of differentiated cells as donors, including striated muscle cells, adult skin cells[11] and blood cells[12]. Thus the principle of totipotency or multipotency was established.

For many years, attempts to transplant nuclei in mammals were unsuccessful, probably because the recipients were fertilized eggs. Nuclear transfer to unfertilized eggs, as in amphibians, has been remarkably successful. Dolly the sheep[3] was obtained using cultured mammary-gland cells as donors, and similar experiments with adult mice and cows have yielded fertile adults[4,13]. Therefore, totipotency of some adult somatic cells has been demonstrated in mammals.

Nevertheless, the yield of normal animals from nuclear transplantation is low. Starting with cells from adult animals, or with fully differentiated cells from tadpoles, only 0.1–1.0% of all eggs receiving transplanted nuclei are born alive (mammals) or reach the swimming stage (tadpoles). Most nuclear transplants either fail to divide or they develop abnormally, possibly due to problems in reprogramming the transplanted nucleus. In amphibians there is the additional problem that incompletely replicated chromosomes are forced to divide prematurely, becoming broken or lost. In mammals, the importance of synchronizing division of the donor nucleus and recipient cell has only recently been appreciated[14].

An important conclusion to come from nuclear-transfer experiments is that the processes of cell differentiation and ageing do not lead to genetic changes in somatic cells. However, there are a few special exceptions. For example, immunoglobulin genes undergo rearrangements to generate antibody diversity. Moreover, mutations can occur in non-reproductive cells, although it is unlikely that changes affecting both copies of an essential gene occur in more than one in 10^4 cells. Finally, telomeres, the specialized structures at the ends of chromosomes, shorten progressively with age in somatic cells. In mice unable to generate telomeres, however, it takes several generations before this has any noticeable effect[15]. Moreover, sheep made through somatic nuclear transfer have shorter telomeres, but this has had no detectable effects so far[7].

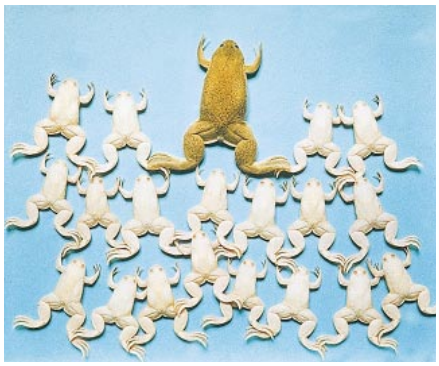

Figure 2 **Cloned frogs. These 19 identical male albino frogs were prepared by nuclear transplantation into unfertilized eggs of the dark green female frog[33]. (Male frogs are about half the size of females.)**

Gene reprogramming

The pattern of gene expression in adult cells is very different from that in embryonic cells. In amphibians, for example, a number of genes expressed in embryos five hours after fertilization are not expressed in differentiated (specialized) larval or adult cells[16]. Conversely, some genes are expressed in adult cells but not in early embryos. When embryos are analysed a few hours after the transfer of adult cell nuclei, gene expression cannot be distinguished from that in embryos grown from fertilized eggs[17]. This means that the exchange of cytoplasm around a nucleus, from that of an adult cell to that of an egg, causes a dramatic switch in gene expression in only a few hours. A nucleus that was once part of an intestine, skin or muscle cell is therefore transformed into that of an embryonic cell.

The situation is probably different for imprinted genes in mammals. Imprinting, which is required for normal development, is a process by which about 30 genes are marked during sperm and egg formation such that they are switched off in the embryo[18]. Imprinted genes are unlikely to be reprogrammed by nuclear transfer, because embryos would not survive if they were. Moreover, imprinting is normally reversed during egg and sperm formation, rather than after fertilization.

Another focus of reprogramming attention is the inactive X chromosome. During early development of female mammals, one of the two X chromosomes is randomly inactivated in those tissues contributing to the fetus[19]. However, in the embryonic tissues that contribute to the placenta, the paternal X chromosome is always the one that is inactivated[19]. These observations prompt a question, so far unanswered, concerning the use of female cells in cloning. Will the inactive X chromosome become reactivated in the tissues of the newly created animal? If not, will embryos reconstructed from a donor containing an inactive maternal X chromosome be viable, given that the maternal X chromosome is usually inactivated in the paternal tissue (which will therefore have no active X-chromosomal genes)?

In amphibians, reprogramming of gene expression is accompanied by massive enlargement of the nucleus and exchange of proteins with those in the cytoplasm. When a nucleus is transplanted to a frog egg, it undergoes 12 rounds of division before new gene activity commences. Does gene reprogramming require the formation of new DNA (DNA replication)? To test this, several nuclei can be injected at once into growing egg cells in which replication does not take place. The transplanted nuclei nevertheless change their pattern of gene expression to conform with that of a growing egg cell[20]. These experiments show that gene reprogramming can occur on the same actual genes as were present before nuclear transfer, and does not require the formation of new DNA. Key molecules found in eggs that may bring about reprogramming include nucleoplasmin and certain embryo-specific histones (proteins around which the DNA is wrapped).

New tissues for old

Damaged or diseased tissues often cannot be repaired by drugs or other medication, and most organs and tissues regenerate very poorly in mammals. In a few cases, artificial materials such as replacement joints, or mechanical devices such as renal dialysis machines, work remarkably well. But ultimately, the most satisfactory remedy is to transplant organs or tissues from other people.

Transplantation works reasonably well for kidneys, hearts and so on, but there are three major disadvantages. First, the supply of such organs is extremely limited, depending largely on donations from accident victims. Second, treatment is very expensive (about £100,000, or US$160,000, for a replacement heart). Third, recipients need to be given immunosuppressive drugs to avoid rejection of the transplanted organ owing to the genetic differences between donor and recipient. Although the use of animal organs has been considered for transplantation, the genetic incompatibility is even greater with these, and even animal organs that have been engineered to contain human immune regulator genes are still targets for rapid rejection.

An alternative strategy involves stem cells; these are cells that can renew themselves and also give rise to a variety of differentiated cell types. Mammalian bone

marrow, for example, contains a range of haematopoietic (blood-forming) stem cells. Some of these can be isolated and encouraged to proliferate using natural signalling molecules such as members of the interleukin family. These stem cells can then be made to progress to form more restricted stem cells (which can form a more limited range of cell types) and, eventually, to form fully differentiated cells such as erythrocytes or granulocytes. This type of blood stem-cell therapy has been practised for many years in humans, but the quantity and quality of available stem-cell types is very limited. The prospects also seem good for stem cells obtained from olfactory placodes. These neural stem cells can be proliferated in culture, and they have been shown to restore function in the mouse central nervous system.

It is also possible for stem cells of one type to generate occasional cells of a different type, although the conditions that achieve this are not known. For example, neural stem cells can generate haematopoietic stem cells when transplanted to mice that have been irradiated to eliminate their own blood stem cells[21]. Stem cells from human bone marrow have been reported to generate functional neural cells[22]. An ideal stem cell is that exemplified by mouse embryonic stem or germ cells, which are derived experimentally from early mouse embryos or germline cells respectively. These cells can be proliferated in culture and, when transplanted to hosts, can differentiate into all types of adult cell. Human cells with several properties of mouse embryonic stem cells have also been described[23,24]. However, all embryonic stem and germ cells are currently obtained by killing normally generated early embryos, raising ethical concerns for human material.

Despite their advantages, transplantation and stem-cell strategies still suffer from the problem of immunological incompatibility, a problem that could be avoided if material derived from a patient's own tissues could be transplanted into them. This is already done with skin for burns patients, but the amount of material is very limited and, for most tissues, it is not yet possible to obtain enough stem cells, if they can be obtained at all. One approach to the problem is to freeze, at birth, samples of cells from the umbilical cord. This tissue is rich in stem cells which, after proliferation and differentiation, might be of use later in life.

Therapeutic cloning

Given the problems of rejection, the lack of identified stem cells for most tissues, and the difficulties of using normal human embryos as a source of embryonic stem cells, an altogether different route is to combine therapeutic cloning with the use of differentiation factors (Fig. 3). Although not yet practicable, this scheme offers a realistic possibility for the future.

The first stage is to use nuclear transplantation to reprogramme the nucleus of a human adult cell and obtain a blastocyst (a very early embryo). This step, in effect, transforms an adult cell into an embryonic cell of the same genetic constitution. By doing a number of such transplants in series, the yield of embryos will be increased. The next stage is to expand this population of embryo cells in culture by promoting proliferation but not differentiation. This has not yet been done with embryos obtained by nuclear transplantation, but it works well for mouse and human embryonic stem-cell-like cells, showing that it can be done in principle.

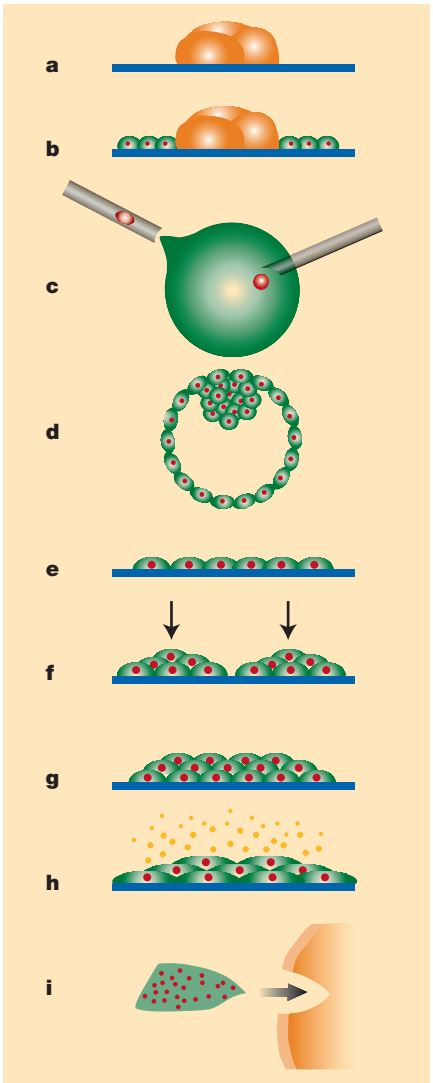

Figure 3 **Steps involved in therapeutic cloning.** a, **An explant is taken from an adult tissue donor.** b, **The tissue sample is grown up in culture (cell proliferation).** c, **Nuclei from the outgrowth cells are transplanted into enucleated eggs (eggs from which the genetic material has been removed).** d, **The nuclear-transplant eggs are cultured to the blastocyst stage.** e, **Embryonic stem-cell lines are derived from the inner cell mass.** f, **The cells are transfected with genes encoding proteins that will cause cell suicide if cells begin to proliferate out of control.** g, **Selected cells begin to proliferate.** h, **Differentiation factors are added and the cells begin to differentiate into specialized cell types.** i, **The replacement tissue is transplanted into the original donor.**

Having obtained a certain number of cells, the next step is to differentiate them in a desired direction. In amphibians this works well when blastula cells are exposed to signalling molecules that act early in development, including bone-morphogenetic proteins, the fibroblast growth factor, Cerberus and noggin. A molecule called activin, which belongs to the transforming growth factor-β family, is particularly useful. Activin is very stable, easy to make in a biologically active form, effective at extremely low concentrations, and it can direct blastula cells towards a wide variety of cell types depending on the concentration at which it is used[25]. Combinations of other secreted factors generate an even greater variety of differentiated cell types. A mass of differentiated cells could then be transplanted back to the original nuclear donor.

Might such extensive manipulations cause some of the resulting cells to become cancerous? Possibly, but this could, in principle, be avoided, at least for those tissues that are not continuously replaced in the body. If a gene (or genes) were introduced to limit the number of divisions that the cells may undergo before their terminal differentiation (a state in which they can no longer divide), cells would be unable to proliferate out of control. Another way to achieve this objective could be to reduce the length of telomeres during *in vitro* culture.

Of course, several steps will need further development before this procedure is of practical use. These include:

- An increased efficiency of nuclear transfer.
- The ability to derive a population of embryonic stem-cell-like cells from nuclear-transplant blastocysts.
- An increased ability to differentiate such embryonic stem cells into functional tissues.
- Methods to screen for any cell lines that may have incurred genetic damage, and that would not therefore be suitable for human *in vivo* use.

This scheme has several advantages. It requires unfertilized eggs rather than embryos, and the nuclear-transplant blastocysts do not need to be able to develop normally (they only need to form the required cell types). Moreover, populations of cells or simple tissues may be useful, avoiding the need to create complex organs. Above all, the newly generated cells would be almost totally compatible with the recipient. We anticipate that any incompatibilities, owing to minor histocompatibility antigens in the transplanted cells, which will differ from those of

Figure 4 **Countries in which anti-cloning legislation has been, or will shortly be, passed. Legislation has been passed in countries shaded green, and is pending in countries shaded yellow. Countries with red stripes are co-signatories to the January 1998 Council of Europe Protocol. This must be ratified by at least five countries. Some (although not all) of this legislation applies equally to both reproductive and therapeutic cloning.**

the patient, should not be significant. This supposition could easily be tested by skin transplantation between a group of cloned animals[13].

Legality and ethics

The publication of Dolly the sheep's existence had an immediate and generally negative effect on public opinion worldwide, and sparked off a flurry of restrictive legislative activity. Most invective was aimed at the prospect of human reproductive cloning; that is, the production of a person using nuclear transfer. Both safety and ethical reasons were cited against such cloning. Belated sober reflection revealed that few of these moral arguments were sound. For example, the ethical objection about the welfare of cloned children has been shown to apply equally well to the case of natural procreation[26]: if the anticipated psychological damage to clones from societal prejudice is enough to ban the practice, then the psychological damage to a mixed-race child from racism should justify a like ban on procreation from mixed-race couples. Clearly the latter would be absurd.

Therapeutic cloning is ethically less contentious because a new person is not produced. However, as for abortion, the issue of the deliberate destruction of a potential person is raised[27]. Therapeutic cloning is illegal in many countries (Fig. 4), where legislation prevents the use of human eggs for therapeutic research. This prohibits therapeutic cloning using unfertilized eggs, even though a nuclear-transplant embryo would be used to make embryonic stem-cell-like cells, and so would have no potential for survival because it would not be implanted.

No such legislation has yet been enacted in the United States, except that federal funds cannot be used for reproductive cloning, nor can they be used in generating embryos for therapeutic cloning. They can, however, be used for research on cell lines derived from human embryos. In the United Kingdom it is legal to use human embryos up to 14 days old for research connected with fertility or contraception. It is illegal to use human eggs for any purpose where the intention is to create an embryo, even if only for cell replacement. Permission to do this (and, hence, to allow therapeutic cloning) could be granted by the Secretary of State for Science under an addition to the present Human Embryology Act.

Perhaps a way round these issues is to use cells or eggs from other species. However, as yet, normal development has not been obtained with nuclear transfers between species. All but one of the human-nucleus to cow-egg transfers attempted died before the embryo had divided five times, and other mammalian combinations behaved similarly[28,29]. It is therefore unlikely that animal eggs could be used as an alternative to human eggs as recipients for human nuclei.

The future of cloning

Live births have been achieved using somatic nuclear transfer in mice, sheep, cows and goats, and the technique would probably also be successful in humans. However, the low efficiency of the procedure may restrict its immediate application to the production of small herds of identical animals. There will be special applications, though, where the production of single animals will be useful. For example, the technique could be used to inactivate prion genes in ruminants[30] (scrapie/BSE research), to disrupt the α1–3 galactosyl transferase gene in pigs[31] (for xenotransplantation), and to knock out the cystic-fibrosis transmembrane-conductance-regulator gene in sheep[32] (creating an animal 'model' in which this disease could be studied). Nuclear transfer is currently the only option for gene targeting in livestock, and one of us (A.C.) has shown that sheep can be generated with targeted changes in particular genes.

Our belief, however, is that the greatest eventual benefit of the new technology will be in therapeutic cloning; the use of somatic-cell nuclear transfer to generate replacement tissues or organs. This would avoid the risks of tissue rejection by supplying a person with new tissue of exactly their own genetic type. All of the main steps in the therapeutic cloning procedure have been achieved individually, albeit at a low efficiency. We now need to improve the success rate for generating nuclear-transplant embryos from adult tissues, to find ways of generating embryonic stem-cell cultures from nuclear-transplant embryos, and to control more accurately the pathways of stem-cell differentiation and the formation of whole organs *in vitro*. As soon as any new scientific technique works at all, it is almost always improved in both efficiency and ease of operation. This seems likely to be the case for cloning technology too. ■

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