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Whither Cloning?

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The statement has been endorsed by the Council of the Royal Society.

BRIEF HISTORY

The first successful cloning experiments in vertebrates arose from the desire of embryologists to know whether the process of cell differentiation from an egg involved permanent or stable changes in the genome. One idea was that, as cells differentiate, the genes no longer needed (such as skin genes in intestine) could be lost or permanently repressed. The other idea was that all genes are present in all cell types, and that cell differentiation involved the selective activation and repression of genes appropriate to the cell type. This fundamental question in development was evident to early embryologists who, in the 1920s, carried out an experiment proving that nuclei of the first eight cells of a newt are genetically equivalent.

The first successful transplantation of nuclei from early embryo cells was achieved by Briggs and King (1952) working with the American frog *Rana pipiens*. When they took nuclei from more advanced embryos they found they could no longer obtain normal development in the way that they could from early cell nuclei. This led to the view that some loss or permanent inactivation of genes might accompany development and cell differentiation.

Later work by Gurdon at Oxford with the frog *Xenopus laevis* led to the opposite conclusion. This is because nuclei from the most advanced stages tested, including tadpole intestine, yielded normal frogs (Gurdon, 1962a). Nuclei of earlier larval intestine could also be transplanted successfully to yield sexually mature adult frogs (Gurdon and Uehlinger, 1966). This showed that a cell, in this case tadpole intestine, can undergo complete differentiation and yet retain all genes necessary for the formation of a normal adult, sexually mature individual. This was the first direct evidence that cell differentiation involves the selective activation and repression of genes and that they are not lost or stably inactivated.

In the course of the work with *Xenopus*, clones of genetically identical adult frogs were obtained. Photographs of such clones were first published in 1962 (Gurdon, 1962b). In later work, it was found that nuclei of cells cultured from adult organs could be transplanted to yield growing larvae which were developmentally normal at that stage. However, none of these reached adulthood. It was known that nuclear transplantation in Amphibia is commonly accompanied by genetic damage to transplanted nuclei. This is probably because the cells of differentiated tissues divide very slowly (once every day or two, or less frequently), but when transplanted they must complete chromosome replication and cell

division within 1.5 hours to conform to the rapid division cycle of an activated egg. Random genetic damage resulting from this gross asynchrony of donor cells and recipient eggs probably accounted for the failure to obtain entirely normal individuals from the cells of an adult.

DEFINITIONS

Embryo transfer Ever since the 1950s, artificial insemination and cryopreservation of sperm proved a breakthrough which is without equal in modern animal breeding. In contrast, realisation of the genetic potential of the female has been slow to develop, primarily because of the small number and inaccessibility of available oocytes (mature female germ cells) and eggs. Embryo transfer involves the recovery of the early embryo from the reproductive tract and placement into the uterus of a suitably primed recipient. The technique has a long history. It was first reported in rabbits by Walter Heape of Trinity College, Cambridge in 1891 and has been refined so that today it provides the foundation on which all other technologies aimed at utilising the genetic potential of the female are based. It has a plurality of uses in cattle including the rapid multiplication of rare or 'exotic' genotypes and the propagation of selected lines from deep-frozen banks of selected embryos which had been previously evaluated by testing their progeny.

Cloning means the production of a precise genetic copy of a molecule (including DNA), cell, tissue, plant, animal or human. In the present context it refers to the process of producing individuals genetically identical to some other living or dead individual. It has been promoted recently as another way to expand the reproductive technologies available to livestock breeders.

Cloning by embryo splitting involves the division of the preimplantation embryo into equal halves which produce two genetically identical embryos. It has been used predominantly in sheep and cattle. It occurs naturally in many species, including humans, to give identical twins.

Cloning by blastomere dispersal begins with the mechanical separation of individual cells prior to the formation of the blastocyst. (Blastomeres are the individual cells produced when the fertilised egg divides into 2, then 4, 8 and 16 cells; a blastocyst is formed later when a fluid-filled cavity appears with an outer layer of support cells and a cluster of cells on the interior). Some blastomeres derived from the 8-cell egg of certain species (as in sheep and pigs) will develop into normal individuals after enclosure in an egg membrane and transfer into a suitable recipient. In the nine-banded armadillo this process occurs naturally at the 4-cell stage, each blastomere giving rise to a complete embryo and placenta.

Cloning by nuclear transfer (or substitution) involves the complete removal of genetic material (chromosomes) from a matured oocyte or an egg to produce an enucleated cell (cytoplast). It is replaced by a nucleus containing a full complement of chromosomes from a suitable donor cell (the karyoplast) which is introduced into the recipient cytoplast by direct microinjection (in amphibians) or by fusion of the donor and recipient cells (in mammals). This technique will be referred to here as *embryo reconstruction*. Both nucleus and cytoplasm are transferred into the recipient cells but subsequent development of the embryo is thought to be controlled by the interaction of the recipient cytoplasm and the nuclear genes of the donor chromosomes. If this produces a normal animal, the result demonstrates that the donor nucleus is totipotent (i.e. after nuclear transfer to an enucleated egg the zygote so formed subsequently develops and produces all the fully differentiated cellular

tissue of the normal animal). Nuclear transfer is thus the basis of asexual reproduction which has value for the production of cloned offspring or for research purposes. Within mammals successful nuclear substitution followed by the birth of live young has been reported previously in cattle, sheep, pigs, goats, rabbits and mice mostly using embryonic blastomeres. It has been successful only when early embryos were used, probably because the nucleus can pass more readily through a process of reprogramming and respond to its new cytoplasmic environment and control subsequent development.

We can see from this brief description that the term *cloning* has been applied rather loosely to a range of different procedures. In some plants and invertebrates cloning by budding or splitting is common as a form of reproduction, and is very widely used in forestry and horticulture. In the case of the Roslin experiment (Wilmut et al.,1997) which produced the sheep 'Dolly', the term nuclear substitution (or transfer) is a useful description of what took place. Nuclear substitution results in individuals in which the nuclear genes of offspring are identical to the donor, so that in the case of Dolly she shares the same set of nuclear genes as her adult donor.

RECENT DEVELOPMENTS

It was generally believed prior to the Dolly experiment that a donor nucleus originating from an adult mammalian cell would already have been irreversibly programmed. At least in the case of a cell taken from a primary culture of sheep mammary tissue this does not seem to have been the case. It is this aspect of the experiment, the transfer from, and hence reproduction of an adult with known and visible characteristics (rather than from an embryo) which has given rise to the great excitement.

Dolly resulted from fusion of an adult donor cell which came from a Finn Dorset ewe and an enucleated egg of a Scottish Blackface ewe. These breeds are phenotypically distinct, so that the visual appearance of Dolly neatly complements the all-important DNA fingerprint data in determining that she is indeed derived from a Finn Dorset nucleus and not a Scottish Blackface one! The procedure carried out was as follows. Eggs of Scottish Blackface sheep were first enucleated. An adult cell from a Finn Dorset was then fused with each egg by passing an electrical current which also activated the egg. After a period of culture, the embryos were transferred back into a Scottish Blackface recipient ewe. The adult donor cell came from a primary culture of mammary gland cells and was forced into a so-called period of quiescence before being fused into the recipient egg. Alongside the adult cell experiments there were run two parallel experiments which have received much less attention, presumably because the (cultured) donor cells came from embryos and fetuses respectively. Most of the reconstructed embryos in all three experiments were maintained in sheep oviducts for several days before being transferred to the recipient uterus.

Full-term lambs were obtained from all donor cell types, but the success rate of live lambs born compared to reconstructed embryos transferred at around the blastocyst stage was very low. Only one successful live birth - Dolly herself - resulted from 277 attempts with adult cells fused to eggs (couplets). Two lambs were born from 172 fetal fibroblast-derived couplets and 4 from 385 embryo-derived couplets. In all cases the gestation period was extended beyond the normal length of approximately 147 days, though birth weight of the lambs was within the normal range.

The mammary gland cell used in the Dolly experiment came from a mixed heterogeneous culture. As the authors acknowledge, it is not clear whether there was something exceptional about the particular nuclear donor cell which was important to the successful

culmination of the experiment - was it a mammary cell, a stem cell or some other? The very low success rate of live births to couplet transfers (1 out of 277) implies that the behaviour of different cell lineages should be examined systematically in order to establish the best differentiated lines for the type of reprogramming that is being attempted.
Wilmut and his colleagues believe that the novelty of their cloning procedure lies in enforcing the quiescence of the donor cell before transfer to the recipient egg. The donor cells are forced into the quiescent phase of the cell cycle by a standard serum starvation system involving a reduction of the serum levels in the culture medium. The aim is understandable, namely, to induce the changes that would facilitate the reprogramming of gene expression in the reconstructed egg, that is to reprogramme the nucleus to its totipotent state.

A disturbing finding was the high incidence of deaths *in utero*; in some instances this occurred at late stages of gestation which is uncommon in this, as in many other mammalian species. This finding has been confirmed by Wells and colleagues in New Zealand using fetal cells (Wells et al, 1997). The reasons for this large loss during pregnancy are unknown and require investigation. A recent press announcement from a Wisconsin biotechnology company of the birth of a calf 'Gene' produced by fusing an enucleated egg to a cultured fetal cell is instructive in this respect. Death *in utero* was the inevitable fate of all embryos reconstructed from the Wisconsin cell until a serial nuclear transfer strategy was introduced. After the reconstructed egg had been in culture for several days, the aggregate of cells was dispersed and a single cell was transferred into another enucleated egg which was then placed into the uterus of a surrogate cow. In one of 18 cases pregnancy continued successfully to term; at the time of the announcement it was claimed that a further improvement in the success rate had been achieved (Nature Biotechnology 15:833, 1997). Clearly, the use of a second round of nuclear transfer complicates the procedure, but the results suggest a possible way to reduce the high incidence of pregnancy failure.

EMERGING SCIENTIFIC QUESTIONS

Scientific discovery is secured by the confirmation and repeatability of new findings. The Roslin group have undoubtedly provided a new impetus to research in this area and corroboration of their result with an adult donor nucleus is eagerly anticipated. Their work has identified important new questions and re-emphasised others which demand to be answered - is there an ideal donor cell type for nuclear transfer? what are the underlying mechanisms by which the programming of somatic cells can be modified? which are the critical molecular mechanisms that determine cell-cycle synchronisation between the transplanted nucleus and host cytoplasm? what is the nature of the signals required to activate the newly constituted egg? what is the basis of the greatly enhanced pregnancy failure rate that is observed whether reconstituted embryos come from embryo, fetal or adult nuclei and what is required to correct it?

Repeating the Dolly experiment with suitable variations will be costly in time and resources but, as a further contribution to basic research, such experiments could give greater insight into the processes of cell commitment and ageing, and into the question of whether they can be reversed. Since Dolly was derived from the adult nucleus of a 6-year old sheep it remains to be seen if she has a shorter lifespan than normal, whether she is fertile and whether she or her progeny have an increased susceptibility to cancer or an increased rate of abnormalities arising from accumulated somatic mutations and chromosomal damage.

A major step forward would be to identify a more manageable, faster growing, mammalian species which would allow critical studies of the molecular biology of gene activation in the

reconstructed embryo and so illuminate the relationship between gene expression of the nucleus derived from the adult donor cell and mitochondria derived partly from the recipient egg. One obvious approach would be to develop this technique in mice. In addition to their advantages of defined genetic constitution and short generation interval this species also benefits from the existence of well characterised embryonic stem cell (ES) lines which would provide an ideal supply of nuclei for transfer. The ES cell lines, with their unique property of dividing regularly whilst still maintaining their original totipotent state, can be genetically manipulated, subjected to somatic cell selection procedures and maintained by cryopreservation. The nuclei of these cells can be evaluated using molecular techniques to determine such characteristics as viability and sex. Despite all these advantages no success has yet been achieved in mice using nuclei from any stage more advanced than the 8-cell stage. However, donor karyoplasts which have been rendered quiescent have not, as yet, been transplanted into mouse cytoplasts and the results of such experiments are awaited with interest.

Animal husbandry Aims of developing ES cell lines for livestock include the preparation of genetically selected and identical embryos of predetermined sex. Such cell lines would also be suitable for the introduction of genes into the germline. The group at Roslin, as well as others in Europe, USA, Canada, Australia and elsewhere, have actively sought without success to derive ES cell lines comparable to those available to rodent biologists. A promising alternative to the use of well defined ES cells was reported last year by the Roslin group (Campbell et al, 1996). In that study nuclei from partially differentiated sheep embryonic cell lines were successfully used to produce viable offspring (Megan and Morag). These results were confirmed in the recent Roslin study paralleling the Dolly experiment. Embryo multiplication by nuclear substitution could provide a rapid route through the female line to the multiplication of high quality breeding stock or disease-free animals for the national herds in more and less developed countries. Such a procedure has been proposed for producing replacement stock that are free from genes coding for prion proteins associated with transmissible encephalopathies.

An unanswered question concerns the cause of the large size of some offspring produced in some experiments involving embryo reconstruction followed by culture *in vitro*. The extended gestation period may be one reason for the increased size of the fetus but the converse seems not to be true since large fetuses were observed at all stages of gestation. The way the embryos were cultured may be a contributing factor but from the Roslin experiments there were insufficient data to compare development after culture in vitro with that in ligated oviducts.

REPERCUSSIONS

The birth of Dolly has provoked worldwide discussion and a plethora of reactions ranging from panic to euphoria. We believe it is now time to evaluate the difficulties that lie ahead in transforming the advance signalled by the arrival of Dolly into practical applications that may benefit humankind.

A principal motivation behind non-human mammalian cloning is to form new transgenic animals of medical importance. This can be done by genetic modification of the cell line from which the donor nucleus comes. This is expected to lead to the more efficient preparation of new types of animal that not only produce proteins of therapeutic use in humans but also organs amenable to transplantation. The production of therapeutic pharmaceutical proteins remains a major and promising objective but overly optimistic

expectations have also been raised about treatments for ageing and cancer, the regeneration of new cells and the supply of tissue from cloned embryos.

While the technology that underlies the birth of Dolly theoretically expands the options that will be available to address some of the most intractable problems of modern medicine, there are several caveats. The long lead time required for the development of these techniques should not be underestimated. The scientific and technical hurdles are not inconsiderable before any major application could become available. Nuclear substitution may one day serve as a therapeutic measure to combat the very serious consequences of abnormalities of mitochondria (a component of the cell that provides energy and has some of its own genes), the mother's adult nucleus being transferred to a normal recipient egg containing normal mitochondria. However, little is known at present about the degree of risk involved. We agree with the Opinion recently presented to the European Commission by the Group of Advisers on the Ethical Implications of Biotechnology (1997) which says that any intention to produce genetically identical fetuses or babies ('reproductive' cloning) raises serious ethical questions about instrumentalisation of human beings. On the other hand, the use of nuclear substitution for research raises no new ethical questions other than those inherent in all human embryo research, which has already been faced in the United Kingdom, where the Human Fertilisation and Embryology Act of 1990 forbids the culture of human embryos beyond the first 14 days, and also forbids the replacement in the uterus of any embryo used for research.

So far as animal husbandry is concerned, the production of elite stock derived from banks of embryos with desired and tested genotype could accelerate the multiplication of these animals. The questions of animal welfare and the maintenance of genetic diversity in livestock populations are ones that will need to be kept constantly under review if this approach is to be found acceptable by public, scientists and welfarists alike. Another application of cloning that has been mooted concerns the preservation of species that are endangered or resistant to breeding in captivity. By the use of cryopreserved banks of cloned embryos a species might be secured against the eventuality that numbers fall to disturbingly low levels, though this would require appropriate host mothers of another species. Yet the rate of adoption of cloning in animal husbandry will be seriously impeded if its efficiency is not dramatically improved: three or four sheep were required for the separate stages to produce a Dolly and we have already referred to the huge losses during pregnancy. In short, there is little evidence that widespread cloning is just round the corner!

MORATORIUMS or PROHIBITIONS?

Cloning Dolly sent ripples around the world concerning the implications for so-called 'human cloning' and what the law would allow or disallow. In the US there is no law at State or Federal jurisdiction that bans cloning of human beings, consistent with the Fourteenth Amendment to the United States Constitution that guarantees the right of procreative autonomy. Nonetheless, the American National Bioethics Advisory Commission (NBAC) was instructed to undertake a thorough review of the legal and ethical issues associated with cloning and concluded that it was morally unacceptable to produce a child by cloning using the above technology. A Bill in response to the findings of the NBAC has recently been submitted (Cloning Prohibition Act, 1997) which prohibits cloning, stating that *it shall be unlawful for any person or other legal entity, public or private, to perform or use somatic cell nuclear transfer with the intent of introducing the product of that transfer into a woman's womb or in any other way creating a human being.*
The European Parliament has recently passed a resolution on cloning (The European Parliament Resolution on Cloning, 1997) the preamble of which asserts: *the cloning of*

human beings, whether experimentally, in the context of fertility treatment, pre-implantation diagnosis, tissue transplantation or for any other purpose whatsoever, cannot under any circumstances be justified or tolerated by any society, because it is a serious violation of fundamental human rights and is contrary to the principle of equality of human beings as it permits a eugenic and racist selection of the human race, it offends against human dignity and it requires experimentation on humans...each individual has a right to his or her own genetic identity and human cloning is, and must continue to be, prohibited.

Whereas in Europe and the USA the thrust of legislation is to ban all forms of human cloning leading to the birth of a baby, embryo research on such clones has not been banned in all countries. In the UK, human cloning by embryo splitting is banned by the Human Fertilisation and Embryology (HFE) Authority for clinical use but not for research. The HFE Act at present essentially consists of two shields of protection. The first is that only institutions with a licence may use human embryos for clinical treatment, or conduct research on human embryos; the second is that a licence may not be granted where the treatment or research involves nuclear transfer from any cell in the body, embryo or adult to an enucleated embryonic cell.

The technique announced by the Roslin Institute does not use an embryonic cell but an unfertilised egg as a recipient cell for the implanted nucleus. However, the application of the HFE Act could be amended to extend to a human embryo produced by nuclear transfer. This would outlaw the unacceptable cloning of humans without the requirement for subsequent amendments to the Act on account of scientific advance, but retain the possibility that scientific research could still be pursued on cloned human embryos within the first 14 days after fertilisation allowed by the Act of 1990. The Opinion of the EC Group of Advisors on the Ethical Implications of Biotechnology (1997; *vide supra*) also recommended recently that any attempt to produce a genetically identical human individual by nuclear substitution from an adult human or child cell (reproductive cloning) should be prohibited. This conclusion was reached to counteract the frankly selfish (elderly millionaire vainly seeking immortality) or the more understandable (couple seeking replacement for a dead child, perpetuation of outstanding talent or a fully compatible donor for a dying child). The basis for rejection of these possibilities was ethical on the grounds of using human embryos as mere tools of biomedicine (instrumentalisation) or of eugenics. We believe they should in any case be rejected on safety considerations because the hazards and potential risks associated with the procedure are largely unknown and unexplored.

CONCLUSIONS

The Council of the Royal Society recognises that research into cloning in mammals could lead to significant new insights into the function and control of cells and how they develop, age and undergo pathological change within different tissues of the body. Contrary to public perception, however, cloning is still a highly unpredictable laboratory procedure.

With respect to human cloning, Council supports the view that reproductive cloning of humans to term by nuclear substitution is morally and ethically unacceptable and believe it should be prohibited. We should, however, bear in mind that there might be some special case, such as that referred to above on the possibility of correcting maternal mitochondrial abnormality, but this would still involve a further ethical debate.

We propose that provision for the culture of cloned human embryos up to the 14 day limit specified in the Human Fertilisation and Embryology Act (1990) is justifiable on the grounds of biomedical research but that it should be permitted only with a specific licence issued by the Human Fertilisation and Embryology Authority. We further recommend that any

modification to existing legislation, or any new legislation, should be carefully drafted so as not to outlaw the potential future benefits that could be derived from research on cloned embryos.

With respect to animals, Council supports the view that cloning is acceptable on the grounds of biomedical advances, improved food production in the case of livestock, and preservation of endangered species. All work that is designed to produce cloned animals should be conducted within the provisions of the Animals (Scientific Procedures) Act of 1986, include detailed studies of the welfare of cloned animals (including their cognitive capabilities) and prospective analysis of any danger resulting from a loss in variation to the gene pool together with the development by relevant institutions of strategies for maintaining genetic diversity.

The Royal Society favours a wider and well informed public debate of the scientific, technical, ethical and moral issues to ensure the involvement of lay and specialist participants from different generations and groups. Council believes that this is a matter of some urgency.

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