DOLLY: THE SCIENCE BEHIND THE WORLD'S MOST FAMOUS SHEEP

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Abstract
'The Roslin Institute near Edinburgh is one of the world's leading centres for research on farm animals. Its main expertise is in animal genetics, with the results benefiting the livestock breeding and production industries in particular. Its work was, however, little known to the general public. A single lamb changed all that. **Dr Harry Griffin**, Assistant Director at the Institute, explains.....'

Dolly was the first mammal cloned from a cell from an adult animal. She was derived from cells that had been taken from the udder of a 6-year old Finn Dorset ewe and cultured for several weeks in the laboratory. Individual cells were then fused with unfertilised eggs from which the genetic material had been removed. Two hundred and seventy seven of these 'reconstructed eggs' - each now with a diploid nucleus from the adult animal - were cultured for 6 days in temporary recipients. Twenty nine of the eggs that appeared to have developed normally to the blastocyst stage were implanted into surrogate Scottish Blackface ewes. One gave rise to live lamb, Dolly, some 148 days later.

DOLLYMANIA

Dolly was born on 5 July 1996. It took Ian Wilmut and his colleagues a few weeks to complete the experimental work and several more months before they had written a paper and had it accepted by *Nature*. Publication was confirmed for 27 February, 1997 and by then plans were well advanced to handle the expected media interest. We knew that *Nature* was featuring Dolly in the weekly press release that it distributes each Friday. The information in these releases is 'embargoed' until 7 o'clock on the Wednesday evening before publication and this is intended to provide journalists time to research their stories more carefully before going to press. However, ideas of a relaxing weekend were shattered by two phone calls late on Saturday warning us that the *Observer* would be running the story the next day.

A quick visit to the newsagents first thing on Sunday confirmed our story was on the front page. The *Observer*'s science writer Robin McKie had resisted taking a sensational line but he had raised fears about human cloning. Our main aim then was to ensure that the other papers have some solid facts to report. Two of the team at Roslin and four at our PR agency *De facto's* office in Basingstoke worked throughout the day to answer calls from around the world and set up schedules for reporters, TV crews and photographers desperate to visit Roslin.

On Monday, Dolly provided a major story in most of the papers and Roslin was besieged by reporters and TV crews from all over the world. ABC, NBC, CNN and CBS all beamed live interviews across the Atlantic *via* satellite vans in the car park. Phones rang incessantly and extra spokesmen were drafted in to help. Dolly quickly became the most photographed sheep of all time and was invited to appear on a chat show in the US. Astrologers asked for her date of birth and the shares of our collaborators PPL rose sharply. President Bill Clinton called on his bioethics commission to report on the ethical implications within 90 days and Ian Wilmut was invited to testify to both the UK House of Commons and the US Congress. Dolly Parton said she was 'honoured' that we had called our sheep after her.

In the first week after the story broke, we estimated that together the Roslin Institute, PPL and *De Facto* answered more than 2000 telephone calls, talked at length to close on 100 reporters and provided access to Dolly to 16 film crews and more than 50 photographers from all over the world. After all this, the actual publication of the paper in *Nature* on Thursday 27 February seemed an anticlimax.

'Science Breakthrough of the Year'

For developmental biologists, Dolly's existence challenges one of the fundamental tenets of developmental biology. Most scientists had thought that differentiation - the gradual process of specialisation that allows the fertilised egg to develop into the hundreds of cell types that make up the whole animal- was irreversible. After all, even over a 90 year lifespan a liver remains a liver, a nerve cell a nerve cell. The production of a live lamb from a cell taken from the udder of a 6-year old ewe demonstrated that differentiated cells are not immutable: it was possible to take a differentiated cell and turn its biological clock back to zero. To start its life all over again. And for this, Dolly was voted 'Science Breakthrough of 1997' by the editors of the prestigious US journal *Science*, ahead of NASA's Pathfinder mission to Mars and advances in cyclotron design.

At first Dolly was a clone alone, but in recent months clones of adult mice have been reported from Hawaii and cloned calves from both New Zealand and Japan. These additional reports demonstrate that Dolly is not an 'anecdote' and that it should be possible to clone from a variety of different cell types and species.

Cloning of humans

The intense media interest was not with sheep. The immediate assumption was that cloning of humans was just around the corner and that seemed to trigger an explosion (at least in the media) of fears about the future. However, in contrast to the barrage of calls from the press, the relatively small size of our postbag suggested that the general public was much more sanguine. They seemed to know that the scenarios that the media were imagining were unlikely to be realised and could, as a consequence, be enjoyed in safety. Just like '*the X files'*.

Much of the media speculation was based on science fiction rather than good sense, with the *Times* and a Dr Patrick Dixon taking our award for the most outrageous list of 'reasons' for cloning humans. A common misunderstanding was that clones would be somehow less than human. However, most of us have already met a human clone - an identical twin - and no one would seriously suggest that we should produce twins to order as sources of spare parts. Others seemed to believe that cloning would produce an identical photocopy, rather than a child that would grow up with a personality and behaviour all of its own.

Those speculating about potential uses in humans need to recognise that the technology is very much in its infancy. Our experiments in sheep produced several lambs with developmental abnormalities that died in late pregnancy or soon after being born. A clone will inherit somatic mutations from the donor and this in turn may lead to premature ageing or a higher incidence of cancer later in life. These risks alone mean that it would be grossly unethical to perform similar experiments in women now, however desperate they might be to have a child.

Experience with cloning in farm animals may identify ways of reducing risk but this is likely to take many years. In the meantime, society as a whole has time to contemplate which uses of the technology might be acceptable and which would not. Views will inevitable change over time: the first test tube baby, Louise Brown, was born in 1979 and *in vitro* fertilisation is now commonplace. Attitudes will also vary between countries: many feared cloning would be exploited by megalomaniacs but in our view it is far more likely that the ethical boundaries will be tested by the *in vitro* fertilisation clinics in the US or the Far East.

Nuclear transfer

Cloning by nuclear transfer is not itself novel. The technique was first reported in frogs in 1953 and has been used widely since in amphibians to study early development. This work showed that the first few cell divisions after fertilisation produce cells that are totipotent (i.e. they can develop into all of the cell types that make up the whole animal). As the embryo develops further, the cells lose this property and the success of nuclear transfer rapidly declines. Some nuclear transfer experiments using cells from adult frogs produced viable embryos, but these never developed beyond the tadpole stage.

Nuclear transfer in mammals proved to be more difficult. The cloning of mice using nuclei from very early embryos was reported in 1977 but this work was not repeatable and interest among developmental biologists waned. Research on nuclear transfer in cattle continued, stimulated by the potentially large commercial benefits of multiplying elite embryos. Artificial insemination allows each bull to have thousands of offspring but each cow can only produce 5 or 6 calves in a lifetime. Multiple ovulation embryo transfer (MOET) and cloning by embryo splitting have been used to partially redress this imbalance, but these techniques have limited potential for further development. By contrast, nuclear transfer has, at least in principle, the ability to produce an unlimited number of identical animals.

By the middle of the 1980's several research groups from around the world had produced cloned sheep and cattle by transferring nuclei directly from early embryos. In 1985 Steen Willesden in the US had produce live calves by nuclear transfer from embryos that had progressed to the 64- and 128-cell stage and this was the first suggestion that nuclear transfer in mammals was possible from at least partially differentiated cells.

In the early 1980's the then Animal Breeding Research Organisation initiated a programme designed to produce transgenic sheep and cattle that would secrete human proteins in their milk. Using the beta- lactoglobulin promoter, John Clark and colleagues were able to direct expression of the transgene to the mammary gland. This success led to the setting up in 1987 of PPL Therapeutics and in 1989 the production of Tracy, a transgenic sheep that secreted 35 g of a human protein- alpha-1-antitrypsin- in her milk. Over the same period other groups had developed other transgenic livestock, including genetically modified pigs for use as sources of organs for transplantation to human patients.

At this time the only way of producing transgenic livestock was by pro-nuclear injection. This procedure involves the introduction of 200-300 copies of the transgene into a recently fertilised egg which is then implanted in a surrogate mother. Only 2-3% of eggs give rise to transgenic offspring and only a small proportion of these express the added gene at sufficiently high levels to be of commercial interest. It was also only possible to add genes.

If animals can be derived from cells in culture, then it is possible to carry out much more specific genetic modifications, including the removal or substitution of specific genes. This has been achieved in mice using embryo stem (ES) cells but to date no one has yet succeeded in obtaining ES cells from cattle, sheep or pigs. After learning of Willesden's success, Ian Wilmut thought that nuclear transfer might provide an alternative.

The major breakthrough came in 1995 when Keith Campbell, Ian Wilmut and colleagues produced live lambs - Megan and Morag - by nuclear transfer from cells from early embryos that had been cultured for several months in the laboratory. The key element in this success was the induction of quiescence in the donor cells. However, at this stage they did not know if they had also stumbled on a particular amenable cell type simply by chance.

Additional experiments were performed to test if successful nuclear transfer technique was restricted to embryo-derived cells or could be carried out with a wider range of cell types. Nuclear transfer was carried out

from embryo-derived cells, foetal fibroblasts and - in collaboration with PPL Therapeutics - cells from an adult ewe. Four lambs were born from embryo cells, two from the foetal cells and one, subsequently named Dolly, from an adult cell. Their identity was confirmed by DNA testing and the results published in *Nature* on 27 February 1997.

Practical applications of cloning

The ability (*via* nuclear transfer) to derive live animals from cultured cells provides an alternative way of producing transgenic farm animals. Moreover, the ability to manipulate many millions of cells at once opens up the possibility of much more specific genetic modifications, including the deletion or substitution of specific genes or the introduction of the single letter changes in the whole of the genetic code that are characteristic of many human genetic diseases.

human therapeutic proteins

Human proteins are in great demand for the treatment of a variety of diseases. Whereas some can be purified from blood, this is expensive and runs the risk of contamination by AIDS or hepatitis C. Proteins can be produced in human cell culture but costs are very high and output small. Much larger quantities can be produced in bacteria or yeast but the proteins produced can be difficult to purify and they lack the appropriate post-translational modifications that are needed for efficacy *in vivo*.

By contrast, human proteins that have appropriate post-translational modifications can be produced in the milk of transgenic sheep, goats and cattle. Output can be as high as 40 g per litre of milk and costs are relatively low. PPL Therapeutics, one of the leaders in this field, recently announced that alpha-1-antitrypsin produced from a transgenic flock is now being used to treat cystic fibrosis patients in phase 2 clinical trials. In the US, Genzyme Transgenics have focussed on goats and their lead product, tissue plasminogen activator, is also in clinical trials.

An immediate advantage of producing transgenic animals by nuclear transfer is that it uses less than half of the experimental animals than does pronuclear injection. It is also possible to specify the sex of the offspring and thereby substantially reduce the time taken to generate a production flock. PPL and the Roslin Institute have already used this approach to produce Polly and Molly, two transgenic sheep that secrete the human blood clotting factor IX in their milk.

At present such transgenic animals produce a human protein in addition to the normal complement of milk proteins. This restricts yield and with some proteins there would be a major advantage in removing one or more of the endogenous milk proteins. This is the case with serum albumin where the total demand for treatment of burns and other trauma is estimated at over 600 tonnes each year. In this case production would be in cattle and the aim would be to replace the bovine albumin gene with its human equivalent.

nutraceuticals

There are a number of potential opportunities for altering the nutritional content of milk. For example, cow's milk is ideal for calves but not for premature infants. Gene targeting using nuclear transfer will allow milk to be produced in which one or more of the normal cow's proteins have been replaced by human proteins, thereby improving its nutritional quality for these special 'consumers'. Other people have a immune response to specific proteins in milk or are intolerant of lactose and gene targeting would allow the creation of herds of cows that produced milk lacking the problem components.

xenotransplantation

Over the past 20 years transplantation of hearts and kidneys has become almost routine. There is an shortage of suitable organs for transplant (about 5000 each year in the UK) and many patients have to suffer prolonged dialysis and/or die as a result. Transgenic pigs are being developed as sources of organs to meet the shortfall. The pigs produced so far have an added human protein such as compliment inhibitory factor that coats the pig tissues and is intended to prevent immediate rejection of the transplanted heart or kidney.

The ability to remove genes could have a major impact on the success of such xenotransplants. Surprisingly, most of the antibodies in our blood that would the react against a pig organs recognise a single carbohydrate linkage, galactose alpha (1,3) galactose. This sugar residue does not appear to be functionally important because it is not present in humans and monkeys. Elimination of the glycosyltransferase responsible for attaching this sugar residue by targeting the relevant gene in pigs is therefore expected to greatly reduce hyperacute rejection of transplanted organs.

animal models of disease

Mice in which specific mutations have been deliberately introduced have often proved very useful as models for studying human genetic diseases. In some cases, differences between mice and humans means that the effects of the introduced mutation are not the same as in the human genetic disease. This is the case for the cystic fibrosis mutation, where differences in chloride channel mean that the knock out experiment in mice has little effect on lung physiology.

Nuclear transfer will extend the range of species in which gene targeting will be possible and thereby provide better models to test treatments for human diseases. Lung physiology in sheep and humans is very similar and the gene targeting to introduce the cystic fibrosis mutation in sheep would be expected to produce a similar phenotype in homozygous animals to that in man. The deliberate creation of a genetic disease in a large animal raise public concerns and the ethical justification of creation of such a model would need to be well argued.

cell therapy

Intact cells are already used to treat patients suffering from a number of diseases, including leukemia and Parkinson's disease. In most cases these cells have to be obtained from close relatives to avoid problems of immune rejection.

The fact that Dolly was cloned from a cell taken from an adult ewe shows that even differentiated cells can be 'reprogrammed' into all the cell types that make up an intact animal. The only way that we can perform this dedifferentiation step now is by 'incubating' cells in the cytoplasm of an unfertilised egg but when we know more about the mechanisms involved, then it may be possible that human cells can be reprogrammed without the use of a human egg. This would allow the patient's own cells to be used in cell therapies, thereby avoiding the time, expensive and uncertainty of tissue matching. Cells would removed from the patient, converted into the desired cell type in the laboratory and then reintroduced into same patient for treatment.

cloning in farm animal production

In addition to providing a route to gene targeting in livestock, nuclear transfer could be used to deliver what is the popular image of cloning: that is the production of, at least in principle, unlimited numbers of genetically identical animals.

There are major practical hurdles to overcome before this could be a routine procedure in livestock

production. We first need to show the techniques can be used in cattle and pigs since it is probably only in these species that the benefits are likely to justify the cost. Non-surgical means would be needed for embryo transfer and success rates would have to be dramatically improved. Previous experience with new technologies such as artificial insemination and multiple ovulation embryo transfer suggest that it may be 10-20 years before this could be possible

Nevertheless, the main advantage of cloning would not be in selection programmes but in the more rapid dissemination of genetic progress from elite herds to the commercial farmer. At present this is achieved through artificial insemination - which supplies only half the genes- and by limited use of embryo transfer. This process is not that efficient and in dairy cattle, the performance of the average cow is probably some 10 years behind the best. With cloning, it would be possible to remove this difference. Farmers who could afford it would receive embryos that would be clones of the most productive cows of elite herds and thereby lift the performance of their herds to that of the very best within one generation. This would be a one-off gain, since from then on the rate of genetic progress would return to that of the elite herds.

In this scenario, breeding companies would sell cloned embryos in much the same way as they now sell semen. Farmers would choose cloned embryos for high merit beef bulls or dairy cows from catalogues that described the genetic merit for a series of economically important traits, including fertility, health and longevity. The cloned embryo would be delivered to the farm much in the same way as semen straws are today, perhaps from breeders overseas.

A major risk would be loss of genetic diversity but this could be avoided by systems that ensured that breeding companies produced a limited number of clones of each genotype and restricted the number of each of the clones that could be sold to any one producer. Although the herds of some producers might consist entirely of cloned animals, the fact that they were clones of different elite animals may actually increase genetic diversity on some farms.

genetic conservation

Although cloning is associated in most people's minds with a loss of genetic diversity, the techniques that were used to produce Dolly will also provide new approaches to genetic conservation. With increasing commercial pressures, many indigenous breeds adapted to local conditions are under threat from imported breeds that are being reared in intensive farming systems. The local breeds may contain valuable genes that confer heat tolerance or disease resistance and there is an urgent need to prevent their extinction. Current methods of conservation involve storage of frozen semen or embryos but are time consuming and costly. As a consequence, the future of only a small proportion of endangered breeds is being addressed.

The new techniques developed at Roslin may provide much simpler and more effective means of conserving breeds. Blood samples, skin biopsies or even hair follicles might be suitable sources of cells that could be grown briefly in the laboratory and then frozen in liquid nitrogen for long term storage or transfer to other endangered populations.

To some these applications seem much less glamorous than the prospect of the cloning of human beings. In reality, they have the potential to provide radical new treatments for a wide variety of serious diseases that affect many millions of people world-wide. By contrast, the cloning of human beings- if and when it happens- is likely to remain a marginal activity with little impact on society beyond the immediate participants.

Further information on cloning, including references to work cited, is available on the Institute's web site on