After Dolly, a Pharming Frenzy

New successes in using DNA from fetal cells to clone transgenic animals have boosted a new biotech business, but low success rates still need to be improved

BOSTON—Births are usually announced on a newspaper's society or personal pages, not on the front page. But that convention didn't apply to Dolly and Polly and—just last week—George and Charlie. These white-faced lambs and Holstein calves made headlines as the products of cloning technologies that have generated fascination and fear—a reaction fanned this month by the improbable claims of a physicist who says he plans to clone adult humans within 2 years (*Science*, 16 January, p. 315). But the technologies have done more than spawn an ethical debate about the prospects for human cloning: They have also galvanized efforts to create transgenic livestock that will act as living factories, producing pharmaceutical products in their milk for treating human diseases and, perhaps, organs for transplantation.

That was always the main intention of Dolly's creators, Ian Wilmut, Keith Campbell, and their colleagues at the Roslin Institute and PPL Therapeutics in Roslin, Scotland. But in the year since the announcement of Dolly's birth, a dozen other groups have been adapting the technique used by the team in Scotland. Some want to clone animals bearing working copies of transplanted genes. Although key problems remain to be solved, these efforts—many of which were reported here at last week's annual meeting of the International Embryo Transfer Society—have already resulted in the birth of sheep containing a human clotting factor gene and calves containing foreign marker genes. Experiments in which the nuclei of pig cells have been fused with cow eggs have also given tantalizing results.

This work is invigorating the "pharming" industry: Underwriting the cloning frenzy are biotech and pharmaceutical companies eager to cash in on its potential for creating transgenic livestock. "There is a huge industry that is organizing itself around [the new cloning] technology," says James Robl, a developmental biologist of the University of Massachusetts, Amherst.

There is, however, a crucial difference between these experiments and the original Dolly breakthrough—a distinction that has sometimes been lost in the public discussion of the implications of these new results. Dolly was cloned by taking nuclei from adult mammary gland cells, starving them of nutrients to reset their cell cycles, then fusing them with sheep eggs whose own nuclei had been removed. But this procedure was very inefficient—producing only one success out of the 277 eggs that took up the new DNA. The later experiments all use nuclei from fetal cells,

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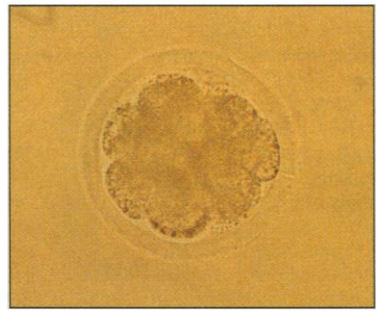
Cash calves. These calves prove that nuclear transfer can lead to transgenic cattle.

which have proved more efficient at generating viable offspring than adult cells. Indeed, so far the Dolly experiment has not been exactly replicated, and some scientists have even questioned whether Dolly is in fact the clone of an adult (see sidebar, letter on p. 635).

Animal geneticists have jumped on the technology because it potentially offers a far more efficient way to produce transgenic animals than previous techniques, which involve the injection of foreign DNA into newly fertilized eggs. The success of an egg injection is not known until after the offspring is born. For example, using egg injection, PPL Therapeutics took years to develop a flock of 600 transgenic sheep, as only about 4% of the lambs carried the desired gene.

In contrast, nuclear transfer technology allows researchers to select as nucleus donors only those cells that express the transplanted gene. Moreover, in theory, those cells could provide as many clones as needed in a single generation. "In one fell swoop, you get what you want," says PPL research director Alan Colman. Indeed, Will Eyestone of PPL's Blacksburg, Virginia, facility told last week's meeting that egg injection "may well become old-fashioned."



N. FIRST

Pow ... or cig? This embryo resulted from the transfer of the nucleus of a pig cell into a cow egg.

From sheep to cows to pigs

Campbell, who recently moved from the Roslin Institute to PPL's labs 300 meters down the road, Wilmut, and their colleagues were the first to announce that they had been able to produce transgenic animals with cloning technology. They reported in December that they had produced three cloned sheep, two of which are still alive, carrying the human factor IX clotting protein (*Science*, 19 December 1997, p. 2130).

Now, Advanced Cell Technology has achieved in cows what the team in Scotland did with sheep: Robl and his colleague Steven Stice announced at last week's meeting the birth of two calves carrying a foreign gene. To produce these transgenic animals, the researchers first grew bovine fetal fibroblast cells in the laboratory and then added an antibiotic-resistance "marker" gene. Only the cells that took up the gene survived exposure to an antibiotic added to the culture dishes. The researchers then fused nuclei from the survivors with enucleated cow eggs, employing a variation on the technique used by Wilmut's group. About 40% of the resulting embryos continued to develop once inside foster mothers, and two calves—George and Charlie—were born in mid-January. A third has been born since the announcement, and more are on the way. "[They are] the first transgenic cloned calves, and that's great," says Campbell of PPL, which is also doing nuclear transfer work in cattle. The three calves show "the phenomenon and the technology are not restricted to one species," adds nuclear transfer pioneer Kenneth Bondioli of Alexion Inc., a biotech company in New Haven, Connecticut.

That demonstration has been eagerly awaited. Transgenic cows, which produce 9000 liters of milk per year, should be better factories for therapeutic proteins than sheep or goats. "Milk is cheap, and we have an incredible dairy infrastruc-

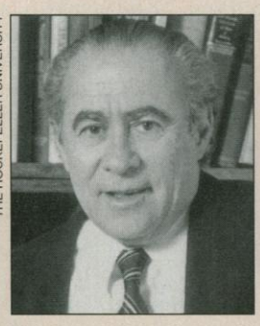
Where's the Beef?

In a perfect world, important scientific discoveries are impeccably documented and quickly replicated. But on page 635, two prominent biologists say that was not the case for Dolly, arguably the most famous lamb in history because she was reportedly cloned from adult cells. In a letter to the editor, Vittorio Sgaramella from the University of Calabria in Cosenza, Italy, and Norton Zinder of Rockefeller University in New York City ask for more convincing evidence that the experiment that produced Dolly worked as claimed. If in fact it hasn't, it would mean that human cloning, which for most conceivable purposes would start with adult cells, is not the immediate threat some worry about.

Because the mammary cells used to produce Dolly came from a pregnant ewe, Zinder and Sgaramella question whether she might have been cloned not from an adult mammary cell but from a contaminating fetal cell. And while Ian Wilmut, the embryologist at the Roslin Institute in Roslin, Scotland, where Dolly was cloned, and his colleagues cite evidence that that could not have happened, they may never be able to prove their assertion conclusively. Because "none of us expected to get Dolly," says embryologist Alan Colman of PPL Therapeutics in Roslin, which collaborated in the work, "we didn't do what we should have done" to document the genetic composition of either the ram that impregnated the ewe or the fetus she carried. Consequently, Dolly's DNA can't be compared with theirs. But the Roslin group also says that some of the other data Zinder and Sgaramella want, concerning whether Dolly's DNA has the mutations and other changes expected in an adult, will be available as soon as the analyses are completed.

Still, even if Dolly is an adult clone, no one has yet exactly replicated the experiment that produced her. Few laboratories, Wilmut's included, have even tried, mainly because the emphasis now is on using DNA from fetal cells, rather than adult cells, to commercialize the nuclear transfer technology used to create Dolly (see main text). Others, including Neal First's team at the University of Wisconsin, Madison, and Mark Westhusian's group at Texas A&M University in College Station, have tried to clone cows from adult cells but failed. None of the embryos survived to birth, they note. Similarly, in Boston last week at the annual meeting of the International Embryo Transfer Society, a team of researchers from Germany and Austria reported it had tried to use heifer udder cells as nuclei donors, but no embryo lived past 40 days of gestation.

Two other teams, one led by James Robl and Steven Stice, developmental biologists at the University of Massachusetts, Amherst, and the other at the biotech firm Infigen in DeForest, Wisconsin, say they have calves in utero that were cloned from adult cells. However, neither team is confident enough that these calves will make it through the final months of their 9-month gestation to reveal the tentative due dates.



THE ROCKEFELLER UNIVERSITY

RICK KOZAK

Raising doubts. Norton Zinder *(left)* wants more proof from Ian Wilmut *(right)* and his colleagues.

But other results from the First team support the Roslin group's finding that adult DNA can be induced to support embryonic development. They used cow oocytes as universal recipients for nuclei obtained from the ear cells of adults from four other species: rats, sheep, pigs, and monkeys. Although only about 34% of eggs receiving rat nuclei began dividing, almost 86% of those with monkey DNA and 52% with pig DNA were activated, Wisconsin's Maissam Mitalipova reported at the embryo transfer meeting.

Dividing eggs continued to develop, with many expanding to 130 cells and reaching the stage where they needed to be implanted in a womb. These developing embryos also contained a protein, not found in the ear cells themselves, that is usually produced only in cells capable of developing into whole new organisms. "It means that if you can do [nuclear transfer] with fetal fibroblasts, you can do it with adult cells," says First. Robl agrees. Getting another clone from an adult cell is "a matter of time," he says. "If you do enough [transfers] and get lucky, you can do it." –E.P.

ture," points out Carol Ziomek, an embryologist with Genzyme Transgenics in Framingham, Massachusetts.

Indeed, that potential has already spurred a gold rush. In October 1997, Genzyme Transgenics awarded Advanced Cell Technology a 5-year, $10 million contract to develop transgenic cows that will produce albumin, a human blood protein used in fluids for treating people who have suffered large blood losses. And earlier this month, Pharming Holding N.V. in Leiden, the Netherlands, formed an alliance with ABS Global, an animal breeding company in DeForest, Wisconsin, and its spin-off company, Infigen Inc., to develop transgenic cattle that produce the human blood proteins fibrinogen, factor IX, and factor VIII in their milk.

Other efforts are aimed at expanding the utility of pigs, particularly in biomedicine. A few companies and research groups hope to use pig organs or tissue to help meet the large unfilled demand for transplant organs. The goal is to genetically modify the animals' tissues so they are less readily rejected. Also, because a pig's physiology is more like a human's than is a mouse's, some animal scientists argue that pigs could be good models for studying human diseases if their genetic makeup could be modified so that they develop appropriate symptoms.

But this work has been lagging, partly because researchers have had trouble getting pig oocytes to start dividing after the nuclear transfers. Moreover, researchers are still working out a suitable way to keep new embryos alive until they can be placed into female pigs for continued development.

At the meeting, several teams reported progress solving these problems. At the University of Missouri, Columbia, Randall Prather has worked out a new way to activate cell division using a chemical called thimerosal as the initial trigger. And reproductive physiologist Neal First's group at the University of Wisconsin, Madison, offered a more radical potential solution: Avoid the hard-to-activate pig egg altogether by transferring nuclei from adult pigs into bovine oocytes (see sidebar). "Instead of using a pig oocyte, perhaps you could use a sheep or cow oocyte," Robl suggests. It is unclear, however, whether such cross-species embryos would ever come to term.

Reducing the body count

In spite of the rapid advances in nuclear transfer since Dolly's debut, some big obstacles still remain. At each step along the way some—often many—individuals don't survive. That low efficiency doomed an earlier version of nuclear transfer when it made its commercial debut a decade ago. At that time, several companies, including Granada

Inc., based in Houston, were going great guns using nuclei from very early embryos to clone hundreds of calves to make large herds of genetically superior beef cattle. But by 1991, Granada had shut its doors. "We couldn't make as many calves as we wanted to," recalls Bondioli, who worked there. And too often, calves were oversized and unhealthy, with lungs that were not fully developed at birth.

Researchers see the same trends in the few cows and sheep produced by the newer cloning procedures. Large numbers of deaths occur around the time of birth. For example, PPL and Roslin lost eight of 11 lambs in their first experiment with transgenic clones. But it's not the nuclear transfer procedure itself that's at fault, says Robl. Animals produced by in vitro fertilization and other procedures involving the manipulation of embryos have similar problems, albeit at a lower frequency.

"Something that you do to the embryo ... leads to a problem 9 months later," says George Seidel Jr., a physiologist at Colorado State University in Fort Collins. His data and other observations suggest that in problem calves the placenta does not function as it should. As a result, cloned calves have too little oxygen and low concentrations of certain growth factors in their blood.

While some researchers are experimenting with different nutrient solutions or making other subtle changes in their nuclear transfer techniques to make embryos and newborns thrive, others are frantically trying to hone the genetic manipulation techniques. Researchers currently have no control over where the foreign genes end up in the chromosomes or how many copies of the gene become part of that cell's genetic repertoire.

Developing that control would enable them to knock out specific genes, say the one encoding the pig protein that elicits a strong, immediate rejection response to pig organ transplants. "The Holy Grail for many is finding a way of getting targeted disruption of genes in livestock as we have in mice," explains Colman, who is confident that even this tough molecular biology problem will be solved quickly. "I expect we'll have targeting solved by next year," he predicts.

Such confidence is required in this fast-moving field, in which progress generally comes through trial and error. Understanding how it all works, say these scientists, will come later. "[There] clearly is at this point in time a pushing forward of the technology," says Alexion's Bondioli. "Have we learned any more biology? Probably not. But [we] have opened up a means to study [it]."

–Elizabeth Pennisi

XENOTRANSPLANTS

No Moratorium on Clinical Trials

U.S. health officials last week said they will allow limited clinical trials of animal-to-human transplantation to proceed, even though some researchers argue that such work poses a risk to public health and should not be permitted without further study. At a meeting on 21 and 22 January, officials from the Food and Drug Administration (FDA), the Centers for Disease Control and Prevention (CDC), and the National Institutes of Health (NIH) outlined plans to allow this research to go forward under stringent safeguards that are now being finalized. They intend to impose rigorous standards to maintain disease-free donor animals, create a national registry of organ recipients, and establish both a tissue bank of samples from both donor animals and recipients and a national policy advisory committee.

Xenotransplantation, which once seemed an implausible alternative to human organ donation, is now being tested as a real possibility. Diabetes patients have received encapsulated pig pancreas cells, and fetal pig brain cells have shown some success in Parkinson's disease therapy. Although previous attempts to transplant whole organs from animals into people have failed when the patient's immune system attacked the organ, clinicians hope to overcome these problems with new immunosuppressive drugs and genetically engineered animals whose organs masquerade as human tissue (see previous story).

But even if it succeeds, xenotransplantation carries some unusual risks. Implanting living cells into an immunosuppressed host gives microorganisms—especially viruses that would not ordinarily leap from an animal into a human—a way past the body's first lines of defense. Once inside, an invader might adapt to its human environment and infect other people.

Those fears were heightened last spring, when researchers discovered that a pig retrovirus could infect human cells. Pigs had been the favored donor animal, in part because scientists thought their diseases would be less likely to infect humans than primate diseases. In October, the FDA ordered a halt to all clinical trials with pig tissues until a test was available to detect the virus in patients. So far, all patients have tested negative for the pig virus, and the FDA has permitted several trials, including tests of therapies for Parkinson's disease and epilepsy, to resume.

SCOTT BAUER

Potential donor. Pigs may provide organs for patients.

Some scientists say the FDA may have moved too hastily, however. In last week's issue of *Nature* and next month's *Nature Medicine*, a team of nine scientists—led by xenotransplantation researcher Fritz Bach and health policy expert Harvey Fineberg, both of Harvard University—call for a moratorium on clinical trials pending a broad public debate.

The Department of Health and Human Services, meanwhile, is proposing a new committee to address such concerns, said Mary Groesch, an NIH staffer. The panel would function like NIH's Recombinant DNA Advisory Committee, she said, sponsoring public workshops and offering advice, but leaving regulatory decisions to the FDA. Groesch said the current situation is "strikingly similar" to the apprehension about potential ecological disasters that caused a moratorium on recombinant DNA research in the 1970s.

The plan to allow this research to continue while experts monitor and discuss the risks won support from some meetinggoers. But others remained skeptical. Bach, for example, said that while he is "comforted" by the plans for a national advisory committee, he would prefer human trials to be halted until the committee is in place. Virologist Jonathan Allan of the Southwest Foundation for Biomedical Research in San Antonio, who opposes the use of nonhuman primates as donors, said he was "baffled, absolutely baffled," that the agencies have left open the possibility of transplanting material from such primates. "You're playing Russian roulette," he warned. If a new retroviral disease emerges, he says, there would be no ready treatment. But CDC epidemiologist Louisa Chapman said the guidelines will impose a practical ban on the use of primates, because they require that donor animals be free of specific diseases known to infect humans—a standard nearly impossible for primates to meet. Officials say they will take such criticism into account over the next few months as they prepare a set of final guidelines for publication—they hope—sometime this summer.

–Gretchen Vogel