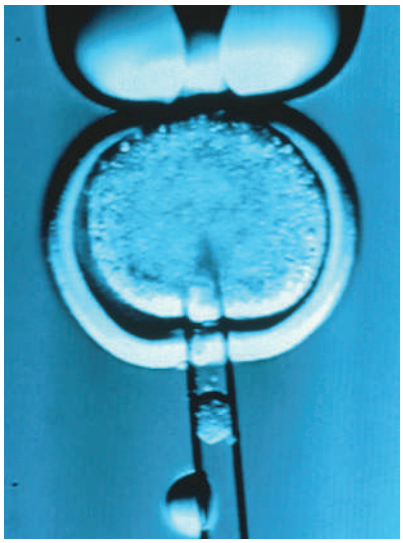
Cloning: what now?

Less than a month ago, investigators at Seoul National University in South Korea announced that cloning researcher Woo Suk Hwang had lied when he claimed his team cloned human embryos with relative ease and produced stem cells from them.

The news was a significant setback for cloning researchers. In this special section, *Nature* looks at how biologists are regrouping. Below, Carina Dennis asks how they can get cloning to work given a very limited supply of eggs. Phyllida Brown looks at whether we will need therapeutic cloning at all, if immunologists can stop our bodies fighting transplants (see page 655). And on page 658, one of Hwang's closest rivals admits it may not continue its cloning quest.



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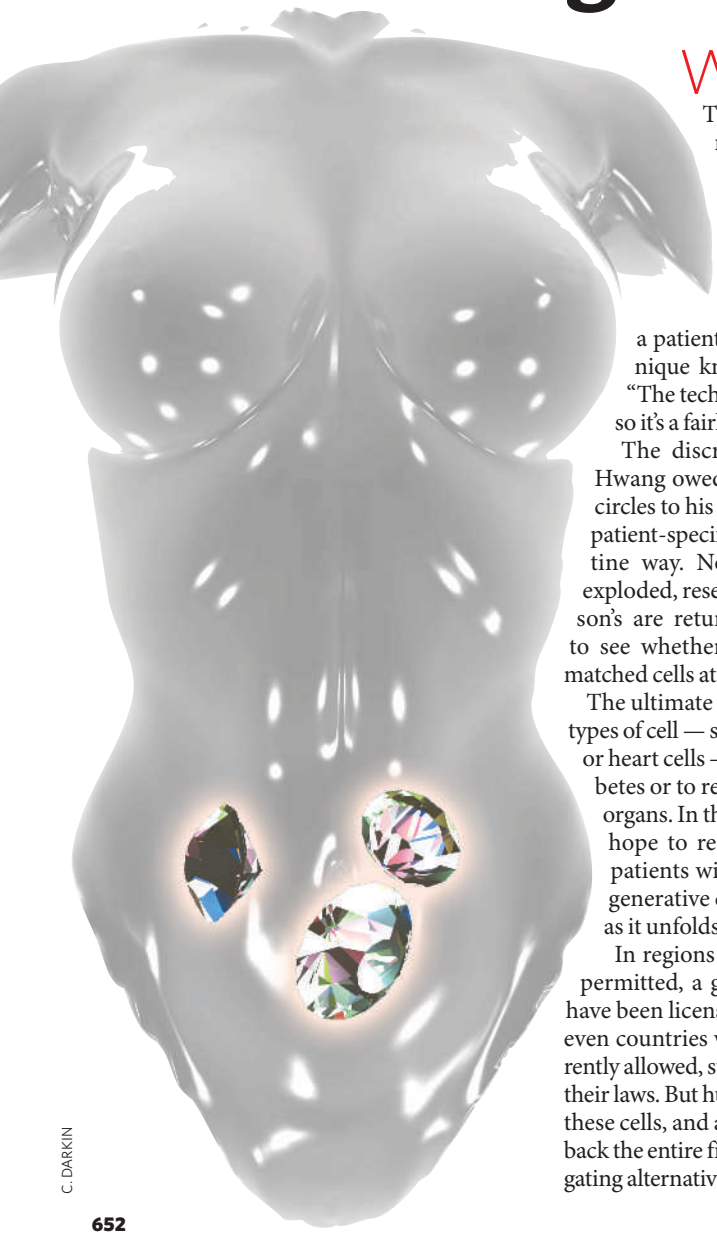
Mining the secrets of the egg



C. DARKIN

Women occasionally offer Alan Trounson their eggs. They approach the stem-cell researcher from Monash University in Melbourne, Australia, after he gives talks to patient focus groups. Trounson wants to treat neurodegenerative disease by using eggs to create cells that match a patient's genetic make-up — a technique known as therapeutic cloning. "The technique is not legal in Australia, so it's a fairly brief conversation," he says.

The discredited researcher Woo Suk Hwang owed his preeminence in cloning circles to his claims to have produced such patient-specific stem cells in an almost routine way. Now those claims have been exploded, researchers with aims like Trounson's are returning to the drawing board to see whether anyone can make patient-matched cells at all.

The ultimate dream is to create specialized types of cell — such as insulin-producing cells or heart cells — to treat diseases such as diabetes or to repair damaged hearts or other organs. In the nearer future, scientists also hope to recreate embryonic cells from patients with diseases such as neurodegenerative conditions, to study an illness as it unfolds and to test new drugs.

In regions where therapeutic cloning is permitted, a growing number of scientists have been licensed to start experiments. And even countries where the method is not currently allowed, such as Australia, are reviewing their laws. But human eggs are needed to make these cells, and a shortage of them could hold back the entire field. So researchers are investigating alternatives such as nurturing immature eggs, growing artificial eggs in the lab and using animal egg substitutes. Each strategy comes laden with its own technical — and ethical — challenges.

To make therapeutic tissues such as heart cells, many researchers start with unspecialized, immature cells called embryonic stem cells. As their name suggests, such cells come from young human embryos, termed blastocysts, that are only a few days old. At the moment, researchers work on stem cells taken from surplus embryos created by clinics doing *in vitro* fertilization (IVF). But if these cells were simply transplanted into patients, the immune system would recognize them as foreign tissue and reject them (see 'Do we even need eggs?' on page 655).

Therapeutic cloning could, in theory, solve this problem. Working in animals, researchers have shown that if they transplant a nucleus from an adult body cell into an egg that has had its nucleus removed, the egg somehow 'reprogrammes' the adult nucleus back to an immature state, where it directs the development of an embryo. The resulting embryo is a genetic clone of the adult from whom the nucleus was taken. If this procedure works in humans, researchers could use cloned embryos to produce therapeutic or research cells that are essentially identical genetic copies of a patient's cells. Such cells should not be attacked by the patient's immune system.

In excess

But cloning is a wildly inefficient process, often requiring hundreds of eggs to produce a single viable clone. Indeed, one shocking revelation of the Hwang affair, was the sheer number of eggs his lab had got through[1]. And obtaining human eggs is not easy. Donation is an unpleasant, invasive process that carries a small risk to a

woman's fertility and can, in rare cases, cause life-threatening side effects. This may make it hard to recruit donors. "We'll have to wait and see how difficult human eggs are to acquire," says Arnold Kriegstein, director of the Institute of Stem Cell and Tissue Biology at the University of California, San Francisco.

Most eggs currently donated to research are leftovers from IVF treatments — the ones that fail to fertilize and would otherwise be discarded. But these eggs typically fail to reprogramme[2], "probably for the same reasons they failed to fertilize," says Alison Murdoch, of the University of Newcastle Upon Tyne, UK. Murdoch and her team have successfully cloned a single blastocyst using excess eggs from women having infertility treatment[3].

Ideally, researchers want healthy, competent eggs. Murdoch now asks women undergoing IVF treatment who produce plenty of eggs — more than 12 in a treatment — whether they would be willing to donate two eggs after the first dozen. "We have calculated that this does not significantly reduce their chances of a pregnancy," says Murdoch.

Greater good

Some researchers expect altruistic donations will be sufficient for research purposes. "My view is that most eggs are likely to come from women who have family members with a disease and want to donate their eggs to advance research on that disease," says Trounson.

But obtaining eggs for clinical use is likely to be a major obstacle, at least in the foreseeable future. "I can't conceive there will be enough eggs to use on a wide scale. In the end, we have no choice but to develop other methods," says Robert Lanza of Advanced Cell Technology. His company, based in Worcester, Massachusetts, has done therapeutic-cloning research using eggs from altruistic donors[4] (see page 658).

"Human eggs are so precious — why practise on them?"
— Huizhen Sheng

And egg donations — especially those given altruistically — create an ethical quagmire. Is it appropriate to put healthy fertile women through such a procedure? Should they be paid for their eggs? These issues divide scientists. "Until we can get the efficiency to a reasonable level, we shouldn't be working in human eggs," says Stephen Minger, a stem-cell researcher at the Wolfson Centre for Age-Related Diseases in London, UK.

The most obvious place to start looking for alternatives to conventional donation is to go direct to the ovary. Although women only ovulate around 500 eggs in a lifetime, their ovaries are packed with thousands of eggs at varying stages of development. What if researchers could somehow get hold of these — from ovarian biopsies, say — and grow them to maturity in the lab?

Biologists are making some headway in culturing eggs that are in the last stages of development. Outi Hovatta from the Karolinska Institute in Stockholm, Sweden, is working on eggs that are on the verge of being ovulated. Researchers are able to coax such eggs, which are collected alongside mature eggs in normal IVF procedures, through the final stages of readiness for fertilization. Hovatta is optimistic that they could work in cloning experiments, which she is about to start. She estimates that altruistic donations of these almost-mature eggs would yield about 300 a year from a collaborating IVF clinic.

But culturing really immature eggs has proved extremely difficult. Egg development in humans is long, extraordinarily complex and not well understood. It begins in the embryo when special embryonic cells make their way to the developing ovary (see graphic, overleaf). Here, they divide many times to generate millions of egg precursors, called primary oocytes. At birth, the ovary contains about half a million follicles: these consist of a primary oocyte wrapped in one or more layers of cells that support the oocyte as it grows and accumulates nutrients needed for the early development of an embryo.

Only after puberty do follicles fully develop, with one follicle growing to full size per menstrual cycle and releasing its enclosed oocyte. Just before ovulation, this oocyte ejects half its chromosomes, getting rid of half the remainder when a sperm makes contact with its own genetic cargo.

In the hands of John Eppig, a researcher at the Jackson Laboratory in Bar Harbor, Maine, culturing eggs through these stages looks almost easy. He can create live mice pups by fertilizing eggs that have been cultured in the lab from ovaries extracted from newborn mice[5]. Although the first offspring of these experiments — dubbed Eggbert — was a sickly creature, subsequent mice look healthy.

But it is a different story with larger animals. "It's tougher in species other than rodents because it takes so much longer for egg development to occur," says Eppig; it takes

SPANNERS IN THE WORKS

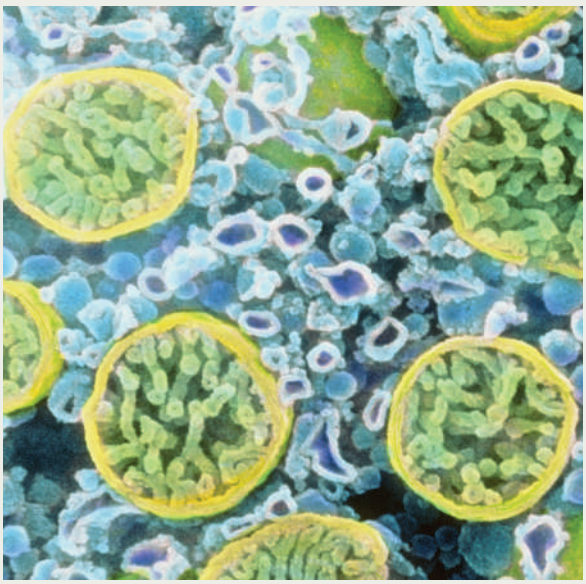
Cloning is notoriously inefficient — it can take hundreds of eggs to produce a single embryo. Researchers trying to work out why can see one possible culprit: the tiny bodies that generate a cell's energy.

These powerhouses, called mitochondria and pictured right, have their own small genomes. But they also need to interact with the products of genes in the nucleus. A mismatch between mitochondrion and nucleus, or even between resident mitochondria and those introduced during the cloning process, could make the cell fail.

"Mitochondria are being totally overlooked — with devastating consequences," says Doug Wallace of the University of California, Irvine.

Fusing cells from two individuals might cause problems, says Wallace, but the biggest problems will arise if researchers try using animal eggs to reprogramme human nuclei. "From our experience, combining the mitochondrial DNA from even a species as closely related as chimpanzees result in incompatibilities."

A recent study suggests that cellular function may not be affected in cells derived from mice clones. But Wallace argues that such cells might be compromised when they move from the carefully controlled lab environment to repairing dilapidated organs inside a body.



P. M. MOTTA, S. MAKABE & T. NAGURO/SPL

more than three months for a human egg to mature. Another factor is the relatively gargantuan size of a human egg, which swells to more than 100 micrometres. The challenge is to ensure that the voluminous egg receives adequate nourishment, as well as figuring out the right factors to coax it along the development pathway.

That said, researchers have had some success with human eggs. Ronit Abir from the Rabin Medical Center at Beilinson Hospital, Israel, has nurtured isolated follicles for several weeks *in vitro*. Controversially, she has also cultured immature eggs from aborted human fetuses to almost the same stage[6]. Aborted fetuses are not likely to be a good source of eggs, given the obvious ethical concerns, including the fact that a fetus cannot give its consent. But Abir says the work could unravel the mysteries of culturing eggs from early development and reveal ways to restore the fertility of cancer patients who have had their ovaries extracted and frozen.

Hovatta's team has been able to grow human primary oocytes in intact ovary slices in culture and has nudged them along several developmental stages. But these efforts to culture primary oocytes have yet to yield eggs that can be fertilized. "In the beginning, I thought it would change everything, but now I see how slow the rate of progress is," says Abir.

Make it up

Others are going right back to the earliest stages and trying to develop eggs from scratch using embryonic stem cells[7,8]. If such cells are left to grow very densely on a culture dish under the right conditions, they clump together and, amazingly, will form egg-like structures.

Researchers have not yet shown that these egg-like cells can be fertilized. But they might be good enough to reprogramme a nucleus, according to Hans Schöler of the Max Planck Institute for Molecular Biomedicine in Münster, Germany. Schöler pioneered the growth of egg-like cells from the embryonic stem cells of mice. The research field is anxious to see whether reprogramming will be possible — Schöler claims to have made one unsuccessful attempt. "We are still working out the conditions," he says. "It's not as trivial as we'd thought."

With human eggs presenting so many difficulties, some researchers are exploring the possibilities of animal eggs, at least for research purposes. "Human eggs are so precious — why waste them to practise on?" asks Huizhen Sheng, from the Centre for Developmental Biology at Xinhua Hospital in Shanghai.

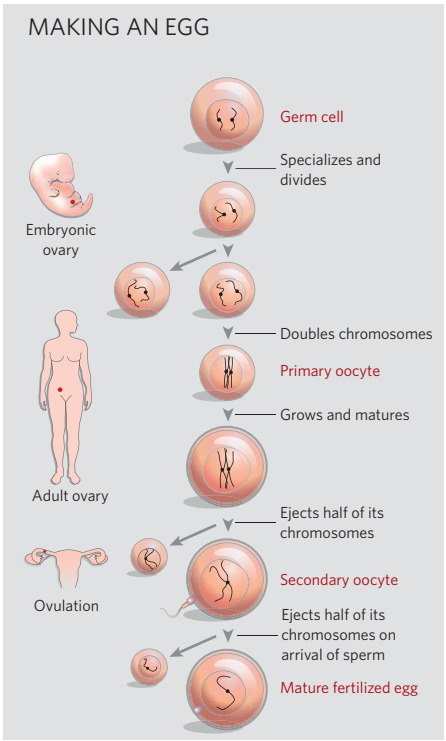
Sheng's lab sparked an international storm when the media reported in 2002 that she was using rabbit eggs to clone human blastocysts. Some newspapers ran headlines about animal–human monsters, fuelling public hysteria. The debate was reignited recently when Chris Shaw, a neurologist at King's College London and Ian Wilmut, the creator of cloned sheep Dolly, based at the University of Edinburgh, announced that they were seeking approval to do similar experiments.

Sheng has published her data[9], but the research community remains unconvinced that her method works. "You have to be uncertain of that work until it is repeated," says Trounson. Sheng attributes certain discrepancies to the lab's culturing methods, a problem that she says has now been rectified.

Creating hybrids of human cells and animal eggs is banned in many countries, under review in others such as Australia, and yet to be tested in the more permissive regulatory environment of Britain. But with scant supplies of fresh human oocytes, many researchers see animal eggs as the only practical alternative for refining therapeutic-cloning techniques. And they could be useful for generating patient-specific lines to study the genetic basis of human diseases. "We are trying to understand disease processes to identify new therapeutic targets. These cells are not for putting back into people," says Shaw.

Mismatched

Still, many scientists are doubtful that animal eggs will yield useful human embryonic stem-cell lines. The main concern centres on mitochondria, the bacteria-like powerhouses of the cell. Mitochondria have their own genomes, which interact with the genome in the cell's nucleus. Mixing nuclei and mitochondria from different species simply may not work (see 'Spanners in the works', previous page). "It's hard enough to keep our nuclear chromosomes in sync with our own mitochondrial DNA and we're the same species," says Irving Weissman of the Stanford School of Medicine, California. And yet scientists are undeterred. "It's an important question and difficult to answer until you have done the experiments," says Shaw.



J. MCLEAN/SPL

Slim pickings: it is hard to harvest quantities of human eggs because the process is unpleasantly invasive.


C. DARKIN

Do we even need eggs?

Nobody likes rejection, but for a transplant patient it can be a death sentence. The risk that a patient's immune system will see a transplanted organ, or graft, as 'foreign' rather than 'self', forces transplant patients on to immunosuppressant drugs that can have severe side effects. Therapeutic cloning, its enthusiasts say, could solve the problem by allowing doctors to grow cells and tissues that are perfectly matched to individual patients. In this approach, a patient's DNA is transferred into an egg which is persuaded to develop into stem cells that in turn generate spare-part tissues. But many researchers now think therapeutic cloning is unrealistic, largely owing to the scarcity of human eggs.

So the spotlight is turning on to different strategies, aimed at persuading the immune system to tolerate foreign tissue. "The field is moving very fast," says Harry Moore of the Centre for Stem Cell Biology at the University of Sheffield, UK. "Ten years ahead there may be no need for cloning, except in certain cases."

There are different ways to increase the success of tissue transplants. One is to develop generic stem cells, cell lines and tissues, and then persuade the immune system to accept them. These could be therapeutic transplants of, say, insulin-producing cells to treat diseases such as type 1 diabetes. Those championing this idea admit it is years, or decades, from the clinic. So more pragmatic approaches are also under development. Instead of trying to make the immune system perfectly tolerant of a transplant, some researchers are aiming to increase its tolerance enough for patients to sharply reduce their dependence on powerful immunosuppressive drugs.

"Ten years ahead there may be no need for cloning."
— Harry Moore

At the moment, most people who have an organ transplant face a lifetime of treatment with drugs that affect the whole immune system, such as cyclosporine and steroids. Although these drugs increase the life of a grafted organ by several years, they often fail to prevent its eventual rejection, and they put patients at risk of infections, cancers and kidney failure.

Drug problem

In studies on small numbers of patients who have had organ transplants, medical teams are discovering that they can manipulate the immune system so that drug treatment can be reduced. For example, Chris Watson and Roy Calne at Addenbrooke's Hospital, Cambridge[1], are among those who gave patients an antibody, Campath-1 (alemtuzumab), at the time of an organ transplant. This antibody depletes lymphocytes, a large family of key immune cells.

Watson and Calne then gave lower doses of immunosuppressive drugs to the patients than would normally be given following a transplant, and no steroids. For the five-year study period, the patients' grafts survived as

Given that eggs are so problematic, some teams are attacking the problem of reprogramming from a different angle. They are trying to see whether other kinds of cells share an egg's ability to reprogramme a nucleus. One such candidate is an embryonic stem cell itself. Recently, Harvard University researcher Kevin Eggan and his colleagues transformed adult body cells to an embryonic state by fusing them with embryonic stem cells[10]. And some experiments have even suggested that embryonic stem cells might be better at certain aspects of reprogramming than oocytes.

But the major drawback of this method is that the chromosomes of the embryonic stem cell used to spark the process are retained. This limits a cell's therapeutic potential because a patient's immune system could recognize the leftover chromosomes and launch an attack. Researchers are working on fixes, however. For example, Paul Verma from Monash University has devised a way of getting rid of the unwanted chromosomes[11], and now has unpublished evidence that mouse cells might be reprogrammed using this approach.

Others are searching for those seemingly magical factors in eggs that allow them to wind an adult nucleus back to an embryonic form. Nobuaki Kikyo of the Stem Cell Institute at the University of Minnesota in Minneapolis, for example, has fished out factors from frog eggs that can repackage chromosomes, dismantle the nucleus's structure and switch on gene activity — all key aspects of reprogramming[12]. But this approach will take time. "Someone might get lucky, but I think it's a long way off," says Keith Latham from Temple University School of Medicine in Philadelphia, Pennsylvania.

There is unlikely to be one single way to mimic the almost mystical reprogramming ability of a human egg. The answer, says Weissman, could be to combine methods — kick-starting the process with one approach, and finishing it with another. ■

Carina Dennis is *Nature*'s Australasian correspondent.

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