Embryology: Out of the Womb—into the Test Tube

The possibility of "creating" human life in the test tube has long fascinated —or repelled—scientists and laymen alike. Because of the progress in manipulating embryos in vitro, many investigators now think that this possibility, with its attendant ethical, moral, and legal dilemmas (see box), will be realized in the near future. Techniques for in vitro fertilization of human eggs, as well as the eggs of common laboratory animals such as mice, rabbits, and hamsters, are available. Moreover, researchers are able to grow mammalian embryos in culture and even to freeze them.

These advances are significant, not just because of their potential application to human reproduction, but also because they permit study of fundamental problems of genetics and development. Furthermore, if applied to the highly practical realm of animal husbandry, they could facilitate the breeding of superior cattle.

The success of the molecular biologists in explaining control of genetic expression in bacterial cells has yet to be translated into a similar understanding of these processes in the cells of developing mammalian embryos. The study of biochemical and physiological events in embryos developing within the uterus of a living animal presents formidable difficulties. The alternative —studying embryo development in vitro—has been hindered by lack of culture systems in which embryonic cells would differentiate normally throughout the entire gestational period.

Recently, however, Yu-Chih Hsu of the Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland, devised a system for culturing mouse embryos from the blastocyst stage to a state of development approximately equivalent to that seen after 9 days of gestation in vivo—or almost half of the 21-day gestation period of the mouse. Previous investigators were able to culture fertilized mouse eggs only to the blastocyst stage (Fig. 1) or to culture embryos older than 7.5 days. (The blastocyst is a hollow ball of cells that forms, in the mouse, 3 to 4 days after fertilization; at the end of this stage—5 days after fertilization—the embryo implants in the uterine wall.) Thus, Hsu bridged a gap in embryo culture that includes the critical period during which implantation occurs.

The requirement for blastocysts to implant before undergoing further development may account for earlier failures to culture embryos beyond the blastocyst stage. Hsu overcame this obstacle by using petri dishes coated with reconstituted rat tail collagen to serve as a substrate for implantation. The embryos did attach to the collagen and develop normally.

According to Hsu, the kind of serum added to the culture medium is critically important to embryo development. He uses calf serum for the first and second days of culture, fetal calf serum through the fourth day; a mixture of fetal calf and human cord serums on the fifth and sixth days; and, finally, human cord serum. He is trying to identify the serum components that are active in the different stages of development.

Most of Hsu's investigations have been performed with embryos resulting from in vivo fertilization. Female mice are treated with hormones that stimulate egg maturation and release (a process called "superovulation" that resembles the hormone treatments producing multiple births in humans) and then mated. After 35 days of gestation the embryos are washed out of the oviducts and cultured. Hsu now says that he has cultured eggs fertilized in vitro; they also attained a state of development equivalent to that after about 9 days of gestation in vivo.

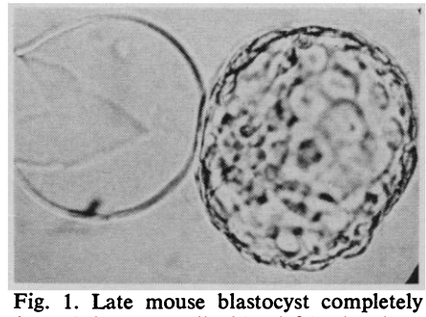

Fig. 1. Late mouse blastocyst completely free of the zona pellucida (left), the clear, noncellular layer that originally surrounded the egg. The blastocyst developed from a two-cell ovum cultured in vitro for 72 hours (× 110). [Source: Ralph Brinster, University of Pennsylvania School of Veterinary Medicine]

A number of investigators have studied the metabolism of mammalian embryos and the conditions needed for their culture. Included among them are John D. Biggers, Harvard University Medical School, Cambridge, Massachusetts; Ralph Brinster, University of Pennsylvania School of Veterinary Medicine, Philadelphia; Wesley K. Whitten, Jackson Laboratory, Bar Harbor, Maine; and David G. Whittingham, University of Cambridge, Cambridge, England. In general, embryo culture requires carefully controlled conditions of temperature (37°C) of *p*H (around 7.4), carbon dioxide, high humidity, an ionic composition similar to serum, a source of amino nitrogen (frequently bovine serum albumin), and an energy source.

The energy requirements of embryos change during gestation. Whitten found that one- or two-cell embryos would not divide with glucose as an energy source. Brinster later showed that pyruvate is the principal energy source for supporting development of such early embryos. The eight-cell stage, however, could use glucose and a number of other compounds. By the time of implantation, energy metabolism of embryonic cells resembles that of other cells.

Brinster thinks that there may be considerable similarity between preimplantation embryos of different mammalian species. He points out that before implantation their development follows parallel courses in both morphology and timing of development, although total gestational periods may vary greatly. Consequently, much of the current information, usually derived from studies on mice or rabbits, may also apply to other species, including humans.

Embryos can be maintained in culture for relatively short times, but they cannot be stored for prolonged periods as is frequently desirable. For example, breeding colonies must now be maintained—a process both time-consuming and expensive—in order to preserve rare strains of mutant mice even though they are not presently needed for research. Last year, Whittingham, in collaboration with Peter Mazur and Stanley Leibo at Oak Ridge National Laboratory, Oak Ridge, Tennessee,

found that mouse embryos could be frozen at temperatures as low as −269°C for as long as 8 days—and survive. Some investigators think that the ability to store frozen embryos might eliminate or reduce the need for maintaining colonies of animals not in use. Since it is possible to transplant mouse embryos into foster-mothers in which they will develop into newborn mice, the frozen embryos could be thawed when needed and grown to term in foster mothers.

Whittingham, Mazur, and Leibo used two criteria of survival for their frozen embryos—development to the late blastocyst in culture and development to living mice in the uteri of foster-mothers. Up to 70 percent of the embryos frozen in the one-, two-, or eight-cell or blastocyst stage fulfilled the first criterion. Almost 1000 of the thawed embryos were subsequently transplanted into foster-mothers. Sixty-five percent of the animals became pregnant. Forty-three percent of the transplanted embryos developed into living fetuses (killed after 18 days of gestation) or newborn, apparently normal mice. The fetuses or pups carried genetic markers—dark eyes and coats—not possessed by their albino foster-mothers. Mazur says that they have now frozen mouse embryos for periods up to 1 year with survival of 80 percent of the thawed embryos.

According to Mazur and Leibo, who are specialists in cryobiology rather than embryology, formation of ice

Speaking of Science

In vitro Fertilization of Human Eggs:

Is in vitro fertilization of human eggs a valid means for alleviating the suffering of infertile couples? Or is it a dehumanizing and illicit intrusion of technology into one of the most profound aspects of human life? These are among the bioethical questions raised by current research in human embryology.

One rationale for much of this research is that it may permit a married couple, infertile because the woman's oviducts are defective, to have their own biological child. The woman could bear the child if an embryo, obtained from in vitro fertilization of her eggs by her husband's sperm, could be implanted in her uterus to develop.

The stumbling block for many who object to this procedure is the unknown element of risk to the embryo—destined to become a human being if pregnancy results. Paul Ramsey of the Department of Religion, Princeton University, Princeton, New Jersey, thinks that in vitro fertilization constitutes unethical medical experimentation on potential human beings. He argues that it is impossible to exclude the possibility that manipulations performed on the embryo outside the womb will damage it and result in production of a deformed or handicapped human. Experiments on animals might prove that the techniques of in vitro fertilization, embryo culture, and implantation were safe for animals. But only human experimentation could prove them safe for the human—and, according to Ramsey, that experimentation is unethical because of the risk involved.

Ramsey thinks that detection of embryo damage is not the answer to this dilemma. Before implantation, some abnormalities may escape detection or the detection methods themselves may be harmful; after implantation, amniocentesis (sampling of uterine fluids plus fetal cells) could identify some defects so that an abortion could be performed, but amniocentesis itself entails an added element of risk.

At present, no one knows whether in vitro manipulations of the embryo involve greater or less risk than does ordinary conception in vivo. But it is known that all conceptions are fraught with risk. Marc Lappé of the Institute of Society, Ethics, and the Life Sciences, Hastings-on-Hudson, New York, pointed out that up to 20 percent of human pregnancies, usually those in which the fetus is abnormal, may abort spontaneously. He also believes that research on the early mammalian embryo during the preimplantation stages indicates that it is quite resistant to damage. Most known teratogenic effects, for example, appear to occur after implantation during organ development.

Lappé thinks that in vitro fertilization can be a legitimate means of fulfilling a couple's desire for a child, if the risks prove to be acceptable and if the parents understand and consent to them. Nevertheless, he has suggested a moratorium on human research until animal experimentation, especially on subhuman primates, demonstrates that the risks of in vitro fertilization are at least no greater than those of normal conception.

Leon Kass, a scientist who frequently writes on bioethical topics, questions whether, in an age when overpopulation is a major concern, there are compelling reasons to proceed rapidly with the development of new means of producing babies—especially since, in his view, these techniques would introduce elements of depersonalization and dehumanization into the act of human procreation. Kass points out that there is an alternate solution to this problem of infertility, an operation for reconstruction of the oviducts. He suggests that additional effort be expended to improve the operation, now frequently unsuccessful, because it can cure the defect that causes the infertility without raising complex ethical issues.

Risk to a potential human being is not the only basis for objections to in vitro fertilization. To some individuals, the termination of fetal life—by whom, at what stage of development, by what method—is one of the central bioethical problems. For embryos would undoubtedly be killed, even in cases where implantation is the goal. More than one egg is fertilized, but only one is implanted.

Although there are analogies to abortion, Kass suggests that the issues involved in the two cases differ in part. Embryos produced by in vitro fertilization are wanted, used, and then deliberately killed; embryos that are aborted are usually the result of an "accidental" conception. Furthermore, André Hellegers, Director of the Kennedy Institute for the Study of Human Reproduction and Bioethics, Washington, D.C., points out that the basis for allowing abortion is conflict between the woman

crystals within cells during freezing usually produces irreversible damage. In their experiments with Whittingham, however, they avoided ice crystal formation by employing extremely slow rates of cooling—0.3° to 2°C per minute—to allow enough time for freezable water to flow out of the cells. They also found that slow warming—4° to 25°C per minute—was necessary for survival. A third requirement was the addition of a protective agent that helps prevent freezing damage, possibly through action on cell membranes.

In addition to using frozen embryos to preserve mutant strains of laboratory animals, or even of endangered species, they may also be useful for transportation of animals. Whitten, for example, recently shipped frozen mouse embryos to Whittingham in England. Whitten said that 57 embryos were implanted into foster-mothers; 21 developed into fetuses (killed before parturition) and 11 into newborn mice. Live animals require special handling during shipment and may also be subjected to quarantine against infectious disease if transported across national borders. Frozen embryos are much less likely to carry diseases, such as hoof-and-mouth disease or rabies, than are adults.

Large animals are particularly difficult to transport. Cattle breeders have been importing from Europe "exotic" breeds of beef cattle for improving their herds. Embryos frozen for transport or storage, or even for preserva-

Bioethical and Legal Considerations

and fetus; the fetus invades her privacy or is a threat to her physical or mental health. For embryos in vitro there can be no such conflict.

Use of in vitro fertilization techniques need not be restricted to treating infertility. They could also be applied to eugenics. This could either be positive eugenics, breeding "superior" human beings by mating eggs and sperm from donors with the desired qualities; or negative eugenics, discarding embryos carrying genetic defects (if these can be detected). Most investigators agree that the consequences of such tampering with human evolution—itself poorly understood—are unknown. The same could be said of the possibility of using these techniques to allow parents to predetermine the sex of their children. All of these applications require destruction of some embryos.

Experiments in human genetics and development could be performed on embryos obtained from in vitro fertilization. For example, controlled mating experiments in the human are not possible. The scientific difficulties (such as long generation time and small number of progeny) are almost as insurmountable as the ethical ones. However, such mating could be achieved in the test tube and the resulting embryos studied in the initial stages of development for expression of a genetic trait such as synthesis of an enzyme. One scientist suggested that these embryos not be maintained beyond the blastocyst stage. Some do not believe that these early embryos—which are barely visible to the naked eye and have not yet differentiated—are human life worthy of protection; others do, however.

Underlying any discussion of bioethics and the legality or morality of scientific research is still another thorny problem—one about which members of the scientific community are highly sensitive. That is the question of regulation of research. Who decides what is permissible and how is the decision enforced? In the case of research funded by the federal government, enforcement, at least, is relatively simple: deny funds for research not meeting the required guidelines.

When Robert Marston was director of the National Institutes of Health (NIH), Bethesda, Maryland, he commissioned an NIH task force, unofficially known as the Human Investigations Committee, to study the ethical and legal issues of human experimentation and to recommend guidelines for NIH-supported research. The committee report, titled "Draft: Special policy statement on the protection of human subjects involved in research, development, and demonstration activities" was published in the *Federal Register* on 16 November.

A subcommittee of the task force probed (among other things) questions relating to the use of human fetal material, whether derived from fertilization in vivo or in vitro, in research. A scientist who served on the subcommittee outlined some of their considerations on in vitro fertilization. (He requested that his name not be used to prevent a deluge of what he characterized as hate mail.) The scientist pointed out that there were legal issues in addition to moral or ethical issues.

Unlike natural conception, in vitro fertilization requires participation by a third party—the investigator or physician who fertilizes and implants the egg in the recipient. If a defective child develops from that egg, would the third party be legally liable for damages? And would the agency that funded the research be liable? At present NIH prohibits investigators with NIH funding from requiring participants in their research projects to sign waivers that release the institution from liability for damages. Even if a participant did sign such a waiver, it would not prevent him from suing for damages.

Furthermore, the question of who is legally responsible for caring for the child must be considered. The simplest case is that in which the couple is married; the husband donates the sperm, and the wife donates the egg and carries their child. But the use of in vitro fertilization need not be restricated to the simplest case. The transplant recipient and egg donor may not be the same individual. A man other than the husband may donate the sperm. A number of variations are possible. And finally there is the possibility, however remote, that the embryo can be brought to term completely in vitro. Will the child be without a parent?

The issues raised about in vitro fertilization of human eggs are profound and the views on these issues disparate. Nevertheless, virtually everyone expressed the opinion that only a continuing dialogue between scientists and public would permit a thorough exploration of the issues and an ultimate consensus.—J.L.M.

tion of unpopular breeds, would facilitate the breeding of superior cattle and the practice of animal husbandry in general. In fact, the Agricultural Research Council's Unit of Reproductive Physiology and Biochemistry, Cambridge, England, was midwife to the birth, in June of this year, of Frosty, a bull that developed from a deep-frozen embryo. The Cambridge group, under the direction of Ian Wilmut and L. E. A. Rowson, transplanted, into 11 foster-mothers, 21 embryos that had been frozen at −196°C and then thawed. Two implanted into the uterus of the same recipient, but only one survived to term. Thus, the process, while apparently feasible, is not yet ready for routine application.

Because of growing demands for beef, animal scientists are examining ways to increase production by increasing the number of high-quality animals. Most cows can produce only one calf per year, or six or seven in a lifetime. This number can be increased by transplanting embryos from superior breeding animals to healthy but otherwise undistinguished foster-mothers. The donor is first "superovulated" to stimulate egg release from her ovaries. This can provide up to 30 eggs (instead of just one) to be fertilized by artificial insemination of the donor. The embryos are removed surgically and transplanted to the foster-mother. The recipient must be in the stage of estrus when implantation can occur.

The process may appear expensive, but is well within the limits of economic feasibility. A number of companies provide the service commercially. According to Casey Ringelberg of Modern Ova Trends, Norval, Ontario, the average fee for each calf produced by this procedure is $2500 to $3000; however, breedable heifers of breeds like Limousin, Simmental, or Chianina sell for $20,000 to as much as $100,000 per animal—and the breeder gets a tax break because he can deduct the cost of producing the animal as a business expense.

Ringelberg points out that the efficiency of the process could still be increased—for example, by freezing embryos for transport or storage until the recipient was in the right stage of estrus. A major handicap is the lack of an in vitro fertilization technique for bovine eggs. Despite successes with common laboratory animals—and even with the human—in vitro fertilization of bovine eggs has not yet been accomplished. Such a technique would mean that eggs and sperm, collected from donors anywhere in the world, could be used to prepare embryos that could then be transplanted into foster-mothers.

In vitro fertilization of eggs from other species is now done routinely in several laboratories. Although investigators had tried to fertilize mammalian eggs in vitro for almost a century, progress was slow until sperm capacitation was discovered in the early 1950's. Capacitation is a still poorly understood process that sperm undergo in the female reproductive tract before they can penetrate the egg.

In vitro Fertilization

Development of in vitro fertilization techniques enabled investigators to study the mechanism of fertilization in systems much simpler than the living animal. For example, M. C. Chang of the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts, has studied fertilization, both in vitro and in vivo, in the mouse, hamster, rabbit, and rat. In addition to defining conditions necessary for fertilization in vitro, Chang has used in vitro conditions to capacitate sperm. He found that the sperm of some species could be capacitated in media with well-defined compositions that did not include fluids from the female reproductive tract. A medium containing bovine serum albumin gave good capacitation of mouse sperm.

Capacitation of rabbit sperm appears to have more stringent requirements. However, Benjamin Brackett of the University of Pennsylvania School of Medicine, Philadelphia, found that he could achieve this by incubating the sperm with uterine fluid before using them for in vitro fertilization. More recently, Brackett, in collaboration with Gene Oliphant of the University of Virginia, Charlottesville, found that increasing the ionic strength of the incubation medium can produce capacitation of rabbit sperm in the absence of uterine fluid.

Brackett says that it is frequently difficult to select suitable criteria to prove that a sperm has actually fertilized an egg. Some eggs can divide, when appropriately stimulated, even though they have not been fertilized (a process called parthenogenesis) or they may undergo degenerative changes that resemble those of a fertilized egg. The ultimate criterion is transplantation of the resulting embryo to a foster-mother and its subsequent development to a fetus. This criterion has been satisfied for the mouse and rabbit, but not for the human—although it has been tried.

Several investigators have reported in vitro fertilization of human eggs. Among these are R. G. Edwards of the University of Cambridge and Patrick Steptoe of Oldham General Hospital, Oldham, England. They have also attempted to transplant the resulting embryos into the uteri of women (the donors of the eggs) and to bring them to term. So far, no such "pregnancy" has lasted longer than 21 days. Edwards and Steptoe believe that the principal problems are in the implantation technique and in duplicating the correct hormonal conditions for implantation. Carl Wood of Monash University, Victoria, Australia, reported doing a similar experiment, also without success.

The goal of these investigators is to circumvent a certain type of infertility. Some women have healthy ovaries and a uterus but cannot conceive because their oviducts are blocked or defective. But if her eggs are removed by laparoscopy, fertilized in vitro with her husband's sperm, and returned to her uterus, such a woman may be able to bear her own child.

Although Brackett has attempted in vitro fertilization of human eggs, he believes that additional experimentation on laboratory animals, especially nonhuman primates, is desirable. Not only would this allow time to clarify and solve the biological problems but also to confront the bioethical issues. Brackett is studying in vitro fertilization of rhesus monkey eggs but progress has been slow.

A better understanding of the mechanism and requirements of fertilization can be applied to prevention of conception as well as to treatment of infertility. It may be possible to interfere with sperm capacitation, or otherwise alter conditions within the female reproductive tract and prevent union of sperm and egg. Whatever its applications, current research on fertilization and development impinges on the most profound questions of life and birth.

—JEAN L. MARX

Additional Reading

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