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MEDICALLY ASSISTED CONCEPTION

An Agenda for Research

Report of a Study by a Committee of the

INSTITUTE OF MEDICINE (U.S.)
Division of Health Sciences Policy

NATIONAL RESEARCH COUNCIL
Board on Agriculture

National Academy Press
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This report has been reviewed by a group other than the authors according to procedures approved by a Report Review Committee consisting of members of the National Academy of Sciences, the National Academy of Engineering, and the Institute of Medicine.

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TABLE OF CONTENTS

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Medically Assisted Conception: An Agenda for Research : Report of a Study
http://www.nap.edu/catalog.php?record_id=18586

ACKNOWLEDGEMENTS

The committee appreciates the generous contribution of time and knowledge of those who presented papers at the committee's workshop at the Arnold and Mabel Beckman Center, Irvine, California, August 21-23, 1988. The thought-provoking papers contributed to the workshop, and the presence of exceptional scientists and clinicians stimulated outstanding discussions. The committee gained invaluable knowledge and insight into issues in research in reproductive and developmental biology that provided the basis for its deliberations. I want to extend the gratitude of the committee to all who attended the workshop. By offering those resources that we all value -- time and knowledge -- the committee's work was greatly facilitated.

Kenneth J. Ryan
Chairman
Committee on the Basic Science Foundations
of Medically Assisted Conception

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PREFACE

This report is the result of a study by a committee of the Institute of Medicine (IOM) and the National Research Council's Board on Agriculture. The study results from a 1984 meeting of an ad hoc group convened by the National Academy of Sciences to discuss aspects of genetic engineering of the human germline. It was noted that because of a policy that, in effect, prohibited the use of federal funds for research involving human embryos, the clinical practice of in vitro fertilization and embryo transfer (IVFET) was in danger of outstripping its scientific foundations. Moreover, the United States had not systematically reviewed the current state of knowledge and practice of IVFET. In 1986, the IOM's Board on Health Sciences Policy convened a meeting of experts in the fields of human and animal research, clinical practice, law, ethics, and public policy to consider some of the issues raised at the earlier meeting. This group was asked to advise the IOM on whether it should pursue a study in any of the areas in which difficult issues had been raised by the practice of medically assisted conception—namely, professional, social, legal, and ethical issues, in addition to questions of science policy. The group identified several topics to which the IOM could make a substantial contribution. An important result of the meeting of this group was to highlight the scientific contribution of research relating to animal uses of medically assisted conception, and the lack of communication between those working to further human clinical IVFET and those working in the animal sciences. This study of the basic science foundations of medically assisted conception results from the recommendations made by this group of experts.

Scope of the Study

A committee was appointed whose membership included individuals with expertise in the clinical practice of in vitro fertilization, research in animal and human reproductive and developmental biology, and physiology. The committee was asked to examine the basic science foundations of medically assisted conception, and develop an agenda for basic research in reproductive and developmental biology that would contribute to advances in the clinical and agricultural practice of IVFET. This research would also be applicable to other concerns in human and animal reproduction such as male and female infertility and contraception. Because of limitations on research using human embryos, the committee was also asked to identify animal systems that provide useful models for specific aspects of medically assisted conception. In addition, the committee was asked to address ways of diminishing barriers to progress in reproductive research and consider ways of fostering communication between investigators oriented to human clinical questions and those interested in studies of animals.

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SUMMARY

This study was prompted by a concern that, because of a policy that in effect prohibits the use of federal funds for research involving human embryos, the clinical practice of in vitro fertilization and embryo transfer (IVFET) was pushing to the limit of its scientific foundations. In addition, there was a perception that animal science had made substantial progress in the development of IVFET but, because of a lack of communication among those working to further human clinical IVFET and those working in the animal science area, the knowledge developed in one sector was not being conveyed to the other. As a result progress in each sector was seen as being slowed.

An Institute of Medicine committee was appointed to examine the basic science foundations of medically assisted conception, to develop an agenda for basic science research that would contribute to advances in the clinical and agricultural practice of IVFET, to suggest animal systems that provide useful models for specific research areas, to identify ways of diminishing barriers to progress, and to recommend ways of bringing together the veterinary and human reproductive research workers.

The centerpiece of the study was a workshop at which clinicians and investigators reviewed the status of assisted conception and the related basic research in humans and animals, and suggested productive areas for future research. This workshop brought together representatives from the human and animal research worlds to enable them to exchange ideas, enhance their understanding of ways in which they can contribute to each other's work, and participate in a joint activity that could establish continuing ties.

Social Concerns That Can be Addressed By IVFET

Research directed at pushing forward the frontiers of medically assisted conception has the potential to provide benefits beyond the limited number of couples whose infertility may be solved by IVFET. There are expectations that such research would enable practitioners to identify genetic defects in embryos without damaging them, and to determine the sex of embryos without damaging them, so that those with sex-linked genetic diseases can be identified at a very early stage. Major areas to which advances in basic reproductive biology would make large contributions include:

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o Infertility. The magnitude of the problem of human infertility is reflected by the number of women with "impaired fecundity" — 4.4 million or 8.2 percent of women of childbearing age in 1982 (National Center for Health Statistics, 1985). By one estimate, more than half of the 4.2 million women who have been surgically sterilized for non-contraceptive reasons, and half of the 4.4 million subfecund women would like to become pregnant. Furthermore, one million women between the ages of 15 and 44 who were or had been married reported at least one medical visit for infertility in 1982 (Fuchs and Perrault, 1986). Although data cannot describe the emotional toll of infertility, the communications media are beginning to portray some of the distress. The lengths to which couples will go in attempting to conceive and the formation of nationwide support groups for childless people are indicators of the pain of childlessness. Although IVFET is a solution for only limited numbers of infertile couples, research that advances the practice of IVFET also has the potential of advancing other forms of infertility treatment.

Numerous infertility treatments exist, including education to give couples sufficient knowledge of reproductive biology, surgical repair, artificial insemination, and the use of drugs to induce ovulation. Two major new technologies are IVFET and gamete intrafallopian transfer (GIFT). These are complicated technologies. The simple description that follows will facilitate understanding of the research agenda developed by the committee.

For IVFET, eggs are removed from the woman either during a natural cycle or after growth and maturation of oocytes has been stimulated by such drugs as human menopausal gonadotrophin. This latter method has the advantage of allowing more than one oocyte to be harvested. The egg is placed in a petri dish together with washed sperm that have been treated to ensure capacitation. If fertilization is achieved, the process of cleavage starts, and somewhere between the 2- and 16-cell stage, the embryo is transferred to the uterus. Pregnancy is established when the developing embryo implants itself into the wall of the uterus. More than one embryo may be transferred to the uterus.

For GIFT, growth and retrieval of eggs are performed in a manner similar to that used for IVFET. Semen is collected and placed in a catheter with the eggs, and they are then transferred to the fallopian tube. Fertilization takes place in vivo.

Sometimes donated sperm, eggs, or fertilized zygotes are used in assisted conception. For example, excess zygotes collected from a patient undergoing IVFET can be fertilized and implanted in a recipient uterus that has been synchronized with the donor's cycle.

o Contraception. Advances in the basic science that would improve the clinical practice of assisted conception would, at the same time, help in the search for better contraceptive technologies. Despite widespread use of such contraceptive methods as sponges, surgical sterilization, intrauterine devices, and birth control pills, there remain unresolved

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problems of safety and efficacy. The search for improved forms of contraception is spurred not only by the desire of individuals to gain control over their reproductive lives, but also by the social cost of unwanted pregnancies and the problems caused by fast-growing populations in countries unable to provide an adequate standard of living for the present population.

o Agriculture. The application of assisted conception techniques has made rapid inroads in the domestic cattle industry. Artificial insemination is the norm, with 70 percent of dairy cows conceiving in this manner in 1985. In less than two decades, a multimillion dollar IVFET bovine industry has developed. About 25 percent of embryo transfers in 1984 were of frozen embryos. Artificial insemination has resulted in genetic improvements in dairy cattle that have doubled milk production per cow in thirty years (First, Crister, and Robl, 1985). Embryo transfer technology increases the rate of production of valuable cows. The adoption of new reproductive technologies to enhance the production of food-producing animals has the potential for lowering the cost of food and quickening the process by which animals genetically suited to difficult climates can be created.

o Biodiversity. Advances in reproductive technologies may sustain biodiversity by improving the reproductive efficiency of endangered species.

o Primates for Research. A limited number of primates are in captivity and available for research, and there is a possibility that the capture of more may be halted because of concerns for the future of the species. It will be increasingly important to maximize the reproductive capabilities of the primates available to science.

Barriers to Progress in In Vitro Fertilization and Embryo Transfer

Since the birth of Louise Brown in England in 1978, in vitro fertilization with embryo replacement has become an established method of treatment for certain types of infertility that do not respond to alternative methods of treatment. However, the chances of success in IVF are relatively low. In 1985, 14.1 percent of stimulation cycles resulted in clinical pregnancies. In 1986 this figure rose to 16.9 percent (Fertility and Sterility, 1988). But, the proportion of women entering treatment who attain a live birth is far lower -- only 8.9 percent of oocyte retrievals ended in live birth (Journal of the American Medical Association, 1988). Why are the odds for successful IVFET so low? The state of clinical practice of IVFET today is limited by lack of knowledge of some of the basic reproductive biology involved. This is caused, in part, by the many ethical questions raised by research in pursuit of the needed information. Difficulties in resolving these issues have caused the research to be deprived of federal funding.

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Ethical and Social Issues

Some of the ethical or social issues that arise from the various forms of assisted conception are unrelated to decisions about the progress of research. Examples of these are questions about the protection of the rights of gamete donors, gestational parents, and social parents; the ownership of cryopreserved embryos; and the sale of gametes and embryos.

Some ethical questions have a direct bearing on research, and have had important consequences for the funding of research. The major questions focus on the status of the embryo at each stage of its development. How the embryo is regarded dictates what is morally acceptable to do to it.

At one end of this spectrum of thought is the position taken by the Roman Catholic Church. The Vatican's Instruction on Respect for Human Life states that "from the first moment of its existence until birth . . . no moral distinction is considered between zygotes, pre-embryos, embryos or fetuses" (cited in Fertility and Sterility, 1988b). Therefore, the absolute sanctity that is accorded to post-natal human life begins with the zygote. This concept makes it impossible to discard spare embryos or use them for research purposes. At the other end of the spectrum is the position that an embryo is merely biological material like any other group of living cells. The special value that might be attached to that material results from the expectations or aspirations of others (Office of Technology Assessment, 1988).

Midway between these two positions is one that holds that "the human embryo is entitled to profound respect; but this respect does not necessarily encompass the full legal and moral rights attributed to a person" (Department of Health, Education, and Welfare, 1979). Holding this position, the Ethics Advisory Board (established by the Department of Health, Education and Welfare (DHEW) in 1979) concluded that research was acceptable on embryos up to 14 days after fertilization.

The Federal Government and Embryo Research

Policy concerning research on human subjects has been slowly evolving since the 1960s. A study group was convened at NIH to develop guidelines, and a National Advisory Commission on Health Science and Society was proposed by Senator Walter Mondale in 1968 to examine developments in medical research. Following reports of the infamous Tuskegee syphilis experiments, DHEW recommended that Congress establish a permanent body to regulate federally funded research using human subjects.

In the 1970s the abortion issue became linked to the issue of embryo research. After the Roe v. Wade decision legalized abortion under certain conditions, concern developed that women would be pressured into having abortions and the sale of aborted embryos might occur. In 1974, the federal government created the National Commission for the Protection of

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Human Subjects (P.L. 93-348). Until this commission reported to Congress, research on the living fetus was prohibited unless it was used to help that fetus survive. In 1975, DHEW issued regulations based on the findings of the commission. These regulations did not cover embryo research. The commission also recommended establishing an Ethics Advisory Board (EAB) to review requests for research on embryos and in vitro fertilization. However, in 1980, the Secretary of DHHS allowed the EAB charter to expire. Thus, no research could be approved, and federal funding of embryo research was de facto prohibited. As a result, embryo research has relied on private funding from patient care revenues, pharmaceutical companies, and university budgets.

Since 1985, efforts have been made that, if successful, might establish some rules under which embryo research could proceed. However, the chances of such an outcome in the near future appears to be slim. A Congressional Biomedical Ethics Board, composed of six senators and six representatives, has been appointed. This group established a Biomedical Ethics Advisory Committee. In 1988 the Department of Health and Human Services announced its intention to revive the Ethics Advisory Board and publish a proposed charter. A final charter is awaited.

Domestic and Foreign Decisions Concerning Embryo Research

The two professional societies in the United States that represent the physicians most involved in human IVFET have considered ethical questions about the practice of IVFET and embryo research. In 1986 the Committee on Ethics of the American College of Obstetricians and Gynecologists (ACOG) (1986) issued a statement that acknowledged the ethical issues posed by the creation of embryos outside a uterus, the dilemma of surplus embryos, and the acceptability of research using early human embryos. The ACOG committee recommended that human embryos should be used only if nonhuman embryos could not provide the needed knowledge. It also recommended banning research on embryos that had reached the age of 14 days. The American Fertility Society (AFS) also issued a report in 1986, approving experiments on embryos up to 14 days (Fertility and Sterility, 1986). A year later, after consideration of the Vatican's Instruction for Human Life in its Origin and on the Dignity of Procreation, issued by the Congregation for the Doctrine of Faith, the AFS issued another report. This report stated that progressive degrees of respect are due with progressive development of embryos, and that experimentation can be justified and is necessary if the human condition is to be improved (Fertility and Sterility, 1988b).

The government of the United States, since 1979, has not followed the lead of nations that have systematically examined issues related to human IVFET. Since 1979, at least 85 statements have been prepared by

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committees representing at least 25 countries. Four Australian committees found research on early (preimplantation) embryos to be ethically unacceptable. Eleven committees approved at least some kinds of early embryo research. Six of these accept such research only on embryos left over from clinical activities. Five committee statements (including the 1979 DHEW Ethics Advisory Board) would allow the creation of embryos for research purposes. Although the majority of committees favor limiting research on embryos to up to fourteen days, one committee allowed it only to seven days, and one only through the first cleavage (Walters, 1987).

In sum, numerous groups have wrestled with questions related to the ethical problems of embryo or fetal research. Some have based their conclusions on religious tenets, some on an interpretation of scientific knowledge, some on a mixture of both.

Other Barriers to Scientific Progress

Other factors besides ethical considerations are slowing the progress of research in areas of reproductive biology related to assisted conception.

Deficiencies in the Science Base Papers presented at the committee's workshop and the research agenda developed from that workshop indicate deficiencies in the scientific underpinnings of reproductive biology, and identify many areas in which further research efforts would make major contributions to improvements in medically assisted conception. The deficiencies are on three levels: basic science knowledge; knowledge needed to improve the technologies being used for medically assisted conception, such as cryobiology; and knowledge needed to improve both human and animal clinical practice of IVFET.

Research Funding Approximately $155 million annually is spend on research in reproductive processes. Federal agencies are the principal support for research. In 1986 they provided $109 million for research in reproductive processes (National Institutes of Health, undated). Federal funds for research relating to agricultural animal reproduction are available from the U.S. Department of Agriculture.

Funding for basic research in reproductive biology is undoubtedly constrained by the lack of vocal and focused advocacy groups. Lacking such a voice a major increase in federal support is unlikely.

Lack of Communication Among Researchers Discussion with the scientists and clinicians at the committee's workshop revealed an underuse of available mechanisms for communications among the individuals involved with various aspects of research in reproductive biology -- basic, clinical, animal sciences, etc. Also revealed was a desire for greater

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communication to allow cross-fertilization of ideas and development of ongoing relationships among investigators pursuing similar approaches to problems.

Sources of Research Material for Experiments with Humans and Other Primates The committee's workshop provided many excellent examples of instances in which information about reproductive physiology derived from animal models has been useful in understanding human physiology. However, animal models cannot suffice for investigating all central questions; progress is some areas requires the use of human tissue. An example of this is investigation of reasons for developmental failure of human embryos.

Although specific primates are good models for some aspects of human reproductive physiology, there are only a limited number of monkeys of desirable species in captivity and many of them are presently being used for AIDS research.

Research Agenda

A workshop was held August 21-23, 1988 at the Arnold and Mabel Beckman Center in Irvine, California. Overviews of the experience gained by the clinical practice of IVFET and of the practice of assisted conception in food-producing animals directed attention to unanswered questions that will require basic science research for their resolution. These questions reflect important gaps in our knowledge of the biology of all the stages of reproduction from the development of male and female gametes to the process of embryo implantation. The topics listed below are areas in which further research was recommended by workshop participants and committee members. Work in these areas is expected to increase understanding of the biology of reproduction with the hope that increased knowledge will eventually lead to improvements in the practice of IVFET in humans and other animals, or to advances in contraception. Research areas are listed here in summary form and apply both to lower animals and human beings unless specifically noted. The complete summary of the workshop is contained in Chapter Two of the full report.

Basic Science

Male Gametogenesis

- Definition of the role of cell adhesion molecules in interactions between Sertoli cells and developing sperm cells.

- Understanding the function of differential protein synthesis in different stages of sperm development.

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- Determination of the role of paracrine factors including fibroblast growth factor, somatomedin C, epidermal growth factor, and interleukin-1 on the development and differentiation of male gametes.

- Structural analysis to identify normal and abnormal sperm and the development of markers for abnormal sperm.

- Understanding of the biochemistry of sperm capacitation.

Female Gametogenesis

- Analysis of the effects of superovulation or hormonal stimulation protocols on oocyte development and maturation. This work should also examine differences between species.

- Development of ways to mature oocytes in vitro.

- Investigation of ways to naturally stimulate oocyte and follicular development.

- Investigation into the biochemistry of meiotic arrest and the factors, such as cyclic AMP, purines, calcium, and maturation-promoting factor, that may mediate this process.

- Development of ways to produce or synthesize hormones from non-human primates to be used in ovarian stimulation.

- Definition of the role of ovarian estrogen in oocyte maturation and ovulation and the interactions between estrogen and paracrine factors including fibroblast and epidermal growth factors, insulin-like growth factor, transforming growth factor, and inhibin.

- Definition of the point at which oocytes become sensitive to factors that influence their development.

- Elucidation of the processes that underlie oocyte depletion, to determine why oocytes are lost at a predictable rate throughout life.

- Investigation into ways to augment natural hormone release.

- Investigation into the biochemistry of protein synthesis and modification in ovarian cells.

Fertilization

- Investigation into the biophysics of cell membranes as it relates to sperm and egg interactions at fertilization.

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- Continued investigation to identify the genes for zona proteins in various species, especially humans.

- Further delineation of the role of zona proteins, especially ZP2 and ZP3, in sperm binding.

- Understanding of the biochemistry of the modification of zona proteins in preventing polyspermy.

- Elucidation of the molecular determinants of antibody formation to zona proteins and their possible role in contraceptive strategies.

- Definition of the biochemical mechanisms of the cortical reaction in the egg and the effects of this reaction on zona proteins.

- Determination of the physiological significance of germinal vesicle breakdown and the biochemistry of sperm chromatin decondensation.

- Definition of the molecular events associated with formation of the male and female pronuclei.

- Definition of the molecular events during zygote formation and the first cleavage.

Preimplantation Development

- Definition of the metabolic requirements of early embryos at different stages.

- Determination of embryonic gene expression.

- Assessing the potential of individual embryonic cells and defining the point at which embryonic cells are committed to particular fates.

- Identification of substances produced by early embryos that signal changes in the uterus prior to implantation.

- Improvements in embryo multiplication and embryo splitting, especially for food producing animals.

Implantation

- Definition of the biochemical events that make the uterus permissive to implantation.

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- Definition of the factors released by embryos that cause endometrial changes at the site of implantation.

- Identification of the role of embryo-released factors in suppressing the immune responses of the mother.

- Isolation and analysis of substances released by endometrial cells and their effects on embryos.

- Continued work with in vitro models of human implantation to study the biochemistry and mechanisms of embryo-endometrial interactions, especially the role of extracellular matrix proteins and the biochemistry of trophoblast invasion of the endometrium.

Technological Advances

- Improved cryopreservation techniques, including freezing and thawing protocols for eggs and embryos.

- Improved resolution of ultrasonography for localization and noninvasive harvest of oocytes, eggs, embryos--would have particular usefulness for non-human primates and food producing animals.

- Development of new culture media and methods for in vitro maturation of oocytes.

- Development of safe methods of biopsy of early embryos for preimplantation diagnosis of genetic diseases.

Clinical Research Opportunities

The following areas are those in which a coordinated data collection effort across IVFET clinical centers would help improve the quality and success rates of IVFET nationally and, possibly, internationally.

- Evaluation of hormonal stimulation protocols in terms of number of oocytes harvested, quality of oocytes, and rate of fertilization success.

- Documentation on the incidence of abnormal implantation rates in IVFET practice and correlation of incidence with particular stimulation protocol used.

- Collection of information regarding the incidence of abnormal zygotes and embryos, failed fertilization, and developmental arrest of embryos.

- Analysis of data pertaining to synchronization of embryonic stage with endometrial stage and development of methods to improve synchronization.

- Collection of information on sharing of spare eggs and arrested embryos for research purposes.

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Conclusions and Recommendations

Developing Research Policy

Lack of a mechanism for dealing with ethical disagreement over the use of embryos in research has slowed the rate of progress in research by, in effect, placing a moratorium on the use of federal funds for eight years. This has had undesirable results: the human clinical practice of IVFET is less effective than it might have been had research progressed at a faster pace; other socially desirable goals such as improved contraception, better techniques to preserve endangered species, and more cost-effective methods of producing food have developed at a pace slower than optimal.

The recent appointment of the Biomedical Advisory Committee by the Biomedical Ethics Board, to report to Congress by November 1990 on embryo research issues, could be a step toward a solution. The committee applauds the intention to revive the Ethics Advisory Board of the Department of Health and Human Services to rule on the ethical acceptability of research relating to human embryos, which is required before federal funding of such a research grant can be considered. However, until these groups become fully functional and show evidence of progress, their impact must remain in question.

If these groups can assume leadership roles in resolving the difficult issues of reproductive research, and develop guidelines for research that are based on information provided by science, and on concepts that are ethically acceptable to society, research in reproduction will be able to move forward. But if these groups become paralyzed because of political considerations or an inability to develop a framework for the resolution of differences of opinion, another organization should take over the role. The committee recommends that, if the groups currently being formed fail to come to conclusions concerning embryo and fetal research, a non-governmental organization should be established to develop guidelines for embryo and fetal research that are based on the most advanced knowledge that science can muster, and with serious consideration of the expressed values of society. The group should be composed of individuals with expertise in the relevant scientific disciplines, representatives of the lay public, and experts in the legal, ethical, and social issues. The organization should be housed in an institution that would allow it to conduct its deliberations free from any undue pressures from political and special interest groups. A model for such activities can be found in the Voluntary Licensing Authority of Great Britain.

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Basic Science Foundations

The number and range of topics included in the research agenda indicate the exciting potential for productive scientific exploration. The committee believes that fundamental research to enhance the basic science foundations of reproductive biology should be stimulated and supported. This includes studies of human beings, laboratory animal models, and food-producing animals. The knowledge that would be generated is fundamental to an understanding of how to reverse infertility, to new approaches in the area of contraception, and to increasing the world's food supply.

It is important that male as well as female reproductive biology be studied and that investigators make use of some opportunities that are largely ignored today. These opportunities occur as a result of clinical activities as well as research activities.

The committee recommends that a vigorous program of funding for a basic science agenda in reproductive biology be maintained in a coordinated fashion by an appropriate office in the National Institutes of Health.

Applied Research

Research needs to be stimulated concerning technologies used in medically assisted conception in food producing animals and in human beings. Lack of support in these areas is leading to inadequate scientific underpinnings for safe and effective clinical practice. An example of a technique used, but not carefully evaluated for possible detrimental effects, is freezing eggs or zygotes. Further experiments should be conducted to assess the effects on safety and viability of this technology which is standard practice in many IVFET clinics. Other areas of technology that need to be developed include less invasive ways to retrieve oocytes, ways to mature oocytes in vitro, and ways to assess the quality of spermatozoa or eggs to be used for fertilization.

The committee recommends that applied research into technologies used in medically-assisted conception be undertaken to provide a firm foundation for the safe and effective practice of in vitro fertilization and embryo transfer. Such applied research should be coordinated by the appropriate office at the National Institutes of Health.

Clinical Research Opportunities

Perhaps the most obvious missed opportunity is the failure to learn from the diverse experiences of the approximately 160 clinical programs that provide IVFET. In addition to scientific questions, there are

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questions to do with the organization of clinics and the outcomes of procedures, the answers to which would enable practitioners to work more effectively, and policy makers to make decisions on the basis of the best available information.

Clinical IVFET centers can provide unique opportunities for important studies. For example, human oocytes that fail to fertilize in vitro could be used to investigate the phenomenon of failed fertilization. Research that seeks to understand the basis of reproductive failure, and its relationship to hyperstimulation should be encouraged. Coordinated studies utilizing the mass of material and experience from IVFET centers could begin to answer these and other questions.

The committee applauds the activities of the various professional societies that have issued non-binding statements about the quality of practice of IVF. The American Fertility Society has also provided a voluntary registry for centers.

The committee believes that a mechanism is needed to monitor and evaluate clinical practice so that existing information that is relatively easy and inexpensive to collect can be disseminated. This would enable clinicians to build on the broadly based experience of the community and help ensure that patients have access to information about developments in IVFET and to well-informed physicians. The committee recommends that a mechanism for multi-centered data collection be established to monitor and evaluate human and veterinary practices of medically assisted conception in order to improve the safety, effectiveness, and quality of clinical practice. A cooperative group composed of the relevant professional societies should be established to fund and initiate data collection under the direction of an inter-society council composed of representatives of each participating organization.

Improving Communications

The IOM Workshop on Medically Assisted Conception brought together researchers from basic science, clinical practice, and animal sciences. The resulting interaction was viewed as extremely helpful by investigators from each of these communities. The committee recommends that a mechanism (or multiple mechanisms) be found for fostering continued communication between researchers in diverse areas of reproductive science. The initiative should come both from NIH research administrators who could sponsor additional workshop opportunities, as well as from the professional societies, either individually or through an intersociety council.

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CHAPTER 1

IN VITRO FERTILIZATION AND EMBRYO TRANSFER AND SOCIAL CONCERNS

The scientific advances that today permit clinicians and veterinarians to use such procedures as drug therapy, laser surgery, artificial insemination, in vitro fertilization, and embryo transfer to combat human infertility or improve the productive capabilities of valuable animals are the results of the work of generations of investigators. Sometimes these achievements came about because of research aimed at resolving specific problems related to human infertility or toward enabling livestock owners to improve the return on their investments. More often, however, scientific advances result from the inquiries of scientists investigating fundamental biological processes in the absence of specific applications. Thus, the work of investigators in areas such as physiology, morphology, endocrinology, molecular biology, developmental biology, and biochemistry have contributed to improvements in the treatment of infertility and to progress in assisted conception in humans and other animals.

Just as the findings of scientists pursuing answers to basic science questions often contribute in unexpected ways, the work of those pushing the frontiers of medically assisted conception has applications beyond the limited number of couples with infertility problems who can benefit directly. There are expectations that, in the future, the work being done to improve the practice of in vitro fertilization and embryo transfer (IVFET) will enable practitioners to identify genetic defects in embryos without damaging them, and to quickly determine the sex of embryos so that those with sex-linked genetic diseases can be identified at a very early stage. The stress of abortion later in a pregnancy could be avoided. This chapter briefly outlines some major areas to which advances in research in basic reproductive biology and IVFET would make large contributions.

Infertility

Infertility is defined in many ways. Most often the word is used to denote the inability of a woman to conceive after some months (12 to 24) of intercourse without contraception, or the inability to carry a pregnancy to term. According to a summary by the National Center for Health Statistics (NCHS), some 8.2 percent of women of childbearing age (4.4 million) suffered from "impaired fecundity" in 1982—the latest data available. This category includes over 800,000 women who said that it was impossible for them to have a baby because of accidents or other unexplained reasons. Also included are 2.9 million women defined as

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"subfecund" who said that it was physically difficult for them to conceive or deliver a baby, and 650,000 sexually active women who reported that they did not use contraception and did not become pregnant within 36 months. Defining as infertile couples those who were continuously married, had not used contraception, and had not conceived during the preceding 12 months, NCHS reported that 2.3 million women were infertile in 1982. This represents a decline from 11 percent of women in 1965 to 8 percent in 1982. However, problems of infertility are not diminishing for all sections of the population. The overall decline masks an increase in infertility among women under 30 years of age, and an increase from approximately 700,000 to nearly one million infertile couples with no children (National Center for Health Statistics, 1985).

Although these figures give a rough approximation of the fecundity of women in 1982, they do not indicate the number of men and women who want a child and are experiencing difficulty conceiving. More than half of the over 4.2 million women who have been surgically sterilized for non-contraceptive reasons, and half of the 4.4 million who have impaired fecundity, say they would like to become pregnant (Fuchs and Perreault, 1986).

The magnitude of the problem of human infertility is further reflected in data on medical care for infertility. Although not all infertile individuals seek treatment, by one estimate $1 billion was spent in 1980 on medical services for infertility treatment (Office of Technology Assessment, 1988b). In 1982, 1 million women between the ages of 15 and 44 who were or had been married reported at least one infertility visit during the past year (Fuchs and Perreault, 1986). A similar estimate is produced by a survey of primary care and reproductive care specialists in 1987 which indicated that they treated approximately 1.2 million patients for infertility (Office of Technology Assessment 1988a).

Infertility is not confined to problems experienced by women. By one estimate, about half of infertility problems are due in whole or in part to problems of the male (Fuchs and Perreault, 1986). Indeed, over 80 percent of the women seeking artificial insemination did so because of the infertility of their male partner, and approximately 65,000 children were conceived by artificial insemination during a 12-month period in 1986-1987 (Office of Technology Assessment, 1988a).

Although the emotional toll of infertility cannot be appreciated from these data, in recent years the communications media have begun to portray some of the distress of childlessness, and groups such as Resolve, begun in 1973, have responded to the emotional support needed by childless couples. Equally telling are the lengths to which individuals will go in attempting to conceive. The many months spent undergoing diagnostic procedures can be followed by additional efforts of drug therapy, surgery, and finally the physical, financial, and psychological stress of IVFET.

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IVFET is a possible solution to infertility for only a small number of couples. By one estimate, as much as 10 to 15 percent of infertile couples who could not be successfully treated by other means could be offered hope through IVFET or by a related technology, gamete intrafallopian transfer (GIFT) (Office of Technology Assessment, 1988b). Indications for use of IVFET are quite specific and include tubal disease that has not responded to other therapies, endometriosis, oligospermia, cervical mucus abnormalities and unexplained infertility. Indications for GIFT are more limited because it can only be used when fallopian tubes are normal. As knowledge expands, assisted conception of all sorts is expected to become applicable to a wider range of indications. IVFET can be applied regardless of whether fallopian tubes are present, therefore it is possible that IVFET will become a major therapy relative to the large number of individuals with infertility problems (Jones, 1989). However, research that advances the practice of IVFET will at the same time promote understanding of human reproduction and has the potential of advancing other forms of infertility treatment as well as providing better methods of contraception. As noted by a recent comprehensive study of infertility, "Even as infertility treatments become more sophisticated and complex, basic knowledge of the male and female reproductive process remains lacking. Further research stands as a prerequisite in order for dramatic improvements in infertility treatment to occur" (Office of Technology Assessment, 1988b).

Treatments for Infertility

Infertility can be treated in a variety of ways, including ensuring that the infertile couple know how to pinpoint the time of ovulation, eliminating causes of infertility such as infectious diseases or endometriosis, evaluating sperm seminal fluid, using fertility drugs to induce ovulation, performing surgical repair procedures in the male or female, and employing artificial insemination. Before assisted reproduction technologies are attempted a standard evaluation is conducted which includes hormonal evaluation, endometrial biopsy, hysterosalpingogram, diagnostic laparoscopy. Alternate therapy such as microsurgical corrections of tubal disease or endometriosis might be attempted. At least two noncoital reproductive technologies have been introduced in the last ten years. The major technologies are in vitro fertilization and embryo transfer (IVFET) and gamete intrafallopian transfer (GIFT). These technologies for establishing a pregnancy are reviewed in detail in a variety of recent publications (Office of Technology Assessment, 1988b; Seibel, 1988). To facilitate the understanding of the research agenda proposed in this report, a brief review of the steps utilized during IVFET, GIFT and some other methods of assisted conception follows.

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In Vitro Fertilization and Embryo Transfer

IVFET can be used to overcome infertility caused by numerous conditions including tubal disease, endometriosis and oligospermia. A first step in IVFET is to prepare the women for removal of eggs (oocytes). Two methods are used to accomplish this. Sometimes oocytes can be obtained during a natural cycle of a woman by determining the time of the marked increase in the luteinizing hormone level in the blood, which precedes ovulation by about 1 1/2 days. Using a natural cycle, however, frequent blood samples must be analyzed to exactly pinpoint the increase in this hormone level. Only one mature egg is usually obtained by this method. Alternatively, follicular growth and maturation, which leads to ovulation, can be induced by the use of various fertility drugs such as human menopausal gonadotrophin. The subsequent development of ovarian follicles can be monitored by ultrasound and by measuring blood estrogen levels. By this method, which is most commonly used today, more than one oocyte is stimulated to develop and can be obtained for fertilization.

Just before the timed ovulation would occur, oocytes are removed from the ovary either laparoscopy or by needle aspiration guided by ultrasonography. The eggs, with their adherent nurse cells, are placed in a petri dish so that their state of maturation can be assessed using the state of dispersion of the attached cells as a marker. Fertilization of the mature egg is accomplished by incubation for approximately 24 hours in the petri dish with washed sperm that have been treated to ensure capacitation. Fertilization is defined by the visible presence of two pronuclei in the newly formed zygote.

The first cleavage of the zygote occurs approximately 1 1/2 days after insemination. A catheter is used to transfer the dividing embryo into the lumen of the uterus at somewhere between the 2- and 16-cell stage. To supplement the natural luteal phase, hormones such as progesterone are sometimes administered after transfer of the embryo, (or embryos if more than one oocyte has been fertilized) to the uterus. Pregnancy is established when the developing embryo implants itself into the wall of the uterus. Implantation can be documented by a measured increase in blood levels of human chorionic gonadotrophin.

Sometimes, a greater number of mature eggs are harvested than can usefully be implanted. Increasingly, those excess eggs are fertilized and preserved by cryopreservation for subsequent use.

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Gamete Intrafallopian Transfer

In 1985, Asch et al. (1985) reported on gamete intrafallopian transfer (GIFT) as a new treatment for infertility. In 1987, the Lancet noted that GIFT had been readily accepted in to clinical practice (Lancet, 1987). GIFT involves the transfer of eggs and sperm into patent fallopian tubes so that fertilization may take place in vivo. Follicular growth of oocytes and retrieval are performed in a manner similar to that used for IVFET. Semen is collected and placed in a catheter with the eggs, which are then transferred to the fallopian tubes. In 1987, GIFT was achieving a higher success rate than IVFET. Although this might have been due to the better conditions of in vivo fertilization compared to in vitro, it may also have been due to patient selection. GIFT requires that at least one fallopian tube be patent and that a sufficient number of normal sperm can be obtained (Lancet, 1987). By 1987 there was a report of successful use of GIFT with donated oocytes (Craft et al. 1987). Gift can be used when infertility is caused by such factors as endometriosis, premature ovarian failure, oligospermia, and unexplained infertility (Office of Technology Assessment, 1988b).

Donated Gametes or Concepti

Sometimes the donation of spermatozoa, eggs, or in some cases fertilized zygotes, are necessary. Excess eggs collected from one female donor patient undergoing IVFET can be fertilized and implanted in a recipient uterus which has been synchronized with the donor's cycle. Artificial insemination using donor spermatozoa is a common technique. The results of one survey indicate that each year about 30,000 babies are born from artificial insemination using donor spermatoza (Office of Technology Assessment, 1988a).

Two less frequently used methods of treatment for infertility that also involve the manipulation of eggs or embryos are tubal ovum transfer and embryo lavage and transfer. Egg stimulation and harvesting are undertaken as in IVFET and GIFT. The egg is than reinserted below fallopian tube blockage or other damage and fertilization takes place in vivo (Office of Technology Assessment, 1988b).

In embryo lavage and transfer fertilized eggs are flushed out and removed by a special catheter. They are then transferred to a recipient whose cycle has been synchronized to be ready for the introduced egg. This technique is becoming less frequently used, partly because of fears about transmission of virus and the risk of retained embryos resulting in pregnancy in the donor.

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Contraception

Advances in the basic science that would improve the clinical practice of assisted conception, such as improved understanding of the mechanics of egg implantation, would be likely, at the same time, to help in the search for better contraceptive technologies.

Contraceptive methods range from rhythm methods, the contraceptive sponge, birth control pills, and intrauterine devices to surgical sterilization and barrier methods, including condoms, spermicides, cervical caps, and diaphragms. More than half of American women aged 15 to 44 years used some form of contraception, at a cost of $2.4 billion in 1982 (Fuchs and Perreault, 1986). Despite this widespread use of contraception, there remain unresolved problems of safety, efficacy, and acceptability for each type of contraceptive.

The search for improved forms of contraception is spurred not only by the desire of individuals for control over their reproductive lives, but also by the social costs of unwanted pregnancies and the pressures of fast-growing populations in countries whose economies are unable to provide an adequate standard of living for their present population. Countries have several options of how to cut their rate of population growth, according to a study by the Office of Technology Assessment (1988c), but the only solution that is both morally tenable and feasible is to lower fertility rates. Contraceptive use is by far the most important means of attaining that goal.

Agriculture

In 1890, Walter Heape wrote the first paper on transfer of a fertilized egg, stating, "In this preliminary note I wish merely to record an experiment by which it is shown that it is possible to make use of the uterus of one variety of rabbit as a medium for the growth and complete fetal development of fertilized ova of another variety of rabbit." (Heape cited in Adams, 1982). It was not until 1932 that a successful transfer in a larger animal, a goat, was reported. Only after the second World War was the potential for application of the techniques for livestock improvement and production realized (Adams, 1982). In 1981, it was reported in Science that a "multimillion dollar industry centered on recovery, in vitro culture, and transfer of bovine embryos has evolved over the last decade." This fast growing ectivity had developed in less than a decade into a $20 million a year industry (Seidel, 1981). By 1985, assisted conception was the norm in dairy cows, with 70 percent fertilized by artificial insemination; 100,000 embryo transfers were performed in the United States in 1984, and 200,000 worldwide, of which 25 percent were with frozen embryos. Artificial insemination resulted in genetic

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improvement in dairy cattle to an extent that milk production per cow doubled in 30 years (First, Critser, and Robl, 1985). The major use of embryo transfer technology in the food-producing industry is to increase the rate of reproduction of valuable cows. The techniques of superovulation, recovery of embryos, storage in vitro, and transfer to a recipient cow enable some cows to be the egg donors for 50 calves in a year. IVFET can also be used to enable infertile but genetically sound cows to reproduce. The new technologies make the export of cattle breeding stock more economical because it is cheaper to transport embryos (frozen or unfrozen) than mature animals. Moreover, the resulting calves have immunity to local pathogens (received via the foster mother's colostrum), which imported animals lack (Seidel 1981). In 1988 the possibility arose of further improvements in the reproductive efficiency of food-producing animals; the Granada Corporation claimed that techniques for cloning animals were nearing commercial application (Schneider, 1988).

The development of procedures for the control of reproduction in domestic animals has come from universities, nonprofit research institutions, and commercial organizations. Growth of commercial interest has been recent and rapid. In 1986, more than 115 commercial companies and 100 veterinary practices offered embryo transfer services. Some of these commercial organizations also contribute to the research effort by establishing research laboratories (Dresser and Leibo, 1986).

The adoption of the new reproductive technologies to enhance the production of food-producing animals has potential for lowering the cost of food and for increasing the speed with which animals genetically suited to difficult climates can be created. The impact of artificial insemination on the productivity of cows has already been seen. There is reason to expect that further advances in reproductive technologies could improve production of other food animals.

Biodiversity

Advances in reproductive technologies are potentially important in sustaining biodiversity by improving the reproductive efficiency of endangered species. The new reproductive technologies are being studied by zoo researchers interested in conservation of species. These researchers see IVFET and artificial insemination as a way of improving the reproductive processes of endangered species. Breeding of animals is a new role for zoos, which have in the past regarded themselves mainly as a place to display animals. However, as zoos become the last repository for some endangered species, and as genetic diversity is lost because of inbreeding, maintaining diversity has become an important goal. Thus, the development of reproductive technologies that can be used for endangered exotic species takes on a new urgency as it is increasingly realized that captive breeding programs can prevent extinction (Dresser, 1988).

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Primates for Research

The ongoing battle between wildlife preservationists and scientists who use primates for research purposes is a final illustration of the far reaching implications of developments in reproductive technologies.

The U.S. Fish and Wildlife service has been asked to put chimpanzees on the endangered species list. Some of those making this request believe that the use of chimpanzees in biomedical research is one cause of their endangerment. This assertion is disputed by officials at the National Institutes of Health. If the chimpanzee is declared endangered, new prohibitions on capture, transport, and use of this species will be imposed. The question remains whether, with such restrictions, the 950 chimpanzees in government facilities at the present time are enough to meet the needs of biomedical research, especially in light of their important role in AIDS research (Science, 1988).

It is here that the potential of new reproductive technologies might play a role. With a limited number of available animals it will become increasingly important to maximize the reproductive capabilities of the 350 chimpanzees that have been set aside for breeding in government facilities.

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CHAPTER 2

ADVANCES IN THE PRACTICE AND SCIENCE BASE OF MEDICALLY ASSISTED CONCEPTION

This chapter is based on papers presented at a workshop on Basic Science Foundations of Medically Assisted Conception sponsored by the Institute of Medicine (IOM) and the Board on Agriculture of the National Research Council. It was held August 21-23, 1988 at the Arnold and Mabel Beckman Center in Irvine, California. For the workshop, the organizing committee developed a program that explored the recent advances in reproductive and developmental biology that apply to medically assisted conception. This chapter represents a report of science topics selected by the committee. It is therefore not a comprehensive review of recent advances in reproductive research. The workshop also initiated an interchange of ideas among those involved in patient-related clinical practice, animal IVFET, and those working in basic research as it applies to humans and other animals. Thus, this chapter indicates research areas that promise improvements in the practice of IVFET. First are some developments in human and animal IVFET; subsequently, the processes and recent advances relating to gametogenesis, fertilization, preimplantation development, and implantation are discussed. This chapter summarizes each talk given at the workshop. The full papers contributed by each author are found in Appendix A.

Developments in Human In Vitro Fertilization [1]

Eastern Virginia Medical School, one of the premier centers for the clinical practice of in vitro fertilization, has a pregnancy rate of 18.3 percent, based on the number of attempts used to stimulate the ovaries with either follicle-stimulating hormone (FSH) or human menopausal gonadotropin (hMG), and a pregnancy rate of 37.6 percent based on the number of patients. Both patient age and the cause of female infertility have an effect on the outcome of in vitro fertilization. For women infertile because of tubal ligations or endometriosis, the rate of viable pregnancies obtained by IVFET at Eastern Virginia is 16.4 percent. Although the pregnancy rate did not vary according to whether infertility was due to tubal ligation or endometriosis, age has an effect. After the age of 40, it becomes more difficult to stimulate the ovaries to produce mature eggs.

Because couples often seek in vitro fertilization to overcome male infertility, the evaluation of semen is important. Currently, this evaluation is descriptive and relatively imprecise. Factors such as number of sperm, sperm motility, and general shape of sperm components(e.g. head shape and tail shape) are important variables in achieving pregnancy. For example, if 14 percent of the sperm are "normal," and there are 50,000 sperm per cubic centimeter of ejaculate, in

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vitro fertilization produces ongoing pregnancies in 30 percent of all attempts. However, even if there are 100,000 sperm per cubic centimeter, but only 4 percent are "normal," the ongoing pregnancy rate drops greatly. Abnormal sperm often fail to fertilize an egg because of their inability to penetrate the egg's protective covering, the zona pellucida, therefore workers have tried a technique known as "zona drilling" in which holes are produced in the zona pellucida to permit direct access of sperm to the oocyte plasma membrane. Thus far, this technique has not been successful. Another technique, known as zona splitting, has been used at Emory University, Atlanta. By this technique, the zona pellucida is split mechanically. Pregnancies have been reported by use of this method. Basic science questions need to be answered in order to develop better markers for normal and abnormal sperm and to improve the performance of sperm in IVFET.

Cryopreservation (freezing) of in vitro fertilized embryos[1] is another new and promising technique that will be discussed in greater detail later in this chapter. If embryos are frozen for later use, the stage of their development at the time they are placed in the uterus can be matched with the stage of the uterine wall (endometrium), increasing the likelihood of a successful pregnancy. Before cryopreservation became an option, embryos had either to be placed in the uterus or discarded. In order to avoid the ethical dilemma of what to do with excess embryos, more than the optimal number of embryos were sometimes transferred into the uterus. By allowing the preservation of embryos for later use, the technique of cryopreservation reduces not only the chance of multiple pregnancies, but also the number of times a woman's ovaries might be subjected to hormonal stimulation to produce oocytes for additional attempts of IVFET. The use of cryopreservation has resulted in increased rate of pregnancy at the Eastern Virginia clinic. Nevertheless, basic science research is needed to assess the necessary parameters for successful cryopreservation and the possible deleterious effects of freezing on the embryo.

In cases of ovarian failure, failure of in vitro fertilization, poor quality of eggs, genetic abnormality, or inaccessible ovaries, the only option available to women wanting to bear a child is to use eggs from a donor to perform in vitro fertilization with the husband's sperm. The donated eggs most often come from IVFET patients who have received hormonal stimulation and produced more eggs than necessary for their own use. Sometimes, however, women who are to undergo a tubal ligation agree to ovarian stimulation before the surgery so that eggs can be harvested simultaneously. These eggs are then donated for in vitro fertilization and transfer. Donors are phenotypically matched with recipients and are screened for psychological problems and infectious diseases. Patients who

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[1] The term embryo in this chapter often refers to in vitro fertilized eggs that developed to two- to four-cell staged embryos. Although there are more technically precise terms for various early stages of development, the more precise terms are used here only when the distinctions are important to the concepts under discussion.

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http://www.nap.edu/catalog.php?record_id=18586

conceive through egg donation may need supplemental hormone therapy to replace the estrogen and progesterone normally released from the ovaries. In such a patient, however, the placenta produces detectable estrogen by week 6 of the pregnancy and progesterone by week 7. Fertilized eggs from oocyte donation, if transferred to the uterus by day 17-19 of the recipient's cycle, resulted in a 36 percent pregnancy rate and 80 percent of those pregnancies going to term. One benefit of oocyte donation, in addition to overcoming the problems mentioned above, is that a time period of optimum implantation can be achieved such that the stage of the embryo and endometrium are synchronized. This technique has been found to increase the success rate of IVFET.

Good quality embryos are critically important to the success of IVFET. Experience at many clinics shows that a number of factors determine the quality of an embryo. Some important factors include the way ovaries are stimulated with hormone supplements and the synchrony between the age of the embryo and the endometrial stage. More research is needed to understand fully those factors and to establish unambiguous and unbiased criteria with which to distinguish poor quality and good quality embryos.

Because fertilization is more likely with mature oocytes than immature ones, research to learn how to recruit a more synchronous population of follicles is important. Such follicles produce a more synchronous population of oocytes capable of responding to the maturational stimuli. Failing the recruitment of mature oocytes, research is needed to develop reliable methods of maturing oocytes in vitro. All of these areas, if improved by increased knowledge of the cell biology of early reproductive events, should greatly increase the ability to identify couples with high probability of success and may increase the success rates of IVFET in human clinical practice.

Developments in Assisted Conception in Food-Producing Animals [2]

There are critical differences between the goals of assisted conception in animals and human beings. In human beings, the goal is to increase the reproductive ability of those with impaired fertility or, perhaps in the future, to avoid the transmittal of genetic disorders. In contrast, the goal of assisted conception in food-producing animals often is to increase the yield of milk or meat. Because the ethical barriers are lower and the financial stakes higher, a number of advanced technologies are available for use in animals that are not available in the clinical practice of human IVFET. Techniques in commercial or research use include artificial insemination, superovulation, embryo transfer, freezing of embryos, sexing of embryos, multiplication of embryos by bisection and cloning, in vitro fertilization, and the modification of embryos by gene transfer. All western European dairy cows and 60 percent of U.S. dairy cows are impregnated by artificial insemination (AI). AI allows bulls, selected for their transmission of milk producing genes, to achieve up to 500 inseminations from one ejaculate. Thirty years of AI has helped to double the milk production of each cow.

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Another technique used widely in humans as well as in other animals is superovulation, which involves ovarian stimulation by the administration of hormone supplements. In both animals and humans, superovulation is unpredictable and often unsuccessful. There has been some success when cattle are given priming doses of follicle stimulating hormone (FSH), and when highly purified forms of FSH are used, but the reasons for those effects are unclear. In addition, studies of cattle and other species have found that the oocytes produced by superovulation sometimes exhibit abnormal characteristics. Increased understanding of hormonal cycles and the effects of hormones on the cell biology of maturing oocytes would likely have direct application to the solution of some of these problems.

The process of embryo transfer in cattle starts with superovulation of a cow, and subsequent mating to a desirable bull. The resultant embryos are collected nonsurgically and transferred to another cow whose estrous cycle has been synchronized with that of the donor cow. Although the success rate with embryo transfer is respectable (about 60 percent of transfers result in pregnancy), the technique has not produced great increases in milk or meat production. The production of many identical copies of embryos from cattle with highly desirable traits may improve this situation. For this reason, embryo freezing and embryo multiplication procedures are receiving a great deal of attention in the commercial breeding of cattle.

Embryo freezing, in concert with embryo transfer, has been generally successful in terms of pregnancy rates. Freezing embryos allows for the storage of rare breeds and preservation of a cattle surplus. However, embryo multiplication has greater potential for the production of large numbers of highly desirable cattle. Two methods of embryo multiplication are used—embryo bisection and nuclear transfer. Embryo bisection is performed at a very early embryonic stage and yields at least two cell masses which are genetically identical. The bisected embryos can then be transferred by normal procedures to a recipient cow. There is a species-specific limit on how many bisections of a given cell mass can be done without compromising viability; with cattle, the maximum yield is four embryos from one. In nuclear transfer, a blastomere (embryonic cell), or its nucleus, from a valuable embryo is placed into an oocyte from which the nucleus has been removed. The transferred embryo nucleus promotes development of a multicellular mass that can be used to make a number of copies or clones. There is a great deal of interest in this method both in academic and commercial research. Since those techniques allow more precise selection of desirable traits, both have the potential to effect rapid changes in the prevalence of animals with those traits.

While the techniques discussed above are frequently and successfully used, other methods of assisted reproduction are also being investigated. One of the newer approaches is embryo sexing. Sexing of embryos is of particular importance to the dairy industry since only female offspring are needed for milk production. Sexing is done by three methods. The

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first is karyotyping of embryos by bisecting them and using one half for cytogenetic analysis. The chief disadvantages of this method are the damage that often occurs to the embryo and the variable reliability of the karyotypic identification of sex. The second method employs antibodies to male-specific antigens on the embryos to identify the male embryos. The accuracy of this immunological method is greater than that of karyotyping and may become widely used, depending on the availability of antisera. Finally, DNA hybridization techniques have been adapted from molecular biology to sex embryos by labeling specific parts of the male Y chromosome obtained by embryo biopsy.

There are compelling reasons to develop more fully IVFET procedures in food-producing animals. Such techniques provide a large number of embryos for transfer, research, and embryo multiplication. However, as in human IVFET, the success of in vitro fertilization and embryo transfer in the cattle industry suffers from a lack of basic knowledge of the cell biology and biochemistry of early gamete maturation and fertilization. Among the areas in which research is lacking is oocyte maturation. As in humans, IVFET in cattle is more successful if it begins with mature rather than immature oocytes. The biochemistry of sperm capacitation and entry into the eggs also needs to be better understood. A number of chemicals have been identified that are necessary for those processes, but precise identification of the role each chemical plays in fertilization is needed for further progress. Finally, being able to maintain the growth of an embryo in culture for a longer period than is now possible would be helpful. Such an ability would increase the chances of a favorable match between the embryonic and endometrial stages.

Gene transfer, if developed further, also shows potential for enhancing the productivity of cattle. With the development of techniques by which genes can be microinjected into embryos, the ability to alter the phenotypic characteristics of food-producing animals has become a possibility. In an early demonstration of transgenic technology, workers injected mouse eggs with the gene for human growth hormone. The resulting mice grew to almost twice the size of normal mice. Such technology could be used in cattle to alter the genes for skeletal muscle in order to produce higher quality meat products, or to alter genes in such a way that biologically active substances would be secreted in milk.

There are many areas in which the results of basic research would further the practice of assisted conception and embryo transfer in both human beings and other animals. These include oocyte maturation, sperm morphology, the biochemistry of fertilization, cryopreservation, ovarian stimulation, and molecular genetics. In addition, topics such as membrane biochemistry, hormonal control of testicular and ovarian function, gene expression in early development, and the cell biology of implantation are identified as areas of exploration that would make major contributions to the success of human and animal IVFET.

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http://www.nap.edu/catalog.php?record_id=18586

Induction of Gametogenesis and Superovulation [3]

Comparative research on ovarian stimulation of non-human primates and domestic animals such as cattle, horses, sheep, and pigs raises interesting issues. Some work highlights the differences between the normal estrous cycles of animals such as cattle, and the cycles of human and non-human primates. Yet there are problems that are common among species in the stimulation of follicular development and the induction of mature oocytes. Ovarian stimulation and superovulation are exogenous interventions into a highly regulated physiological process. The normal physiology is not always easily manipulated. Developing strategies to circumvent the differences between normal and induced ovulation is a major goal of research proposed for this area.

Non-Human Primates Non-human primates exhibit a menstrual cycle that closely approximates that of humans. In monkeys, the hormonal events of the cycle include a geometric increase in serum estradiol, midcycle surges of luteinizing hormone (LH), follicle stimulating hormone (FSH), and a later increase in serum progesterone after LH and FSH decrease in concentration. These hormone levels are precisely synchronized in the normal cycle and lead to the development of one follicle, the dominant follicle, from which ovulation occurs. One of the purposes of superovulation is to encourage more than one follicle to develop fully, thereby producing multiple oocytes for in vitro fertilization. In monkeys, one of four chemicals is administered in combination with human chorionic gonadotropin to stimulate ovulation. These are human menopausal gonadotropin, human FSH, pregnant mare's serum gonadotropin, or porcine FSH. However, monkeys sometimes produce antibodies that block the activity of hormones obtained from other species activity, and ovarian stimulation fails. Thus, there is a need to develop stores of monkey hormones to be used for ovarian stimulation in this species. The response to the administration of hormones to stimulate ovulation is also not uniform in non-human primates. In general, estradiol levels are higher than in a natural cycle, the LH surge is lacking, LH concentration remains high over a long period, FSH is low, and progesterone remains high in concentration. However, in some subjects there is a premature, spontaneous LH surge that is not synchronized with follicular maturation; this surge stops the follicles and oocytes from maturing. Even if such an LH surge does not occur, there is a wide range of responses to gonadotropin administration. It is impossible to predict the response of any individual animal. The LH surge and the heterogenous responses, however, can often be prevented by additional administration of gonadotropin releasing hormone. Another difficulty often observed with superovulation protocols is an overstimulation of prolactin secretion. These departures from the normal hormonal levels raise important questions relating to the efficacy and unknown effects of ovarian stimulation.

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Despite the problems of ovarian stimulation in non-human primates, reasonable success rates have been achieved. Nevertheless, there are additional barriers to progress in this field. The supply of non-human primates is severely limited. In addition, these animals often cannot be used more than once for ovarian stimulation because of the immunological responses to human, equine, and porcine gonadotropins used in the stimulation procedures. Finally, eggs must be recovered from monkeys by either laparotomy or laparoscopy, surgical techniques that are restricted by ethical considerations and formal legal constraints to a low number of repetitions per animal. Although these problems of availability, reuse, and the lack of non-human gonadotropins are more difficult to overcome than the biological problems of overriding normal physiology, numerous questions about the basic biological processes remain unanswered. Resolution of the problems associated with the use of non-human primates in this type of research could be facilitated by an increased ability to augment natural hormone and gonadotropin release, by improved resulution of ultrasound imaging to identify matura oocytes and guide their collection by non-surgical means, and by the development of cell lines that could produce larger quantities of non-human primate gonadotropins. Finally, it is important to note that many of the same questions remain unanswered regarding human ovarian stimulation and that particular non-human primate species serve as the best model for human reproductive physiology.

Domestic Animals Extensive use of artificial insemination in cattle has increased the genetic contribution of desirable bulls to the overall supply of cattle. However, there is little likelihood that the desirable characteristics of females will be further increased by augmenting the male genetic contributions. Superovulation, on the other hand, combined with embryo freezing and embryo transfer, has the potential to increase directly the gene pool of desirable female traits by increasing the number of offspring from a valuable cow. There are a number of differences between non-human primates and domestic animals in the ways ovarian stimulation is accomplished. Some of these differences arise from differences in the reproductive cycles of the two groups. In dairy and beef cattle, stimulation is accomplished with the administration of either pregnant mare's serum gonadotropin (PMSG) or porcine FSH. The latter is preferred to PMSG because PMSG has a long half-life and often results in asynchronous ovulations. Porcine FSH is not without problems either since it is contaminated by variably high levels of luteinizing hormone. Human chorionic gonadotropin, routinely used in non-human primates, is not used in the commercial practice of ovarian stimulation of cattle. To control the variability and lack of predictability in the estrous cycles of cattle, current protocols often include an injection of a specific type of prostaglandin (PGF-2 alpha). This treatment causes the regression of the corpus luteum, thereby artificially restarting the cycle.

The success rate of superovulation in cattle has been variable. This is caused by a number of factors including seasonal variability, breed differences, dose and timing of gonadotropin administration, and history

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of previous superovulations. Such differences contribute to difficulty in comparing studies performed on different breeds and, at times, in comparing studies in the same breeds, but done with varied techniques or in animals of varying age. It has been found that, even if all the factors possible are controlled, there remains a great deal of variability in the success rate of superovulation. A number of strategies to improve the success rate are currently being tried. One is an attempt to purify porcine FSH preparations to exclude the contaminating LH and, further, to determine the FSH to LH ratios that are optimum for successful superovulation. Another strategy is to try to neutralize PMSG with antibodies so that the complications that arise from the long half-life of PMSG can be prevented. Because the lack of normal coordination of endocrine events is a common occurrence in superovulation, investigators are also trying to define the sources of asynchrony and develop ways of normalizing a coordinated series of biological events.

Superovulation in domestic animals other than cattle also has had mixed results. In goats, the technique has been tremendously successful. Superovulation of sheep has also been largely successful, and a few innovative strategies have been applied in this species. Sheep are seasonal breeders and attempts have been made, with initial success, to induce ovulation in this species during times when they are normally anestrous. In order to bypass the use of exogenous gonadotropine, workers have employed antibodies to inhibin in sheep. Inhibin is a substance made in an ovarian follicle that suppresses FSH secretion. The blocking of inhibin with antibodies raises FSH levels, thereby increasing the natural stimulus for follicular development. Antibodies against steroids have also been tried in sheep to achieve the same end.

In horses, superovulation has proven to be difficult, and to require nonstandard protocols. The period of estrous or "heat" in mares is unusually long and variable. In addition, there are preprogrammed physiological mechanisms in mares to prevent the development of twins. These two aspects of equine reproductive physiology greatly complicate the successful application of ovarian stimulation techniques. Also of critical importance is the fact that PMSG, even in high doses, does not adequately stimulate the development of follicles in mares. Unlike other animals, where PMSG acts as both FSH and LH receptors, in mares PMSG has only LH activity. Some success has been achieved in mares with administration of porcine FSH, especially if combined with human chorionic gonadotropin. In general, however, progress in successful superovulation in horses lags well behind that in other domestic animals, except for the pig.

The most limited of all commercial applications of superovulation in animals has been in the pig. Although possible, the technique does not confer many of the advantages that it holds for other species. One reason is that pigs deliver litters as opposed to one offspring at a time. Therefore, the need to increase the offspring from one individual, while helpful in certain circumstances, is not as compelling in pigs as in other domestic animals. Nevertheless, superovulation and embryo transfer may

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be able to lower the frequency of diseases by increasing the number of offspring from disease-free or disease-resistant populations. More frequent reasons for the use of embryo transfer in pigs are to obtain disease-free embryos from infected pigs[2] and to introduce new genetic material into specific pathogen-free herds. Thus, further research into the normal reproductive physiology of pigs will be useful.

To improve IVFET in domestic animals as well as in human and non-human primates, more needs to be known about the normal regulatory events of ovulation, including the physiology and biochemistry of the development of a dominant follicle. Improved ultrasonography has potential for increasing the success of superovulation in all species. As with monkeys, developing ways to bypass the use of exogenous agents like PMSG and porcine FSH would be of enormous use in domestic animals. One way of doing this may be by blocking inhibin as has been done in sheep. In order to improve the success rate of assisted conception in animals, many of these research areas must, nevertheless, be pursued in each species separately with realization of the inter-species differences. It is not always possible to predict when the results obtained from one species can contribute knowledge applicable to other species.

Biology and Maturation [4]

This section examines three topics relating to the biology and maturation of oocytes: 1) The study of membrane biochemistry, which has relevance to the complex membrane interactions that occur at fertilization; 2) the study of molecules that are important in maturation events, is also important to understanding the possible reasons for failure in IVFET; and 3) the biological and physical properties of eggs and embryos that are affected by preservation techniques such as freezing, known as cryopreservation.

Membrane Biochemistry The membranes of all cells of the body share certain standard structural characteristics. The basis of a cell membrane is called a bilayer, which is a two-layered collection of phospholipid molecules (lipids containing phosphorus). Phospholipid molecules are polar in that each has one end that is stable in water (hydrophilic), and one end that repels water (hydrophobic). In a bilayer, than, the lipid molecules lins up so that the hydrophobic ends meat each other on the inside of the bilayer. The hydrophilic ends are thus oriented on the outside of the bilayer such that one group faces the intracellular space and the other faces the extracellular space. Such an arrangement makes sense when one considers that both the cytoplasm of a cell and the extracellular spaces are composed largely of water. It is, in fact, a quite natural response of lipids to form bilayers in an aqueous environment; such a process underlies soap bubble and oil droplet formations.

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[2] Embryos can be rinsed free of pathogens or treated with enzymes to destroy pathogens making embryos the safest method of moving germ plasm without pathogens around the world.

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Proteins are part of the membrane also. Some proteins span the thickness of the membrane and act as hydrophilic channels for ions or participate in molecular signal transduction mechanisms. Other proteins are partly embedded in the lipid bilayer and partly exposed to the extracellular space. Attached to such proteins are long, often branching, chains of carbohydrate molecules that also extend into the extracellular space. Finally, large extracellular proteins often associate with or stick to the most external parts of the carbohydrate molecules. Thus, the cell membrane, even in its standard form, is a complex structure made up of different types of molecules arranged in regions that each exhibit special properties. The possible interactions of membrane molecules are immense since the various molecules are mobile within the bilayer. These interactions underly the equally immense range of biological responses of cells including cell-cell interactions, receptor-mediated signal processing, and transmembrane transport of nutrients or toxins.

Reproductive events in which membrane biochemistry is of particular importance include sperm capacitation and fusion of sperm and egg membranes for fertilization. Basic aspects of membrane biochemistry are most often studied using membranes from cells such as red blood cells or membranes manufactured in the laboratory, called liposomes. Such membranes are simpler than the membranes from eggs or sperm and, therefore, easier to study. However, data obtained from these model membranes must be extrapolated to eggs and sperm with great caution.

Mammalian Oocyte Maturation In a normal follicle there is communication between the oocytes and the surrounding nurse cells, called granulosa cells, through specialized junctions (gap junctions) through which small molecules pass from one cell to another. Developing oocytes undergo a type of cell division in which only half the normal number of chromosomes are retained. This process is known as meiosis and has a number of stages. Until just before maturation, however, the growing oocytes remain in a state of suspended or arrested meiosis. It is thought that the granulosa cells help to maintain this arrest of meiosis by producing substances that enter the oocyte through the gap junctions. It is also possible that substances that induce final maturation of the oocytes are produced in the surrounding cells and are transmitted to the oocytes in the same manner. Since the ability to mature oocytes in vitro would be of enormous value in IVFET, the understanding of the substances that either maintain or abolish meiotic arrest is particularly desirable. It is important to note that many of the studies summarized in this section were done with mouse oocytes and these data were compared to these obtained from other mammals including rabbits and rats. Investigators have also studied frog oocytes, but the mouse is the most common animal model. Eventually, of course, certain critical experiments will have to be replicated using oocytes from human or non-human primates.

Several substances are thought to maintain meiotic arrest. One, cyclic adenosine monophosphate (cyclic AMP), is a molecule present ubiquitously in the body. Another, hypoxanthine, is common in the body

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and belongs to a group of molecules known as purines. Guanosine, another purine, is also a potent inhibitor of meiosis and the purine, adenosine, has been shown to augment the activity of hypoxanthine. By examining the effects of some of these substances on each other, a hypothesis has arisen which suggests that hypoxanthine and other purines may increase the level of cyclic AMP, which then inhibits meiosis. Future research, focused upon the molecules of the cyclic AMP pathway inhibiting meiosis, may elucidate ways in which oocytes can be maintained in meiotic arrest in vitro.

While knowledge regarding the inhibition of oocyte maturation is important, knowing how to mature oocytes in culture would have many applications that are described later. In vivo, oocyte maturation is produced by the surge of LH immediately prior to ovulation. The mechanism of action of LH is unclear, however. One might assume that, if cyclic AMP maintains meiotic arrest, then LH somehow causes a decrease in cyclic AMP in the oocyte or the surrounding nurse cells. It has been shown in vivo that administration of LH in combination with human menopausal gonadotropin reduces cyclic AMP in the oocyte, but not in the surrounding nurse cells. This effect is puzzling since the gap junctions between the oocytes and the other cells still appear to be functional, so cyclic AMP should pass through these junctions easily. An alternative hypothesis is that LH acts indirectly by causing some maturation-inducing substance to be produced in the surrounding cells that decreases the cyclic AMP in the oocyte. Since an increase in intracellular calcium has been shown to be essential in somatic cell division, it is possible that a maturation-inducing substance operates in the oocytes by causing an increase in intracellular calcium. The search for these substances and their mechanism of action is a major area for future research.

The study of oocyte maturation has led to extremely successful methods for maturing mouse oocytes in vitro. Such work has shown that the in vitro culture of oocytes and their surrounding cells can be accomplished in ways that allow subsequent fertilization, implantation, and delivery of normal young to occur. Successful in vitro fertilization of oocytes from other species, however, depends upon continued basic research on oocyte nutrition and metabolism and on the factors or substances that control differentiation and maturation. The potential applications of an increased ability to mature oocytes in vitro are numerous. As discussed earlier, such an ability would be of enormous value in the production of agriculturally important animals. These techniques also have important applications in attempts to preserve endangered species. For example, it has been demonstrated that oocytes removed from mice up to six hours after death can be matured in vitro (Lazarus effect). Yet another application can be found in basic science research into genetic diseases through the use of transgenic mice. Such mice are produced by introducing known gene fragments into the genome of a mouse. These gene fragments have been inserted by microinjection into eggs. Alternatively, the fragments, incorporated into a harmless virus, have been injected into neonatal female mice. In this way, such fragments are incorporated into the DNA of developing oocytes in these females. It

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would be extremely helpful to be able to transfer gene fragments by viral infection of oocytes, which could then be matured in vitro for subsequent fertilization. Finally, the ability to mature human oocytes may have some application for human IVFET, since matured oocytes from donors would obviate the need for hormonal stimulation therapy and permit synchronization of the developmental state of the embryo with the state of the uterine lining. The degree to which data obtained from experiments with mouse oocytes can be applied to humans is not known. Therefore, for such applications to be realized in human IVFET, it is probably necessary that basic research be done using oocytes from human ovaries removed for justified clinical reasons and/or oocytes from non-human primates.

Cryopreservation of Oocytes and Embryos Cryobiology is the study of how living tissues can be frozen and stored for later revival. There are a number of uses for cryopreservation of oocytes and embryos in connection with IVFET and other reproductive technologies. Cryopreservation of human and cattle sperm has been a routine and successful procedure for many years. It is thought that, properly stored, frozen embryos can be preserved for many decades before thawing and transfer to the uterine cavity. To date, embryo cryopreservation has been successful in many mammalian species, including mouse, sheep, cow, human, and baboon. Rare mutant animals have also been preserved for later study.

The Mouse Embryo Banks at the Jackson Laboratory in Maine contain many examples of cryopreserved mutant mouse embryos. Such mutants are valuable for research into genetic and other diseases. Cryopreservation allows easy transportation of frozen embryos by researchers who move frozen mice embryos from one lab to another, and for the national and international transportation of cattle embryos.

If proved safe for use in clinical practice, routine Cryopreservation of eggs or embryos could reduce substantially the number of times a woman would have to undergo hormone stimulation, because the eggs harvested could be frozen for later fertilization or fertilized in vitro and the extra embryos stored for future transfer. These stored embryos could be used if the first pregnancy failed or used at a later time so that an infertile couple could have more than one child from one stimulation/IVFET procedure. Thus, such preservation techniques could also minimize the waste of extra eggs and extra embryos produced by superovulation and other IVFET procedures.

Although the specific methods of cryopreservation vary, the basic steps involved are the same. The first is chemical protection of the tissue against damage from freezing. The tissue is then sometimes cooled in sequential steps to a subzero temperature before being rapidly cooled to -196^{o} C for long-term storage. The procedures used for warming and cooling are variable. After rewarming, the chemical protective solutions are removed by rinsing. Protection from damage by freezing is critical to the survival of an embryo because, without protection, the formation of ice crystals causes tissue damage that can not be repaired.

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Cryoprotective solutions, which are made from chemicals such as glycerol or dimethyl sulfoxide, function in a manner similar to antifreeze in a car. They replace the water in the embryo.

Because of the importance of this step in cryopreservation, much basic research is being conducted to find appropriate cryoprotective solutions. Certain specific properties of the solutions are important determinants of the survival rate of eggs and embryos. For example, some chemicals, such as sucrose, cannot enter cells and, therefore, do not protect from freezing damage in the same way, or with the same efficiancy, as other molecules that can enter cells. Glycerol, on the other hand, can permeate a cell, partially replace the water, and impart the cryoprotection necessary. The osmotic characteristics of particular solutions are important also. In the case of glycerol, if one exposes a cell to a solution of water and glycerol, glycerol moves into the cell by osmosis, a process by which molecules in a high concentration will move across a semi-permeable barrier, such as a cell membrane, into a region of low concentration of that molecule. Likewise, water moves out of the cell by osmosis into the surrounding fluid. Eventually, the concentration of glycerol will equilibrate and be the same on both the ineide and outside of the cell. Since glycerol moves into the cell more slowly than water moves out of the cell, the cryoprotection procedure must be carefully controlled so that the cell does not lose water so quickly as to shrink and die.

It is especially important to control the movement of molecules across the cell membrane when thawing ova and embryos. After thawing, it is important to remove the glycerol from the cells and to return the intracellular contents to normal. However, a cell protected with glycerol, if suddenly exposed to a normal saline solution, will take up a great deal of water very quickly. This influx of water causes the cell to swell and the membrane may burst. Although this problem can be avoided by exposing the cells to solutions of decreasing concentration of glycerol in a stepwise fashion, this is not always easy to accomplish. Research has shown that adding the impermeable solute, sucrose, to the saline solution results in a more even exchange of glycerol and water across the membrane and obviates the rapid influx of water into the cell. This one-step method has proven to be of particular use in the application of these techniques under field conditions such as in work with endangered species.

The method used to freeze and thaw the ova and embryos is also important. Until recently, the tissue was first cooled to a few degrees below zero so that ice formation could be controlled. The tissue was then cooled to some intermediate subzero temperature before being rapidly cooled to the storage temperature of -196^{o} C. Research with mouse ova showed that the cooling procedure used could affect the survival rate in important ways. For example, it was demonstrated that, if ova lose a considerable amount of their original volume during cooling, the survival rate is greater. Cells that retain most of their original volume usually do not survive. This is probably because a cell is primarily composed of water from which the damaging ice crystals form. The method used to

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freeze the tissue also determines the subsequent thawing procedure. Thawing is performed stepwise in reverse order to the freezing steps. New freezing protocols have been developed that simplify the multiple step methods. For example, mouse embryos have been successfully frozen in one step after incubation in glycerol and sucrose. This method uses the glycerol to cryoprotect the tissue and the sucrose to dehydrate it before freezing, eliminating the need to slowly cool the tissue before freezing. Variations of this method have also been successful. One novel method is called vitrification, which means freezing without crystal formation. Sometimes, this can be accomplished by extremely rapid freezing. However,in a variation of this approach, embryos are exposed to a solution of chemicals that form a glassy solid substance when cooled. No ice crystals form in the embryos exposed to this solution so freezing can be rapid and done in one step. These new freezing procedures are still being developed, but they have the potential to increase the success rate of ova and embryo freezing.

Improvements in cryobiology would have significant impact. Improved preservation and storage of embryos from valuable cattle and other agriculturally important animals would greatly increase the yield from superovulation and IVFET procedures. The preservation of endangered species and rare genetic breeds of laboratory animals would be enhanced. Finally, successful storage of human embryos that result from IVFET techniques can increase the chances of an infertile couple to have a baby.

Intratesticular and Intraovarian Paracrine Control [5]

Development of male and female gametes occurs over varied periods of time in special body compartments, the ovaries and the testes. In this section, the biochemical control mechanisms that affect gamete development will be discussed. In contrast to hormonal control mechanisms in which bioactive substances are released into the bloodstream and act at distant sites, paracrine control mechanisms involve chemical interactions between neighboring cells. For example, one cell can release an active substance into the extracellular space, the substance then diffuses around other cells in the region and affects the activity of these other cells in some way. These effects most often are mediated by cell surface receptors for the substance released.

Intragonadal Control of Testis Function The testes are composed of a number of different cell types, including germ cells in their various developmental stages (spermatogonia, spermatocytes, spermatids, and mature sperm) each of which displays unique characteristics. Other cell types include Sertoli cells, which are nurse cells for the developing spermatogonia and spermatocytes, and Leydig cells, which release the hormone testosterone, required for sperm maturation. Many bioactive hormones and peptides have also been found in the testis and research has begun to determine the cell sources of these chemicals and their function in spermatogenesis. Some of the molecules found in the testis have been shown to affect cell cycles and cell differentiation. For example, somatomedin C (also called insulin-like growth factor, IGF) and epidermal

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growth factor stimulate cells to begin DNA synthesis for mitotic cell division. Fibroblast growth factor stimulates quiescent cells to reenter the cell cycle in order to replicate and divide. Various hormones and growth factors act as stimulators to cell differentiation, thus causing the cells to exit permanently from the cycle of chromosome replication and mitotic division. Cells are inhibited from exiting the cell cycle by interleukin-1, which is a molecule made by cells of the immune system. The functions of most of the hormones and growth factors in the testis are not known. For a few, however, certain functions are beginning to be described.

In the rat, a common animal model for investigations of sperm development, Sertoli cells start to divide and proliferate around the 22nd day of embryonic life, and complete this proliferation by 20 days after birth. Leydig cells, in contrast, do not start to divide until 2 or 3 days after birth and continue to proliferate until 35 days after birth. By the end of these processes, almost equivalent numbers of Sertoli and Leydig cells are found in the testis, despite very different developmental timetables. A hypothesis has been proposed that these cells may regulate each other by complicated interactions during development. Fibroblast growth factor stimulates replication of Sertoli cells by causing an increase in the number of receptors for follicle stimulating hormone (FSH). As this occurs, the numbers of Sertoli cells increase and release somatomedin C in greater and greater amounts. Somatomedin C increases the number of receptors for luteinizing hormone (LH), which causes the Leydig cells to divide and increase in number. The increasing numbers of Leydig cells leads to an increase in beta-endorphin released by the Leydig cells. A negative feedback occurs such that beta-endorphin decreases the proliferation of Sertoli cells.

Such paracrine interactions are probably not unusual and may occur between other types of cells in the testis. In addition, non-paracrine mechanisms of communication probably occur, including cell to cell adhesion and communication through gap junctions between cells. Very little is known regarding the mechanisms of interaction between developing sperm cells and their nurse cells, the Sertoli cells. Cell adhesion molecules have been found on spermatocytes that, some suggest, promote continued meiosis of the spermatocytes bound to the Sertoli cells. Experiments done in culture have shown that sperm cells synthesize more DNA and RNA when they are cultured with Sertoli cells (especially if FSH is in the culture medium), than if the sperm cells are cultured alone, or alone with FSH. Such data suggest that the Sertoli cells are important to sperm cell development.

Investigators have looked for proteins that can be related to specific stages of sperm cell development. The stages of sperm cell development are quite complex, but can be generally grouped into four main stages. The primordial germ cells are called spermatogonia, and undergo six mitotic divisions before becoming spermatocytes. Meiosis begins in spermatocytes. Further development forms spermatids, which are immature germ cells in which meiosis is arrested. Spermatogonia, spermatocytes,

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and early spermatids are in close apposition to Sertoli cells in the long tubular structures of the testis where sperm are formed, called the seminiferous tubules. The seminiferous tubules open into other tubes through which sperm are transported. As spermatids mature and differentiate into early sperm cells, they are released from the Sertoli cells into the seminferous tubule. An amazing feature of the seminiferous tubule in some species is that, in any given segment of the tubule, the germ cells form a similar pattern of stages. While this arrangement argues for some kind of cellular control of developmental stage, it also provides a definable tissue state with which to experiment.

Scientists have isolated specific segments of seminiferous tubules and extracted the proteins contained in them. Maps of these proteins have shown that the different stages are associated with different proteins. By isolating some of these proteins and producing monoclonal antibodies to them, it has also been possible to determine which cells contain the protein of interest. One such protein has been found in Sertoli cells, and its synthesis and secretion have been shown to be stage-specific. These experiments have also found that such proteins diffuse into the extracellular spaces of the seminiferous tubules, but because of the slow flow rate within the tubules, remain in spaces near developing sperm cells for quite a long time before being degraded by proteolytic enzymes. Therefore, there seems to be strong evidence that Sertoli cell products greatly influence the development of sperm cells.

Continued research into the molecular events of male gamete development would fill some of the large gaps in knowledge about the normal development of sperm cells and would further the search for causes and cures of male infertility.

Paracrine Control of the Ovary Although it is generally agreed that paracrine control is important to the development of follicles in the ovary, much of the evidence is circumstantial. This state of affairs derives from the nature of autocrine/paracrine regulation. It bas proved difficult to manipulate experimentally systems in which the cell of origin of an observed signal is the same as or adjacent to the target cell.

Early experiments to examine the importance of intraovarian chemical control involved removing the pituitaries of rats. Without the pituitary hormones (especially LH and FSH), which normally promote follicular development, investigators could then look at the effects of a number of substances on ovarian follicles. Such experiments found that estrogen, which is synthesized and released from cells in the ovary, was needed for follicular development and that other steroids produced inhibitory effects. Further, it was demonstrated that estrogen could act in concert with or synergistically with LH and FSH to differentiate follicular cells. Later experiments used granulosa cells from pig follicles in culture to show various effects of estrogen. Following a brief inhibitory effect, estrogens stimulated progesterone synthesis in the granulosa cells in a manner that was time-dependent and blockable by pharmacological

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inhibitors to estrogen. Continued research demonstrated that estrogen action was exerted after the generation of cyclic AMP (see earlier section on oocyte maturation for other actions of cyclic AMP).

There are several chemical forms of estrogen that are made by enzyme modification of a basic structure. One type, catecholestrogen, is found in high concentrations in the walls of large ovarian follicles in the pig and probably enhances, by paracrine mechanisms, the effects of FSH in the follicle. This concept arose from culture experiments which found that catecholestrogen with FSH stimulates progesterone synthesis and production of cyclic AMP. However, antiestrogens do not block this effect. Thus, while experiments have shown that estrogen plays an important role in the control of follicular development, its true role in the natural state remains to be determined.

Estrogen is only one of the substances under investigation in terms of paracrine regulation in the ovary. Tissue culture experiments have shown a number of peptides and growth factors to cause proliferation of granulosa cells. For some of these factors, it is known that they have effects on follicular cells and that they are present in the ovary in vivo. However, whether or not the factors are necessary for follicular development and normal ovarian physiology is not known. The most research has been done on insulin-like growth factors (IGFs). IGFs are synthesized by many tissues and have been found to be a powerful local mediator of growth and differentiation. They seem to stimulate all kinds of effects in granulosa cells including increasing enzyme levels, cell numbers, protein synthesis, glucose utilization, and various secretions. Insulin itself and the IGFs seem essential for FSH induction of receptors for LH, for generation of cyclic AMP, and for FSH stimulation of steroids. IGFs also interact with other growth factors in the ovary to increase cell number. Since most of these findings have been from in vitro studies, it is important to demonstrate the presence of IGFs in follicles in vivo. In fact, IGFs have been shown to be present in follicular fluid in in vivo concentrations similar to those that produce effects in culture. Furthermore, the concentration of IGFs increases during follicular growth and under the influence of FSH, LH, and growth hormone. This is important because any substance active in follicular development and local regulation in the ovary would be expected to change in concentration in response to pituitary hormones.

Another growth factor found in the ovary is a type of transforming growth factor called TGF-alpha, which is a molecule related to epidermal growth factor. TGF-alpha increases the rate of cell division of ovarian cells in vitro and has a negative effect on the secretion of estrogen from granulosa cells. It also seems to decrease the effect of FSH on LH receptors, although this effect varies. TGF-alpha has been found in significant concentrations in follicular fluid and is in highest concentration in small follicles. Based on these findings, it has been proposed that TGF-alpha promotes cell replication in the follicles and inhibits differentiation. The other type of TGF, TGF-beta, is a molecule very similar to inhibin and Mullerian-inhibiting substance, both of which

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have effects in the ovary. In general, studies have suggested that TGF-beta augments the actions of FSH to increase estrogen secretion, LH receptor activity, and progesterone synthesis. These effects seem to be dependent on both the concentration of TGF-beta and FSH and seem to vary across species.

Fibroblast growth factor is another factor being investigated for its possible role in regulation of ovarian function. This growth factor is also present in many tissues and has been shown to be particularly potent in stimulating the formation of new blood vessels. In vitro, fibroblast growth factor has effects on cells of the corpus luteum and it stimulates granulosa cells to divide. In addition, the factor seems to support the growth of blood vessels in the corpus luteum. Its effects on synthesis of estrogen and progesterone are probably inhibitory. There are many other peptides that have been found in the ovary, but research on these substances is sparse. Many of them have not even been purified enough to allow analysis of their structure and examination of their effects in vitro or in vivo.

This complex area of research promises to provide useful knowledge about regulation in the ovary and could have far-reaching implications for both IVFET and natural reproductive biology. In order to demonstrate that a particular substance has effects in the ovary, the substance must be shown to be secreted by ovarian cells into the local extracellular space and to produce changes in the activity of neighboring cells. Such minimum criteria have been met for some of the factors discussed above; however, elucidation of the mechanisms involved is critical. Especially important for future research is to determine the biochemical regulation of cell replication and differentiation in the ovary. Also, it is necessary to determine how some of these factors are produced by the cells. For example, are there large precursor molecules produced that are modified inside the cells for specific needs? The interaction of these factors with known hormones is also important and may be of special application in evaluating the effects of superovulation. Strategies to block the activity of these factors could help to determine if any of them are absolutely necessary to normal ovarian function as this is entirely unknown. Finally, it is of utmost importance to relate the findings from in vitro studies to what is actually occurring in the natural state.

Assessment of Gametes [6]

There are many reasons why IVFET techniques in human clinical practice do not result in ongoing pregnancies one hundred percent of the time. Certainly, some of the failure rate is attributable to such things as the varied skill levels of the practitioners, the physiological problems of the infertile male or female, or the difficulty in synchronizing the stage of the embryo with the stage of the uterine wall. Nevertheless, a significant contribution to the failure of IVFET derives from poorly understood physiological responses of oocytes and early

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http://www.nap.edu/catalog.php?record_id=18586

embryos. Between 20 and 40 percent of apparently mature eggs fail to be fertilized by IVFET methods. Of the 60 to 80 percent that are fertilized, most of the zygotes produced fail to establish a pregnancy after transfer to the uterus. Such early reproductive failure raises critical questions for scientists interested in both improving IVFET and understanding the normal physiology of early reproduction.

Experimental strategies have been developed that are beginning to shed light on some of these problems. One such strategy is to examine the degree of chromosomal abnormality in preovulatory oocytes in an attempt to determine whether these abnormalities contribute to either failure to fertilize or failure to develop after fertilization. There has been a variety of studies assessing the number of eggs obtained from woman who had undergone ovarian stimulation, that exhibit a particular type of chromosome abnormality called aneuploidy. A normal oocyte contains 23 chromosomes after meiosis, half of the full human complement of 46 chromosomes. Aneuploid oocytes contain less or more than 23 chromosomes. Comparison between the studies is difficult because the results range from 11 to 65 percent of the oocytes being aneuploid. However, the most exhaustive studies have indicated that the best estimates are likely to be between 20 and 25 percent. The interpretation of such studies is also difficult since the oocytes examined were these that failed to be fertilized. Thus, the possibility exists that the chromosome abnormality is simply an indicator of some other developmental problem. In addition, there are no data on the percentages of normal, unstimulated oocytes which are aneuploid, so how much of the observed abnormality is caused by hormonal stimulation is not known. When human oocytes were matured in vitro, one study found only 1.5 percent to exhibit the chromosome abnormality. This suggests that hormonal stimulation may increase the number of aneuploid oocytes. Finally, in a study of 163 women, a very high percentage of abnormal chromosomes was found in oocytes obtained from particular individual women. The possibility exists, therefore, that some women are especially likely to produce oocytes with chromosome abnormalities.

The extent to which chromosomal abnormalities affect fertilization and pre- or early post-implantation failures is important to know. This is especially true when one considers that sometimes aneuploid eggs are fertilized and the resulting embryos develop to term. Also, findings to date suggest that a significant number of embryos produced by IVFET exhibit some type of chromosmal abnormality. One way to approach those important questions is to develop a focused, multi-center research program to analyze eggs that either failed to fertilize in vitro, or that were produced in excess of the number needed for IVFET methods.

The use of fluorescent stains for chromosomal DNA in such eggs could reveal a variety of abnormalities and answer a variety of questions. Such an approach could determine more precisely the frequency of abnormalities in stimulated cycles. Various stimulation methods could be compared to find ones with the lowest rate of chromosome perturbation. In addition, associations of chromosomal abnormalities with particular patients, or with patients of certain ages, could be made.

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http://www.nap.edu/catalog.php?record_id=18586

While the analysis of oocytes and eggs is important, it is equally necessary to analyze the processes that cause fertilized eggs to develop to varying stages before finally failing to develop. It has been estimated that only 10 to 15 percent of all fertilized eggs develop past a certain stage, and these failures occur at similar frequencies despite differences in ovarian stimulation or in vitro culture conditions. Thus, failures are probably due to processes within the eggs or embryos themselves.

In contrast to the analysis of oocytes, analysis of embryos is an extremely difficult endeavor. Between fertilization and implantation, a myriad of cellular and molecular changes take place in an embryo that have to be coordinated precisely and synchronized both spatially and temporally. Research, then, must focus upon individual stages beginning at the earliest stage of fertilization.

It has been found that 5 to 10 percent of human eggs that appear to be unfertilized actually do contain one or more sperm in the region just outside the egg membrane when viewed microscopically. At higher magnifications with electron microscopy, such oocytes exhibit an absence of binding between the sperm and the egg. Usually the membranes of the sperm form protrusions, called microvilli, which seem to be important for sperm binding to an egg. Sperm associated with these seemingly unfertilized eggs lack this structural feature. Although the reasons for this failure in sperm/egg binding are unknown, a number of possible explanations are likely. For example, cell surface molecules, which function as cell recognition markers, could be absent. Another possible cause is an abnormal organization of specific proteins, called microfilaments, that form the structural framework necessary for microvilli. This possibility is attractive because eggs have been found that lack microvilli except in small restricted regions of their membranes. Since the abnormality has been observed in mature, preovulatory oocytes, it is not likely to be the result of culture conditions. Further, the lack of microvilli is also typical of oocytes from women with a history of failed fertilization by in vitro methods. It may well be necessary to analyze immature but fully grown oocytes from such women to determine if stimulation protocols affect the frequency of this problem. Other research to determine the molecular changes in the egg membrane necessary for fertilization would also be extremely helpful.

There are two other conditions associated with early failure at fertilization. In one condition, the DNA of the sperm fails to decondense. Decondensation is a necessary prior condition to the combining of the male and female chromosomes. While it is possible that there is abnormal packaging of the sperm DNA, it is more likely that there is a problem in the egg. Animal studies have suggested that chemicals in the egg cytoplasm are responsible for causing the sperm DNA to decondense. Clearly, more needs to be known about the process.

Another critical stage in fertilization that can go awry is the migration, juxtaposition, and fusion of the male and female pronuclei, each of which contains the respective chromosomal contribution to the

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resulting embryo. Electron microscopic analysis has shown that failure here is associated with failure of the membranes between the two pronuclei to dissolve. Therefore, the two pronuclei cannot fuse. Very proliminary data using fluorescent DNA stains have shown that, in these cases, DNA replication is incomplete or absent. It is possible that DNA replication is required for membrane dissolution. Research into this problem could proceed in two ways. First, analysis of fertilized eggs that have arrested development at this stage could be done to further knowledge about the mechanisms of such failure. Second, the mouse could provide an animal model with which to study this problem. It has already been demonstrated in the mouse that chemicals, which specifically inhibit DNA replication, do not affect the formation of pronuclei, their migration, or their juxtaposition. Such chemicals do, however, prevent the dissolution of membranes between the pronuclei. These results also occur when DNA replication is prevented by other means, so probably are not simply a side effect of the inhibiting chemicals.

While this discussion has centered upon very early reproductive failures, there remain many other conditions that cause the demise of early embryos. For example, human development is sometimes arrested when the embryo reaches the four-cell stage. This may be due to a failure in the expression of embryonic genes that results in a lack of proteins required for further development. At the 12-16 cell stage, some normally fertilized eggs develop multiple nuclei. Finally, during the first mitotic division of the zygote to form two cells, a chromosome pair sometimes does not separate or disjoin. This results in different numbers of chromosomes in different cell lines of a developing embryo. These are called "mosaics". Research into the causes of these problems can be of benefit to the clinical practice of IVFET as well as to the understanding of human reproduction in general. While animal models, like the mouse, have great usefulness in this research, the use of developmentally arrested zygotes and embryos produced by IVFET is also necessary. Such zygotes and embryos no longer have the potential to develop into a fetus but may, nevertheless, contain information critical to a better understanding of reproductive success and failure.

Fertilization [7]

Fertilization encompasses sperm and egg maturation as wall as the complex cascade of events that occurs once the sperm reaches the egg. Although some aspects of oocyte maturation have been previously discussed, those that directly influence fertilization require further emphasis. The first hurdle for an oocyte is to acquire the ability to continue meiosis. Oocytes from young mice (less than 15 days old) have been shown to be incapable of resuming meiosis if put into tissue culture. Therefore, it seems that there is a growth-related biological process that allows for meiotic competence. Following the breakdown of the germinal vesicle, the chromosome pairs in the oocyte separate with emission of the first polar body and arrest at metaphase II; the polar body frequently degenerates. Resumption of meiosis is initiated by fertilization of the egg.

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Before ovulation the oocytes are contained within follicles and, as was previously discussed, it is thought that the follicular cells inhibit the oocyte from maturing. Many experiments with frog and mouse oocytes have been done to elucidate the molecular mechanisms that regulate maturation. Cyclic AMP has been strongly implicated in maintaining meiotic arrest, presumably by activating an enzyme called protein kinase A. Protein kinase A promotes the structural modification of other proteins by adding a phosphorus group; this process is known as protein phosphorylation. Often, proteins are active or inactive biologically, depending upon whether they are phosphorylated or not. In a sense then, modification of proteins by enzymes is one way in which cells turn molecular signals on and off. The proteins that participate in this cascade of reactions associated with oocyte maturation are not known, but are a subject of great research interest. In addition, it must be emphasized that, in addition to cyclic AMP, other types of molecules present in the follicle are likely to participate in maintenance of meiotic arrest.

A molecule that is likely to be involved in oocyte maturation has been isolated from frog oocytes and is called maturation promoting factor (MPF). MPF, when injected into immature oocytes of many species, causes chromosome condensation and breakdown of the nuclear membrane. Its biochemical properties are interesting since it behaves like a protein kinase and is only activated when it has been phosphorylated. Oocytes from mice have a similar substance that has not been fully isolated. There have been innovative experiments performed to measure this MPF-like activity. In one experiment, mature mouse oocytes were fused with immature ones. The mixture of the two cytoplasms resulted in the breakdown of the nuclear membrane of the immature cell. Further experiments using similar strategies have shown that the MPF-like factor's biological activity changes according to the developmental stage of the oocyte. It has also been suggested that oocytes that do not develop meiotic competence may contain a substance which blocks the activity of MPF. Greater understanding of oocyte maturation and the molecules that are directly involved in this process might result in improved ability to mature oocytes from humans and other animals in vitro. Research that tests this hypothesis might also further in vitro oocyte maturation.

Sperm recovered directly from the testis cannot fertilize eggs; like eggs, these haploid cells must also be matured after formation if fertilization of an ovum is to occur. This maturation takes place during sperm transit through the epididymis, a process that usually takes about 10-14 days. During this time many molecular changes in the sperm, particularly on the surface of the sperm cell, occur. The mechanisms which bring about these surface alterations have not yet been elucidated. Similarly, it is not known which of these modifications are related to the acquisition of fertilizing function by sperm. Identification of the molecules used by sperm for gamete interaction is necessary before the relevant mechanisms can be ascertained. One way that has been used to examine sperm at different stages of maturation in the epididymis is to stain the cells with antibodies that recognize particular sperm antigens.

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In one case, an antibody recognizes a sperm protein in the anterior region of the sperm head. The protein recognized by the antibody, termed M42, is altered subtly during sperm maturation; prior to the protein's alteration, sperm cannot fertilize eggs, whereas after its modification, fertilization can be achieved readily. It is likely that this change in the M42 protein represents only one example of many molecular changes in the sperm during epididymal transit. Continued examination of individual molecules, like M42, may reveal the mechanisms by which the epididymis causes sperm maturation. This information, in turn, could be used productively in two different ways: to promote fertility by learning how to mature sperm in vitro, or to promote contraceptive development by preventing sperm maturation specifically.

After ejaculation, sperm must undergo a second, and final, maturation phase. This process, termed capacitation, occurs normally in the female reproductive tract, but can be provoked experimentally for many species by incubation in vitro. Like maturation in the epididymis, capacitation is associated with a large number of sperm alterations, the functions of which are largely unknown. But at least two changes in the sperm's surface membrane are now recognized for essentially all mammalian sperm as a function of capacitation: changes in lipid composition and the loss of many surface-associated components. Capacitation is a prerequisite for fertilization, and these alterations apparently permit sperm to penetrate through the physical barriers that surround the egg at the time of fertilization. Clearly, the ability to control this process would assist fertility regulation considerably.

In the ideal circumstance, sperm and egg meet in the fallopian tube and fertilization takes place. However, fertilization is an exceedingly complex process in which the sequence of events is of the utmost importance. At the time of fertilization, the egg is surrounded by two kinds of barriers. Enveloping the egg directly is a jelly-like covering, called the zona pellucida, which is surrounded itself by a cellular layer, called the cumulus. The zona plays a major role during fertilization and many of its molecular features have been defined.

There are three important proteins in the mouse zona--ZP1, ZP2, and ZP3. Each protein has sugar sidechains attached to it and is, thus, a glycoprotein. The structure of the extra-cellular zona pellucida is composed of polymers (compounds produced by the combination of two like molecules) of ZP2 and ZP3 formed into long filaments which are cross-linked by ZP1. ZP2 and ZP3 both function as receptors for sperm. ZP3 also induces the acrosome membrane of the sperm to break down. Both ZP2 and ZP3 are chemically modified following fertilization and play important roles in keeping extra sperm from penetrating the egg.

The genes coding for mouse ZP2 and ZP3 have been isolated. Each is a single-copy gens in the mouse genome and each is highly conserved among mammalian species. The zona genes are expressed only in oocytes where their expression is developmentally restricted to the growth phase of

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oogenesis that occurs just prior to ovulation. The determination of the structure and the nucleic acid sequence of the ZP2 and ZP3 messenger RNAs has led to the prediction of the amino acid sequence of the ZP2 and ZP3 proteins. Using the cloned genes, the zona proteins can now be expressed by tissue culture cells. By modifying the cloned zona genes, scientists will be able to produce mutant zona proteins for further analysis of how the zona proteins interact with one another as well as the biological function of individual zona proteins.

Antibodies have been produced which bind either to ZP2 or to ZP3. After injection into female mice, these antibodies localize to the zonae pellucidae surrounding growing oocytes. The presence of these antibodies causes highly effective, long-term, but ultimately reversible contraception. Recent studies have demonstrated that immunization of female mice with a synthetic ZP3 peptide (based on the ZP3 DNA sequence) induces the formation of circulating anti-ZP3 antibodies that bind to eggs and effectively preclude fertilization. Because the zona genes are highly conserved, the development of this contraceptive strategy using homologous (same species) peptide as a vaccine may have wide spread application. This method of contraception is desirable because it would prevent fertilization, not implantation, and would avoid the dangers and side effects of the hormones administered in birth control pills. Nevertheless, the reversibility of the strategy remains to be rigorously documented.

The surface changes that sperm undergo during capacitation permit them to navigate through the cumulus cells. A secretory organelle that is essentially an enzyme bomb located at tha apex of the sperm head must not be disrupted, however. The sperm will use this bomb, called the acrosome, to digest a path through the zona. If the acrosome breaks down prematurely, sperm stick to the cumulus cells and cannot reach the zona surface. The sperm's recognition site for the zona is located on its surface membrane and binds to the ZP3 protein of the zona. The ZP3 molecule then triggers sperm to release its acrosomal enzymes. Particular components within the acrosome bind to ZP2 and the acrosomal enzymes, together with the motility of the sperm cell, allow the sperm to penetrate the zona and reach the egg membrane. This process is remarkably similar across mammalian species. It is likely that factors from the egg itself are not involved in fertilization up to this point since these events occur even if the zona contains no egg, a so-called zona ghost.

Like the antibodies against zona proteins discussed above, antibodies directed against specific sperm proteins can also inhibit fertilization. The sperm protein M42, described earlier, that is altered during epididymal maturation is one of several candidate sperm proteins for this purpose. Sperm incubated with an anti-M42 antibody cannot fertilize eggs in vitro since the sperm are blocked from losing their acrosomes. In a more natural experimental situation, injection of female mice with the same anti-M42 antibody decreases pregnancy levels markedly. Strong evidence suggests that some forms of infertility in women may be caused by the presence of antisperm antibodies. Perhaps the continued examination

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of this experimental system—the induction of infertility with specific anti-sperm antibodies—will assist in explaining the mechanisms underlying such naturally occurring types of infertility.

Once the sperm has penetrated through the zona, it associates with the egg surface rapidly. Membrane fusion between the two gametes is initiated at a specific location, near the middle of the sperm head, and proceeds around the cell so that the entire sperm is incorporated into the egg. Once the sperm has fused with the egg, small packages called cortical granules, which contain enzymes that modify the zona pellucida, move to the egg membrane, fuse with it, and release their contents into the space between the egg and the zona pellucida. The released enzymes modify the ZP2 protein so that acrosome-reacted sperm can no longer bind to ZP2. Modification of ZP3 also occurs that affects sperm binding and the induction of an acrosome reaction. In brief, it seems as though nature has devised backup systems to ensure thet only one sperm enters the egg.

Very little is known regarding the precise biochemical events that take place during the cortical granule reaction. In vitro experiments have shown that activated protein kinase C inhibits fertilization by modifying ZP2 in the same way as occurs in the natural state. Protein kinase C also modifies ZP3 but it only prevents ZP3 from inducing a complete acrosome reaction. Sperm binding to ZP3 is unaffected. Other experiments have implicated other enzymes and co-factors (inositol phosphate, G proteins, and phospholipase C), all of which participate in cascades of biochemical reactions. Much research needs to be done before any of the actual biochemical events can be known. For example, it is not known what chemicals are released by the granules. The precise changes in the zona proteins are not clear. Since some of these chemical cascades produce changes in calcium storage and release, the role of calcium is not clear. Knowledge of these events could contribute greatly to the ability to fertilize eggs in vitro.

Finally, while the block to polyspermy is taking place, changes occur in the egg that allow the male and female pronuclei to combine. When the sperm first penetrates the egg, its chromosomes are condensed, held together by chromatin-associated proteins called protamines. For the chromosomes of the male to combine with the female, the chromosomes must decondense. It is thought that a chemical change in certain molecules of the protamines is necessary for the chromatin to decondense. Such a chemical change can be induced by glutathione, which has been found in high concentration in mature eggs. Furthermore, depending upon the developmental state of the oocyte, glutathione can cause decondensation of oocyte chromatin when applied in vitro. It is possible that the substance that causes sperm chromatin decondensation is contained in the germinal vesicle, since sperm injected into oocytes before germinal vesicle breakdown retain their condensed chromatin. The substance could be a co-factor required for another reaction, could be an activator of some other molecule, could be solely responsible for the effect, or could actually act as an inhibitor of a molecule whose action is to prevent

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chromatin decondensation. Further information about these processes may suggest strategies with which IVFET could be enhanced.

To summarize, the sequence of events for gamete interaction in most mammals leading to fertilization is:

1. The capacitated sperm penetrates between the cumulus cells.

2. The acrosome-intact sperm binds to ZP3 in the zona pellucida.

3. ZP3 triggers the loss of the acrosome (the acrosome reaction), causing release of digestive enzymes from the sperm head.

4. The acrosome-reacted sperm binds to ZP2 and penetrates through the zona matrix.

5. The sperm and egg fuse with tha eventual formation of two pronuclei and restoration of the diploid state.

6. At the time of sperm fusion with the egg, a reaction is initiated that prevents any other sperm from entering (the block to polyspermy).

Preimplantation Development [8]

In this section, a variety of events thet occur between fertilization and implantation are discussed. These include tha special metabolic requirements of early embryos and the first expression of embryonic genes. Finally, tha results of experiments involving micromanipulation of animal embryos and embryo splitting will be described. The short description that follows reviews the main events of preimplantation development in a general way. It is also offered as an explanation of terms that will be used throughout this section.

The major steps in preimplantation development occur in the fallopian tubes. The fertilized egg, or zygote, undergoes mitotic cell divisions called cleavage. The first cleavage results in the formation of two cells, each of which divide in the second cleavage to form four cells, and so on through 8-cell and 16-cell stages until there are about 50 or 60 cells, at which time blastulation occurs. During the cleavage divisions, the cells are called blastomeres and all the blastomeres are encapsulated by the zona pellucida. Blastulation begins about the fourth day after fertilization, just as the developing embryo reaches the uterus. At blastulation, cavities form in the cell mass and the cells separate into two regions, an inner call mass, from which the fetus and some extra embryonic membranes will form, and an outer cell layer, celled the trophectoderm. Coincident with these changes, the zona pellucida degenerates. The blastocyst, as the embryo is called at this time, can be considered to be a polarized structure in that the inner cell mass is attached to the trophectoderm layer at one side of the blastocyst. If cut

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through the middle, the blastocyst would lock like a ring of cells with a clump of other cells stuck to one part of the inside of that ring. This polarization is important for implantation later since the pole of the blastocyst containing the inner cell mass will first touch the lining of the uterus in preparation for implantation in some species (in others, the opposite is true).

Metabolic Substrates and Pathways

Metabolism encompasses the processes by which cells extract energy from their environment and synthesize the necessary building blocks to form large molecules. Thus, metabolism is an exceedingly complex network of biochemical reactions that can be viewed as intersecting sets of interactions. The sets overlap at key points that regulate which set of reactions will be more or less active, depending on the needs of the cell at the time. The regulators at these key points are enzyme proteins. In most cascades of biochemical reactions, one enzyme's activity will be more important than any other's. Such an enzyme is called a rate-limiting enzyme and is often positioned at the key points of overlap between different pathways of metabolism.

In organisms dependent upon oxygen, glucose is used as a basic fuel for metabolism. There are three linked systems that subserve glucose metabolism--glycolysis, the citric acid cycle, and the oxidative respiratory chain. In glycolysis, glucose is chemically changed in a series of enzyme-catalyzed reactions to pyruvate. In the process, molecules of adenosine triphosphate (ATP) are produced. ATP is tha energy source for vast numbers of biochemical reactions. The citric acid cycle uses pyruvate, formed by glycolysis, as a substrate for another series of reactions to produce carbon dioxide and molecules (NADH and FADH) that are required for a particular type of chemical reaction also important in biological systems. Other products of the citric acid cycle are precursors for the synthesis of other molecules. For example, certain amino acids, which are the building blocks for proteins, are formed from the citric acid cycle. Finally, the NADH and FADH from the citric acid cycle are used in the oxidative respiratory chain to transfer electrons to molecular oxygen and form additional molecules of ATP.

Glucose is also necessary in two other systems that synthesize important biological chemicals. Both of these systems intersect with the glycolytic pathway. The first reaction of glycolysis is the conversion of glucose into glucose-6-phosphate (G-6-P) by the enzyme, hexokinase. The G-6-P produced can be diverted when necessary to enter the pentose-phosphate path or to be used to synthesize glycogen. In the pentose-phosphate path, G-6-P is converted to another kind of sugar by a series of reactions and, in the process, molecules of NADPH are formed. NADPH functions as a hydrogen and electron donor in biosynthethic

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reactions. In glycogen synthesis, the G-6-P is converted to glycogen, which is the way cells store glucose for later use. When cells need ATP, for example, glycogen can be broken down to form G-6-P, which can then continue in the glycolytic pathway. Eventually, through the citric acid cycle and oxidative respiratory chain, every G-6-P molecule leads to the production of 37 ATP molecules. For many years, glucose metabolism was not studied in eggs and early embryos, because it was generally assumed that results of studies from other cells would also apply to eggs. With the advent of tissue culture, it became apparent that eggs and early embryos required unexpected nutrients in their culture media, which seemed to violate this assumption. Specifically, it was shown that, for 2-cell mouse embryos, glucose alone would not promote survival in culture. Rather, lactate was critical for these embryos. Lactate is formed from pyruvate when there is little oxygen in the tissue. Exercised muscles, for example, produce a great deal of lactate from pyruvate. Lactate can also be converted to pyruvate, which may then be used in the citric acid cycle. Thus, the finding of a lactate requirement of these embryos suggested that certain pathways of metabolism were restricted, especially the glycolytic pathway. It was later found that maturing oocytes and embryos up to the 8-cell stage needed pyruvate and other citric acid cycle precursors to survive. While the exact requirements have been shown to differ from one animal species to another, it is interesting thet pyruvate is often used in culture media for human and monkey eggs and early embryos despite the lack of experimental data to justify its use.

The nutrient studies described above are not the only evidence that metabolism is restricted in oocytes and early embryos. Morphological studies have demonstrated that the intracellular organelles in which the oxidative respiratory chain takes place are not the same in early cells and embryos of 8 or more cells. These organelles are called mitochondria and are small membrane bound packages of enzymes and other molecules. Inside each mitochondrion is a complex labyrinth of membranes called cristae. It has been known for a long time that the arrangement of the cristae differ among cell types depending on the function of the cell and the metabolic state of the cell. Studies of eggs and early embryos showed a pattern of cristae that included a concentric and transverse arrangement. However, the concentric cristae disappeared by the 8-cell stage in most species. This change in structure correlated well with an observed increase in oxygen consumption by these 8-cell embryos.

Other studies have explored which substrates were taken up by cells. These studies also showed that eggs and early embryos took up pyruvate rather than glucose until they reached the 8-cell stage. To assess whether the cells actually used the pyruvate, studies were done to measure the carbon dioxide produced by metabolism of pyruvate compared to glucose. This is done by labeling the pyruvate or glucose with radioactive tags which, if the pyruvate is metabolized to carbon dioxide, results in the formation of radioactively tagged carbon dioxide. As might be expected, most carbon dioxide was produced from breakdown of pyruvate in early (1 to 2 cell) embryos. At 8 cells, the carbon dioxide was mostly from glucose metabolism.

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While the results of these studies seem clear, it is important to note some possible confounding factors in these experiments. The most important of these is that, since much of the work has been done with cells in culture, the differences between the amount of oxygen available in culture and the amount available in the natural state may have affected the results. The amount of oxygen available can, by itself, cause metabolism to be switched from one pathway to another. It is also important to determine if the enzymes responsible for glycolysis are present, and if glucose is being used in a pathway other than glycolysis. Various studies have compared the metabolic processes at play by using the strategy of starvation and refeeding of cells with the substances of interest. It was found that, if one starves embryos of glucose at any stage, the level of G-6-P decreases dramatically. Upon refeeding with glucose, the G-6-P levels increase in all stages also. This suggests that transport of glucose into cells and the first enzymatic reaction by hexokinase to form G-6-P are both normal across different stages of embryonic development. In contrast, the levels of fructose 1,6, biphosphate (an intermediate molecule formed in glycolysis) do not change with starvation of glucose at any embryonic stage. However, the levels of this intermediate increase substantially with refeeding of glucose in late embryos, but not early ones. Such data provide strong evidence that, like all cells, the rate-limiting enzyme for glycolysis (phosphofructokinase) is the one that catalyzes the formation of fructose 1,6, biphosphate. Moreover, the results suggest that this enzyme operates at a very low level in early embryos and does not reach its normal activity level until after the 8-cell stage. A similar starvation/refeeding experiment has suggested that the presence of pyruvate inhibits the formation of fructose 1,6, biphosphate from glucose and that the addition of glucose resulted in no increase in the metabolites of the citric acid cycle, whereas pyruvate did result in an increase in these metabolites at all stages of embryonic development.

There is much that should be known regarding the control of glycolysis in oocytes and early embryos. It is important to continue to investigate the rate-limiting enzymes at the intersection points between different metabolic pathways. Since the energy charge of a cell determines to a great extent the pathway chosen, it is also necessary to consider changes in the total energy charge of oocytes as they proceed through maturation and embryonic development. The energy charge can be expressed as the amount of ATP molecules available. Often, it is measured by determining the ratio of ATP to adenosine diphosphate, ADP, which is a lower energy state form. It is well known that if the energy charge of a cell is high, the pentose-phosphate and glycogen synthesis pathways are favored. This is because more energy in the form of ATP molecules is not needed. If there are not enough ATP molecules, the energy level is low and glycolysis, citric acid cycle, and oxidative respiratory chains are the favored routes. It is known that, in the mouse, zygotes and early embryos have a high energy charge that begins to fall as development proceeds. Such a finding is consistent with the evidence that these early embryos exhibit a restricted metabolism in the glycolytic and citric acid

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pathways. It is important to know the metabolic requirements of oocytes and early embryos because these requirements are critical to successful maintenance of these cells in culture.

Gene Expression in Early Embryonic Development

The determination of when embryonic genes begin to be expressed for the synthesis of new proteins is important in the detection of genetic diseases in embryos. Of the general population, two percent are at risk to produce children with genetically transmitted diseases. For some the risk is as high as one chance in two. With the development of IVFET techniques, it may became possible to fertilize eggs from high risk couples in vitro to allow for early diagnoses of genetic diseases. In theory, one cell from a multicelled embryo could be removed, analyzed for disease and, if found to be affected, a decision could be made not to implant that embryo. As an alternative, naturally fertilized eggs could be removed from the uterus by flushing, biopsied, and then returned via embryo transfer. For such diagnostic procedures to be realized, however, certain prerequisites are necessary.

First, there must be a realistic chance of maintaining a pregnancy after embryo transfer. Embryo biopsy may adversely affect the chance of a successful transfer. Second, the embryo should not be damaged by biopsy. In cattle, embryo biopsy is a relatively common technique and has been done with embryos at various stages. The optimum stage for biopsy is, nevertheless, important to determine. For example, techniques using 8-cell embryos involve removing the zona pellucida and removing one cell. An advantage of biopsy at this stage is that the embryo can be transferred to the mother when it is well synchronized with the state of the uterine wall. There are some disadvantages at this stage, however. These include the fact that there is only one cell with which to work, and the fact that embryos lacking zonae can stick together during culture or transfer, and can develop into a mosaic or chimera that, at best, is undesirable and, at worst, is fatal to the embryo. In addition to these problems, research has suggested that zona-free embryos do not exhibit normal cleavage and often exhibit cells that vary greatly in size. In later embryonic stages, it is possible to nick the zona and slice off the 5 to 10 cells that herniate out the nick. This approach will likely result in a lower implantation rate, because the optimum time for implantation would be lost. However, the embryos could be frozen for later implantation. Clearly, much more needs to be known about these changes and their effects.

The third prerequisite for preimplantation diagnoses is to have reliable probes with which to identify the presence of genetic diseases. Ideally these probes would make the identification at the level of the genome or DNA, as is possible with new technologies, such as Polymerase Chain Reaction techniques. In situ hybridization is a technique whereby a strand of DNA is made that matches with the strand of DNA one is looking for. To use in situ hybridization, however, the sequence of the gens in

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question must be known. Some of the sequences for human genetic diseases are known, especially the sex-linked diseases. The problem, however, is that many in situ probes are linked to the Y sex chromosome. This causes an unacceptable rate of false negative readings. A procedure with greater potential is to look at the defactive gens products, like hemoglobin, as an indication of genetic blood diseases, or like hypoxanthine phosphoribosyl transferase (HPRT) as an indication of Lesch-Nyhan disease. The last prerequisite is absolutely critical, and that is that the probes must assess the activity of embryonic genes not maternal genes.

Studies in the mouse have suggested that the embryonic genome turns on between the 2- and 4-cell stage. Before this stage, intracellular proteins are determined by RNA which was stored in the oocyte. Investigators at the Medical Research Council Unit of Mammalian Development in London have developed a microassay for the presence of HPRT in embryos that, applied in mouse embryos, has begun to answer some of the questions about the time of appearance of embryonic gens activity. HPRT is an enzyme that is lacking in children born with the sex-linked Lesch-Nyhan disease. The hallmark of the disease is self-mutilation and such children also exhibit strange motor behavior, are often mentally retarded, and usually die by the age of 10. Using the microassay for HPRT, workers have found that the enzyme increases in concentration in 8-cell mouse embryos suggesting that the embryonic genes have become active. So, it seems fairly clear that, in the mouse, gens activity begins at this point. However, before preimplantation diagnoses can be achieved in humans, one must know the timing of human embryonic gene activity.

In Great Britain, research on human embryos is allowed under the control of the Voluntary Licensing Authority. In the Cambridge University clinic, all patients are asked to donete their excess eggs and embryos for research purposes. Of 300 patients, only two refused permission for the research, and these two were still afforded all the clinical services available. Investigators at this clinic have thus conducted a number of studies on human embryos. In a series of experiments, radioactive methionine was added to the culture media of cultured embryos at various stages of development. Methionine is taken up by cells and incorporated into newly synthesized proteins and so is a qualitative measure of protein synthesis. Researchers found new proteins that contained the radioactively tagged methionine were synthesized for the first time between at the 4- and 8-cell stage. In another experiment, when chemicals, which blocked transcription of messenger RNA, were added to the culture medium, they had no effect on unfertilized oocytes or early embryos and development proceeded normally. However, the blockade of RNA, or protein synthesis, from the 4-cell stage caused the embryos to stop developing. While the results suggested that embryonic gene activity, which directs synthesis of new proteins, is important at the 4- to 8-cell transition period, the data are complicated by the fact that embryos have a tendency in vitro to stop development spontaneously at this stage. There is, however, further support for the idea that the embryonic genes are active at this stage. Since earlier experiments had looked at the

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pattern of proteins present in early and late embryos and found that the pattern changed quite clearly, the protein patterns were measured with and without the blockade of messenger RNA. These results demonstrated that, when RNA synthesis was blocked, the changes in protein pattern characteristic of the 8-cell stage and beyond did not occur, but the pattern remained in the early state.

Because the objective of such research was to develop preimplantation diagnostic techniques, the presence of HPRT was also assayed from human embryos. Unlike the findings in the mouse, there was a large variation in HPRT levels in human embryos, and the massive rise in HPRT at the 8-cell stage characteristic of the onset of gene activity in the mouse was not seen in the human. The reasons for these differences and for discrepancies between these data and that of others are not clear. It could be that HPRT is synthesized by the human embryo later than in the mouse embryo, or that the HPRT from tha maternal genes is being broken down as rapidly as new HPRT is produced so that no net increase can bee seen. It is also possible that there are simply unexplained differences in storage, culture conditions, or other variables. Whatever the reasons, the application of HPRT assays to preimplantation diagnosis of Lesch-Nyhan Syndrome in the human has been disappointing. The situation does point out, however, the dangers of extrapolating from animal studies and underscores the need to conduct some research with human embryos that, otherwise, would be wasted.

Regulative Potential of Micromanipulated Embryos

Many of the classic studies on embryonic development involved manipulation of certain parts of animal embryos and subsequent analysis of the effects of that manipulation on final development. Such manipulations included removal of certain parts (limb buds, for example, to study regenerative capacity), transplantation or exchange of parts (as in exchanging upper spinal cord regions with lower spinal cord to see if nerve outgrowth would follow the limb buds in the normal way), and grafting of parts of one embryo to another. Many new technologies allow micro-manipulation of embryos at the level of cells and, sometimes, even individual molecules.

It is clear that the developmental potential of cells gradually narrows as development proceeds and that embryos have remarkable capabilities to readjust to disturbance. Yet little is known about what governs the point at which readjustment can no longer occur because the potential of a given embryonic region has been irreversibly determined. Further, it is expected that that point of determination will very according to the specific region in question and according to the species involved.

It has long been thought that prior to blastulation an embryo is totipotent, meaning that no cell of the developing embryo is committed to any particular developmental fate. In other words, the cells are

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undifferentiated and uncommitted. However, it is becoming evident that embryos of different species, even though exhibiting the same morphological state and the same number of cells, may differ in the degree of determination and potential of each cell. Such findings call into question the long-held assumptions of totipotentiality. Since an embryonic cell is totipotent only if it can develop into an entire organism, experiments on embryo splitting can answer questions regarding the real potential of embryonic cells at different stages and in different species.

If a 2-cell stage embryo of a laboratory mouse is bisected, development proceeds normally. Up to the 8-cell stage, individual blastomeres can be dissected from an embryo and aggregated with another embryo successfully. However, such isolated blastomeres cannot develop or organize into viable fetuses on their own. The findings in the rat are similar. In contrast, rabbit blastomeres isolated from 4- and 8-cell stage embryos have been shown to be totipotent. Other experiments have examined the effects of bisecting mouse embryos exactly in half. While 65 percent of half embryos survive from the 2-cell stage, only about 45 percent survive from later stages. The success at later stages may not mean that all the cells are totipotent; rather, it may mean that each half embryo contained the required number and type of cell to complete development.

Information on domestic species is derived mostly from experiments with sheep and cattle embryos. In sheep, isolated blastomeres have been shown to develop normally from embryos up to the 8-cell stage. However, for unknown reasons, only some blastomeres are capable of such development. One case was reported in which an 8-cell embryo was divided into four equal parts. Transfer of these split embryos into ewes resulted in the birth of four lambs. Sheep embryos, which have been halved, seem to survive well from the 2-cell to blastocyst stage. In cattle, similar success has been achieved with quartered and halved embryos from the 8-cell stage to the blastocyst stage. Compared to the mouse, sheep and cattle exhibit blastulation at a later cleavage stage. Therefore, the number of cells is higher at blastulation for sheep and cattle. It is possible that a higher total cell number may allow for greater flexibility in embryonic adjustment to manipulations such as splitting.

Beyond questions of immediate survival of micromanipulated embryos, there are other questions that have been addressed by investigators. Some studies have assessed the size of embryos after splitting and the birth weight of resulting offspring. While one study in mice found no differences in these measurements between control and halved embryos, others have reported differences at various times of development. An examination was made of blastocyst formation following 2-cell stage embryos that had been bisected. One half of the embryo was discarded and the other was cultured, returned to a synchronized female, or placed in an immature oviduct. Those placed into culture conditions exhibited delayed

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development. A higher percentage of the half embryos developed normally when returned to a synchronized female or an immature oviduct. Such findings indicate that placing the split embryos in vitro somehow compromises their development. However, it is possible that the number of degenerated half embryos were underestimated in the in vivo conditions, since it is more difficult to recover and identify degenerated embryos from the uterus or oviducts.

These studies further showed that viability was correlated with cell number such that the lower the cell number achieved in the first few days after bisection, the lower the chances of survival. Many of the half embryos failed after implantation, indicating that the bisection did not result in a failure to implant. One of the most clear suggestions from these studies is that the cell number at blastulation (which occurs at a particular time, independent of the cell number) is critical and sometimes blastulation results in an embryo with a low number of inner call mass cells. A reduction in the inner cell mass may then cause the demise of some half embryos. This idea was tested directly by another group of studies that used differential stains to compare the number of cells in the inner cell mass to the total number of cells. Embryos with a low cell number at blastulation had a lower inner cell mass ratio and exhibited a lower viability than embryos with a higher cell number at blastulation. These experiments further showed that maintaining the half embryos in vivo, even in a nonpregnant uterus, increased the formation of the inner cell mass, resulting in a higher inner cell mass ratio and higher viability than that achieved in vitro. In a study examining the long-term effects of embryo splitting, genetically identical embryos were either bisected and immediately placed into foster mothers or were left intact and placed into foster mothers. The half embryos were generally less successful and those born had a higher rate of neonatal mortality than did the controls. However, some of the neonatal mortality might have been because the half embryos were born from smaller litters than the controls. Small litters usually result in longer pregnancies, which are also associated with higher neonatal mortality rates. Comparison of other phenotypic characteristics of the neonates born revealed no significant differences between half and whole embryos in measures such as the age at eye opening or growth curves. Thus, by birth, some type of regulation has taken place. The studies taken together argue strongly for the existence of crisis points for half embryos including blastulation and implantation. Further study of the mechanisms of regulation in micromanipulated embryos not only can add to our basic knowledge of reproductive biology, but can also elucidate important requirements for successful splitting of embryos in different species.

Implantation [9]

Implantation, one of the most poorly understood processes in reproductive biology, involves a complex interaction between the embryo and the cells of the uterus. There are also significant species differences in the process of implantation that can be generally divided

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into three types. The type of implantation found in rabbits is called fusion; whereas the type found in rodents is called displacement. Implantation in humans and other primates is called intrusive and is the most important to understand for the purposes of this report.

The general model for implantation in the human begins with the attachment of the blastocyst to the endometrial wall. At this point, the outer layer of trophectoderm of the embryo proliferate and differentiate into two types of trophoblast cells. The cells close to the embryo become cytotrophoblasts and the cells in direct contact with the endometrium fuse with each other to become the syncytiotrophoblast. A syncytium is a large, multinucleated, mass of cytoplasm formed by the fusion of many separate cytotrophoblastic cells into one. The syncytiotrophoblast acts to erode a path into the endometrial tissue that, in turn, allows the embryo to burrow into the wall of the uterus. Some cells of the endometrium are induced to build up large stores of nutrient molecules, which are then released into the extracellular spaces (close to the embryo) as these cells degenerate. The changes in endometrial cells can be visualized with a number of experimental techniques and are called the decidual reaction. Eventually, the trophoblastic cells penetrate deeply enough into the uterine wall to contact maternal blood vessels. The interactions between the trophoblast and these blood vessels results in the formation of the vascular supply of the placenta.

The study of implantation involves the search for and identification of factors controlling uterine receptivity and maternal recognition of pregnancy. Some of these factors seem to be produced and released by the cells of the blastocyst prior to implantation. The understanding of the biology of implantation has far-reaching implications for human and non-human reproduction. Both in the natural reproductive process and in the practice of medically assisted conception, there is a huge gap between the number of successfully fertilized eggs and the number of offspring born. A large part of this gap can be accounted for by the loss of embryos at or around the point of implantation. For example, in human IVFET practice, 60 to 70 percent of the eggs are fertilized, but only twenty to thirty percent of the embryos placed in the uterus result in an ongoing pregnancy. Moreover, it has been estimated that, in couples without fertility problems and not practicing birth control, a pregnancy occurs in one out of three menstrual cycles in which a fertilized egg is present. In sheep and cattle, twenty to forty percent of the fertilized eggs do not survive and, in the pig, the lose rate is thirty to forty percent.

Early embryonic loss is common across species. While some early embryonic loss can be explained by factors such as heat stress, nutritional deficiencies, and genetic abnormalities, it has been proposed that much of this loss results from three other possible causes. First, the uterine environment is probably only narrowly permissive to implantation by an embryo. Second, embryos may fail to signal their presence and, consequently, fail to induce the necessary hormonal and uterine changes necessary to maintain pregnancy. Third, embryos may be rejected as a foreign body by the mother's immune system.

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The idea that the uterus allows implantation only under precise conditions almost certainly underlies the problems discussed elsewhere in this chapter relating to synchrony between the embryonic stage and the uterine stage. Lack of synchrony has been demonstrated to be a problem in all species examined. It has been shown that if precise synchrony cannot be achieved, it is generally better to transfer embryos that are more advanced than the uterine environment. This makes sense if one considers that, following ovulation, the corpus luteum in the ovary begins to produce progesterone, which acts to prepare the uterus for pregnancy.If the corpus luteum degenerates, the drop in progesterone causes the uterine lining to be shed resulting in menstruation. A hormone secreted by the trophectoderm, chorionic gonadotropin, prevents the degeneration of the corpus luteum and, thus, maintains the progesterone secretion. It is reasonable then that an embryo that is transferred to a uterus more advanced than the embryo may not produce enough chorionic gonadotropin to be able to rescue the corpus luteum from degeneration.

There have been attempts to control the state of the uterine lining through the administration of exogenous hormones. Attempts to advance the uterine environment of pigs by administration of progesterone have been unsuccessful. However, because pig embryos secrete estrogen which also affects the uterine lining, exogenous estrogen was tried. This approach worked, but only during a narrow time window and, if given too early, was actually toxic to the embryos. Other research suggests that it is too simplistic to assume administration of one type of hormone would be sufficient to control the uterine environment. Analysis of proteins synthesized and secreted by embryos indicates that there are a number of different chemicals made by embryos that can affect the uterus or the corpus luteum. It is nevertheless probable that some of the embryo-produced proteins cause changes in the secretions of uterine cells that are necessary and/or supportive of an implanting embryo.

A number of proteins are also secreted by endometrial cells, and much research has focused upon isolation of these proteins, analysis of their functions, and mapping the changing patterns of protein synthesis associated with implantation. One experimental strategy has been to label implantation sites in mouse uterus with a dye, pontamine blue. This dye, when injected into the veins of a pregnant mouse, causes implantation sites to be colored blue without staining the rest of the endometrium. After this labeling, investigators can then remove the uterus and maintain explanta of it in culture. Using precursors for protein synthesis that have been radioactively labeled, investigators can label the new proteins synthesized and compare the pattern of proteins from implantation and non-implantation sites. This strategy has shown that the rate of protein accumulation in implantation sites is up to forty percent greater than in other sites. Much of the increase is accounted for by increases in proteins destined to be secreted from endometrial cells. Further work compared the pattern of protein synthesis from natural implantation sites to those that were mechanically induced to look like implantation sites. Although there was an increase in the synthesis of some proteins by the mechanical method, some proteins were not increased. Such a finding

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argues for there being embryo-dependent protein synthesis in the endometrium at implantation sites. As will be discussed in a later part of this chapter, it is known that embryos release certain factors and proteins that directly affect the cells of the endometrium. At this point, however, it is useful to consider some of the proteins found to be released by endometrial cells.

Endometrial protein 15 has been isolated in humans. This protein is only present in the secretory phase and first trimester of pregnancy. The presence of this protein has been shown to change depending upon the hormonal state, but so far no function has been ascribed to this protein. Endometrial protein 14 has been found in human and mouse endometria. This protein is identical in structure to an IGF, which is present in numerous sites of the body. In rabbits, a protein called uteroglobin has been shown to be induced by progesterone and present in early pregnancy or in pseudopregnancy. This protein has a variety of functions including an anti-inflammatory action. Factors isolated from the mouse include epidermal growth factor and a type of colony stimulating factor, both of which increase in response to progesterone. It is surmised that these, and probably other, growth factors may function to control the proliferation of the placenta.

Studies of endometrial proteins in the pig are of special interest since this species does not exhibit the intrusive type of implantation. In fact, the maternal and embryonic blood supplies never come close to each other in the pig as they do in humans and other primates. Such a situation sets up potential problems in bringing nutrients to the developing embryo. Nutrients must be released from the endometrium and diffuse to the embryo. Proteins have been found in the pig that seem to help in this respect. For example, uteroferrin is a protein that carries iron to the embryo. Another type of protein, which increases in response to progesterone, transports water insoluble molecules to the embryo. These are called retinol-binding proteins. Two other proteins--plasmin and trypsin inhibitors--protect the uterine cells from destruction by embryonic enzymes, and lysozyme protects against infection.

In summary, many classes of proteins are produced and secreted by endometrial cells in response to estrogen, progesterone, or embryo-produced factors. While some of these proteins serve nutritive functions in species in which the embryo either invades the endometrium late or not at all, other functions of these proteins are not clear. It is probable that early embryonic loss is, in some cases, due to abnormal expression of uterins proteins. A poor quality environment for the embryo could result from either excessively low levels of necessary proteins or excessively high levels of proteins, which could be toxic to the embryo.

Less research has been devoted to examining the incidence or mechanisms of immunological rejection of embryos. The uterus is not isolated from the immune system, especially in species in which the maternal and fetal blood are hardly separated. An embryo is like a transplanted organ, which must be transplanted to a site that is protected

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from immune cells or an immunosuppressive drug must be given. In some species, an implanting embryo causes what looks very much like an allergic inflammatory reaction in the endometrium, that is, dilatation of blood vessels, proliferation of blood capillaries, and fluid accumulation. In addition, it is certain that cell-surface antigens capable of eliciting an immune reaction on the part of the mother are eventually present on embryos. Thus, the reasons why all embryos are not rejected immunologically are interesting and important areas of research. It has been found that embryos release substances, especially interferon, that, in other tissues, act to suppress or modulate immune cell activity. Therefore, it is possible that the embryo acts to control locally the immune response of the mother by secretion of immunomodulating substances. However, more research is needed before such a mechanism can be established.

The study of implantation in human beings is particularly difficult because there is no in vitro model available. Such a barrier has special importance because of the wide species differences that exist in regard to implantation. Findings from other species cannot be assumed to be true for human beings. However, it is possible to examine some aspects of human implantation by using in vitro methods recently developed. One of these methods involves the isolation of cytotrophoblast cells. It has been found that cytotrophoblast cells placed into culture under certain conditions will proliferate and differentiate into syncytiotrophoblast. Such studies have shown conclusively that syncytiotrophoblast is derived from cytotrophoblasts. In a culture dish, this differentiation takes place in two steps. First, the trophoblast cells aggregate. Then they fuse to become a syncytium. It is likely that this process is mediated by cell-adhesion molecules or CAMs, which have important roles in cell-cell interactions during development of the nervous system and other tissues. The CAMs are produced by the trophoblast cells because blockade of proteinsynthesis prevents aggregation of the cells. It is also known that the process depends on calcium, since it does not occur in culture medium lacking calcium. In contrast to the aggregation step, the mechanisms responsible for fusion of the trophoblast are entirely unknown.

The interactions of cultured trophoblastic cells with various components of extracellular matrix have also been studied. Extracellular matrix is simply the intercellular space and its component molecules, which are usually synthesized by the surrounding cells and secreted into the extracellular space. The exact composition of the matrix varies from tissue to tissue, but it is important in the uterus since interactions between it and the blastocyst occur during implantation. For trophoblast aggregation and membrane fusion to occur, there must be serum proteins in the culture media and/or the dishes must be coated with extracellular matrix proteins. It is thought that the serum is required because it contains matrix proteins, (e.g., fibronectin). If these proteins are added, the serum is no longer required. Collagen, fibronectin, and laminin are structural proteins thet are plentiful in many extracellular spaces.

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The importance of extracellular matrix was underscored by additional experiments that used endometrial explants to co-culture the trophoblast cells. This method is more like the natural situation, since the trophoblast differentiates into syncytiotrophoblast, as before, but now interacts with endometrial tissue. These experiments showed that trophoblast from first trimester and term placentas bind to epithelial cells of secretory endometrium. The cells also bind to cut surfaces of the explants, areas where extracellular matrix was exposed. After 24 to 48 hours, a zone of tissue necrosis developed in the endometrial explants where the syncytiotrophoblast associated with the tissue. Moreover, trophoblastic cells bind to and invade nests of cultured endometrial gland cells. The trophoblastic cells dislodge the endometrial cells and penetrate beneath them in a process resembling intrusive implantation. These experiments suggest that the extracellular matrix always permits attachment and differentiation of the syncytiotrophoblast. That fact has important implications in disease states where the epithelium is eroded and, especially, when the lining of the fallopian tubes is eroded. Such states may cause implantation to occur in either less desirable sites or in totally undesirable sites such as the fallopian tubes.

The mechanisms for trophoblast invasion of the endometrium are not known. There are a variety of proteases (enzymes which break down proteins) that have been implicated, such as plasminogen activator. In some strains of mice, blastocysts are less invasive of the endometrium apparently because they produce less plasminogen activator. Cultured human trophoblasts produce plasminogen activator (urokinase). Urokinase may degrade fibronectin and activate other enzymes (e.g., collagenase). It is possible that the action of proteases like urokinase could be controlled by the presence of call surface receptors for the enzyme, which localize its actions, and by specific plasminogen activator inhibitors,two of which are known to be generated by trophoblastic cells. This control would be important since the invasion of the endometrium must have an endpoint since embryos do not burrow all the way through the endometrial wall.

It is expected that many of the biochemical interactions between the trophoblast and the endometrium would occur through paracrine mechanisms. Substances released by the syncytiotrophoblast and cytotrophoblast could reach a high local concentration in areas of the endometrium. Substances known to have powerful paracrine effects in other tissues may play a role in implantation including protein and steroid hormones. Substances released from both the trophoblast and the endometrial cells, could account for the inductive and interdependent changes in both these tissues. Further research, however, is clearly necessary to answer these questions.

On the basis of the above studies, investigators have proposed a working model for implantation in the human. The first step of this model holds that the trophoblast binds to specific endometrial cell-adhesion molecules. After binding, the trophoblast penetrates the endometrium and

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attaches to the extracellular matrix by mechanisms involving proteases and controlled by specific inhibitors. This model, then, forms an adequate starting point for further research. Knowledge of the biochemistry is particularly deficient and could be furthered by the application of technologies such as monoclonal antibodies and DNA probes for particular cell-adhesion molecules. Identification of the molecular mechanisms of placenta formation may determine the factors that regulate the proteases. Such findings could be of great use in understanding disease states like toxemia in which abnormal invasion of trophoblastic cells is suspected to be a cause. Finally, investigators see a real need for the institution of a national or international registry of IVFET programs to track the incidence of abnormal implantation and correlate those data with the types of ovarian stimulation protocols used and hormone replacement therapy given. In this way, the disturbances of implantation caused by various aspects of the procedures of IVFET could be separated from these which occur naturally.

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Medically Assisted Conception: An Agenda for Research : Report of a Study
http://www.nap.edu/catalog.php?record_id=18586

ENDNOTES

1. Based on talk by Dr. Zev Rosenwaks.

2. Summary of talk given by Dr. Neal First.

3. Summary of talks given by Dr. Robert F. Williams and Dr. Joanne Fortune.

4. Summary of talks given by Dr. Ray Hammerstedt, Dr. John Eppig, and Dr. Stanley Leibo. For additional information on cryobiology, see S.P. Leibo, Cryobiology: Preservation of Mammalian Embryos. In Genetic Engineering of Animals, J.W. Evans and A. Hollaender (Eds.), Plenum Press, New York, 1985.

5. Summary of talks given by Dr. William W. Wright and Dr. James M. Hammond.

6. Summary of talk by Dr. Jonathan Van Blerkom.

7. Combined summary of talks by Dr. Patricia N. Saling, Dr. Richard Schultz, and Dr. Jurrien Dean.

8. Summaries of talks by Dr. John D. Biggers, Dr. Peter Braude, and Dr. Virginia Papaioannou; summary of talk by Dr. Harry M. Weitlauf, which was given in the fertilization section of the workshop, is combined in this chapter with talks included under implantation. For additional information, see Braude, P.R., Bolton, V.N., and Moore, S., Human gene expression first occurs between the four- and eight-cell stages of pre-implantation development. Nature 332:459-461, 1988; and Braude, P.R., Bolton, V.N., and Johnson, M.H., The use of human pre-embryos in infertility research. In Embryo Research: Yes or No, Ciba Foundation Study Group, G. Bock and M. O'Connor (Eds.), Tavistock Press, United Kingdom, pp. 63-82, 1986.

9. Combined summaries of talks given by Dr. Harry M. Weitlauf, Dr. Jerome Strauss, and Dr. R. Michael Roberts. For additional detail see Weitlauf, H.M. and Suda-Hartman, M., Changes in secreted uterine proteins associated with embryo implantation in the mouse. Journal of Reproduction and Fertility. 84: 539-549, 1988.

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http://www.nap.edu/catalog.php?record_id=18586

CHAPTER 3
BARRIERS TO PROGRESS IN IN VITRO FERTILIZATION AND EMBRYO TRANSFER

Since the birth of Louise Brown in England in 1978, in vitro fertilization with embryo replacement has become an established method of treatment for certain types of infertility that do not respond to alternative methods of treatment. In 1988, 169 centers that offered IVFET were identified in the United States. Appreciable demand for IVFET exists despite some disadvantages, such as a higher rate of ectopic pregnancy than occurs with normal conception, high costs ($6,700 at one of the nation's oldest programs (Jones, 1989) that are often not reimbursed by third-party payers, prolonged treatment time, and uncertain results. Data from 41 clinics that reported to the American Fertility Society indicate the success rate is improving. In 1985, 14.1 percent of stimulation cycles resulted in clinical pregnancies. In 1986 this figure rose to 16.9 percent (Fertility and Sterility, 1988a). However, the proportion of women entering treatment who attain a live birth is far lower -- only 8.9 percent of oocyte retrievals ended in live birth (Journal of the American Medical Association, 1988a). Moreover, some women start treatment, but for a variety of reasons fail to reach the stage of egg retrieval. Improvements in the success rate of IVFET are probably not the result of significant changes in the methods of ovarian stimulation or laboratory procedures. Rather, improvement may be attributed to a decline in the average age of women undergoing IVFET, increasing experience of clinician and laboratory personnel, and more rigorous criteria for selection of patients (Van Blerkom, 1989).

Why are the odds for successful human IVFET so low? The answer lies in part in the state of knowledge of reproductive and developmental processes. There are a large number of unanswered questions whose resolution would have major impacts on the success of IVFET. Some of the most basic questions include: How does one identify a viable embryo? What are the effects of cryopreservation of gametes and embryos? How many embryos should be transferred? Why do some embroys fail to continue to develop after apparently normal development? What are the physiological effects of hormonal treatments? What are the factors that control oocyte maturation? What regulates dominance of ovarian follicles and what are the mechanisms of implantation? One of the most important factors that limits the success of medically assisted conception procedures is the natural frequency of egg and embryo wastage. The most reliable and reproducible estimates of inherent developmental failure in gametes indicate that about 20 to 25 percent of meiotically mature human eggs obtained after hyperstimulation have genetic abnormalities, about 7 percent of spermatozoa are genetically abnormal, and about 10 to 15 percent of unfertilized oocytes contain cytoplasmic aberrations or pathologies. In addition, the vast majority of human embryos derived

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from fertilization in vivo or in vitro will not develop to a blastocyst. Developmental failure during cleavage is fairly typical. Even embryos that do develop to the blastocyst stage can contain abnormalities in cell structure or number that are not consistent with postimplantation development (blighted ovum, for example). Collectively, developmental failure in the early stages of embryonic development appears to be the normal situation for the human species.

The state of clinical practice of IVFET today is limited by lack of knowledge of some of the basic reproductive biology involved. The reason for this is, at least in part, due to the many ethical questions raised by research in pursuit of the needed information. Difficulties in resolving these issues have caused the research to be deprived of federal funding.

This chapter first reviews the social and ethical barriers to progress in research, and then focuses on the scientific barriers. Brief note is made some of the ethical issues that are raised by the practice of in vitro fertilization, and the history of federal involvement in considerations of human fetal research is described. Some nations have examined the ethical questions and have issued guidelines or regulations. These will be described. Finally, the major science barriers that have slowed progress in human and animal practice of IVFET will be noted.

Ethical and Social Issues

Some of the ethical or social issues that arise from the various forms of assisted conception are unrelated to decisions about the progress of research. Examples of these are questions about the protection of the rights of gamete donors, gestational parents, and social parents; decisions on the fate of concepti, embryos, and fetuses; the moral status of human potential; the ownership of cryopreserved embryos whose parents have divorced or died; the confidentiality of sperm donors; and questions of the right of a child born of medically assisted conception to know his or her complete parentage. Other fundamental questions revolve around concepts of the right of an individual to reproduce, the sale of gametes and embryos, and whether infertility should be defined as a disease.

Some ethical questions have a direct bearing on research, and have had important consequences for the funding of research. The major questions focus on the status of the embryo at each stage of its development. How the embryo is regarded dictates what is morally acceptable to do to it. The implications for what may be done with an embryo differ according to when human life is thought to begin, whether any biological material containing the potential for human life is considered to be sacrosanct, and other such questions.

The determination of the moral status of the embryo drives such decisions as what level of risk to the embryo is acceptable in the practice of IVFET and research; whether it is possible to "discard"

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embryos, even when placement in a uterus diminishes the probability of other embryos surviving; whether it is permissible to create embryos for research purposes, and up to what stage of development of the embryo should research be allowed, and with what risk to its normal development.

At one end of this spectrum of thought is the position taken by the Roman Catholic Church, which holds that life begins with the union of two haploid gametes to form a diploid zygote. The Vatican's Instruction on Respect for Human Life states that "from the first moment of its existence until birth . . . no moral distinction is considered between zygotes, pre-embryos, embryos or fetuses" (cited in Fertility and Sterility, 1988b). Therefore, the absolute sanctity accorded to post-natal human life begins with the zygote. This concept makes it impossible to discard spare embryos or use them for research purposes. At the other end of the spectrum is the position that an embryo is merely biological material like any other group of living cells. The special value that might be attached to that material results from the expectations or aspirations of others. Those who hold this view also note that a large number of naturally conceived embryos fail to develop after implantation, thus discarding excess embryos created by IVFET can be viewed as a parallel process (Office of Technology Assessment, 1988).

Midway between these two positions is one that holds that "the human embryo is entitled to profound respect; but this respect does not necessarily encompass the full legal and moral rights attributed to a person" (Department of Health, Education, and Welfare, 1979). This is the position taken in 1979 by the Ethics Advisory Board established in the Department of Health Education and Welfare (DHEW) to advise the Secretary on matters concerning embryo research, and to review specific research proposals. Holding this position, the board concluded that research involving IVFET was acceptable on embryos up to 14 days after fertilization. Other constraints on such research included that it should be designed to improve the safety and efficacy of IVFET, and that the information could not be obtained by other means.

The Federal Government and Embryo Research

(Unless otherwise noted, this discussion is derived from a summary of fetal research issues by the Association of American Medical Colleges (1988)).

As mentioned earlier, the Ethics Advisory Board was chartered in 1977 to review applications for federal support of research. This resulted from growing concern about fetal or embryo research, which in turn stemmed from a concern about federal funding of research involving human subjects. Policy concerning research on human subjects had been slowly evolving since the 1960s. A study group was convened at NIH to develop guidelines, and a National Advisory Commission on Health Science and Society was proposed by Senator Walter Mondale in 1968 to examine developments in medical research. This commission was not established,

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but the impetus from the effort helped put in place the later National Commission for the Protection of Human Subjects. Following reports of the infamous Tuskegee syphilis experiments, DHEW recommended that Congress establish a permanent body to regulate federally funded research using human subjects.

In the 1970s the abortion issue became linked to the issue of embryo research. After the Roe v. Wade decision made legal abortion under certain specific conditions, concern developed that women would be pressured into having abortions and the sale of aborted embryos and fetuses might occur. Many states that had constrained embryo research through abortion statutes proposed separate legislation to ban such research. In 1974, the federal government created the National Commission for the Protection of Human Subjects (P.L. 93-348). Until this commission reported to Congress, research on the living fetus was prohibited unless it was used to help that fetus survive. This Commission did not consider the topic of research on the embryo or IVFET. The Commission however recommended establishing an Ethics Advisory Board (EAB) to review requests for conduct of embryo and IVFET research. Without such review, requests could not be processed. In 1975, DHEW issued regulations based on the findings of the commission. These regulations prohibited federally funded research unless the risk to the embryo was no greater than "those ordinarily encountered in daily life or during the performance of routine physical or psychological examinations or tests," or for therapeutic purposes (cited in Association of American Medical Colleges, 1988).

The EAB met for the first time in 1978. In 1980, the Secretary of Health and Human Services allowed the EAB charter to expire and, without explanation, failed to renew it. Thus, federal funding of embryo and IVFET research was, in effect, prohibited. As a result, embryo research has relied on private funding from patient care revenues, pharmaceutical companies, and university budgets. It is estimated that, were the EAB active today, it would receive more than 100 grant applications (Office of Technology Assessment, 1988). In addition, the federal government has lacked a means for controlling the direction of, or practices used in, such research. As was noted in 1979, "Departmental involvement might help to resolve questions of risk and avoid abuse by encouraging well-designed research by qualified scientists. Such involvement might also help to shape the use of the procedures through regulation and by example." (Department of Health Education and Welfare, 1979). This opportunity has been lost for a number of years.

In 1985, two events occurred in relation to the future of embryo research. Congress created a Biomedical Ethics Board. This board, composed of members of Congress, six senators and six representatives, is to examine the protection of human subjects in biomedical research.

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Included in its brief are studies of the ethical implications of embryo research. By 1987, the 12 congressional members of the board had been appointed. The Board established a 14 member Biomedical Ethics Advisory Committee composed of scientists, physicians, clergy, and others. After months of deadlock over disagreements that mainly focused on appointees views on abortion and other ethical issues to do with definitions of human life, the advisory committee was named (American Medical News, 1987). The first meeting did not take place until September 1988, and the date for submission of a report on fetal research had passed (Capron, 1989).

Appointing the leaders and members of this Biomedical Ethics Board and the Biomedical Ethics Advisory Committee has been fraught with delays, as has the initiation of the activities of these bodies. Adequate funding is not assured. Alexander Capron, Chairman of the Biomedical Ethics Advisory Committee notes that "one can be hopeful, but not certain that [these groups] will be able to fulfill their statutory purposes." (Capron, 1989) Whether progress in enabling embryo research to proceed will be made is open to doubt in today's political environment.

In addition to these congressional groups there has been activity in the executive arm of government. The Department of Health and Human Services intends to reactivate the EAB that expired in 1980. In September 1988, notice of a draft charter was published in the Congressional Record. The 60-day comment period has elapsed and a final revised charter is pending.

The EAB, if reactivated, will develop specific guidelines to review NIH research proposals. It seems likely that the guidelines will be influenced by the broader policy formed by the congressional groups.

Domestic and Foreign Decisions on Embryo Research

The two professional societies in the United States that represent the physicians most involved in human IVFET have considered ethical questions about the practice of IVFET and embryo research. The American College of Obstetricians and Gynecologists (ACOG) issued an opinion that IVFET is a clinically applicable procedure that can be practiced if certain standards are assured and the ethical issues are considered (American College of Obstetricians and Gynecologists, 1984). Two years later the ACOG Committee on Ethics (American College of Obstetricians and Gynecologists, 1986) issued a statement that acknowledged the ethical issues that were posed by the creation of embryos outside a uterus,

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focusing particularly on the dilemma posed by surplus embryos and the acceptability of research using early human embryos. The ACOG committee recommended standards to guide such research, including that human embryos could be used only if nonhuman embryos would not provide the needed knowledge. It also recommended halting research on embryos that had reached the age of 14 days. The American Fertility Society (AFS) also issued a report in 1986. This document notes eight technologies that the AFS Ethics Committee found ethically acceptable, including basic IVFET, the use of donor eggs, and the use of frozen sperm. Six procedures were found suitable for clinical experimentation, including the use of frozen eggs, and experiments on embryos up to 14 days (Fertility and Sterility, 1986). A year later, after consideration of the Vatican's Instruction for Human Life in its Origin and on the Dignity of Procreation, issued by the Congregation for the Doctrine of Faith, the AFS issued another report. This report stated that progressive degrees of respect are due with progressive development of embryos, and that experimentation can be justified and is necessary if the human condition is to be improved (Fertility and Sterility, 1988b).

Despite public debate of the issues, statements issued by religious and professional groups, and other evidence of public interest, the government of the United States, since 1979, has not followed the lead of a number of nations that have systematically examined issues related to human IVFET. However, state statutes relevant to embryo research exist. Twenty-five states restrict embryo research, and 19 of those have language that could be interpreted as prohibiting some "pre-embryo" research (Office of Technology Assessment, 1988). Internationally, some governments have issued rules or regulations to control either the clinical practice, level of research, or both. LeRoy Walters, Director of Bioethics at the Kennedy Institute of Ethics, Georgetown University, has reviewed statements on the new reproductive technologies made by committees in other countries (Walters, 1987). He notes that, since 1979, at least 85 statements have been prepared by committees representing at least 25 countries. Walters focused his analysis on the reports listed in Figure 1. His analysis of issues in human embryo research notes that four Australian committees found research on early (preimplantation) embryos to be ethically unacceptable. Eleven committees approved at least some kinds of early embryo research. Six of those accept such research only on embryos left over from clinical activities. Five committee statements (including the 1979 DHEW Ethics Advisory Board) would allow the creation of embryos for research purposes. Although the majority of committees favor limiting research on embryos to up to fourteen days, one committee allowed it only to seven days, and one only through the first cleavage (for details see Figure 1). An additional, important, position statement was issued by the Vatican in 1987. The Vatican found unacceptable IVFET, artificial insemination, and embryo research if it is not for the direct benefit of the embryo on which the procedure is performed.

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In sum, numerous groups have wrestled with questions related to the ethical problems of human embryo or fetal research. Some have based their conclusions on religious tenets, some on an interpretation of scientific knowledge, some on a mixture of both. It should not be surprising that there are substantial differences in the conclusions drawn by these groups. However, to the extent that each has laid out the foundations of its arguments, the debate about the acceptability of embryo research has been advanced.

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Figure 1

ISSUES IN HUMAN EMBRYO RESEARCH

1. Acceptability in principle

A HEW Ethics Advisory Board 1979	B Waller I-IV, Victoria 1982-84	C South Australia 1984	D Demack, Queensland 1984	E Council for Science and Society U.K. 1984	F Warnock, United Kingdom 1984	G Tasmania I-II 1984-85	H Ontario Law Reform Commission 1985
yes	yes	no	no	yes	yes	no[1]	yes

I Australia, Family Law Council I-II 1984-85	J Benda, German Federal Republic 1985	K Spain, Special Commission 1986	L American Fertility Society 1986	M Western Australia I-II 1984-86	N Dutch Health Council I-II 1984-86	O National Ethics Committee, France I-II 1984-86	P New South Wales I-II 1987
no	yes	yes[2]	yes[3]	yes	yes[3,4]	yes[5]	yes

[1]But a National Bio-Technology Committee should periodically review current community attitudes on the subject.
[2]But only with embryos that are nonviable and not implantable.
[3]Early embryos called "pre-embryos."
[4]"By way of exception."
[5]But only after prior approval by, and under the supervision of, the National Ethics Committee.

Source: LeRoy Walters, Unpublished Paper Presented at the Annual Meeting, American Fertility Society, Atlanta, George. October 11, 1988.

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Figure 1 (cont)

ISSUES IN HUMAN EMBRYO RESEARCH

2. Sources of embryos
   a. Acceptability of using left-over embryos from clinical IVF
   b. Acceptability of creating embryos especially for research purposes

	A HEW Ethics Advisory Board 1979	B Waller I-IV, Victoria 1982-84	C South Australia 1984	D Demack, Queensland 1984	E Council for Science and Society U.K. 1984	F Warnock, United Kingdom 1984	G Tasmania I-II 1984-85	H Ontario Law Reform Commission 1985
a.	yes	yes	---	---	yes	yes	---	yes
b.	yes	no	---	---	(yes)	yes	---	yes

	I Australia, Family Law Council I-II 1984-85	J Benda, German Federal Republic 1985	K Spain, Special Commission 1986	L American Fertility Society 1986	M Western Australia I-II 1984-86	N Dutch Health Council I-II 1984-86	O National Ethics Committee, France I-II 1984-86	P New South Wales I-II 1987
a.	---	yes	yes[1]	yes	yes	yes[2]	yes[3]	yes
b.	---	no	no	yes	no	no	no	yes

[1] But only with embryos that are nonviable and not implantable.
[2] "By way of exception."
[3] But only after prior approval by, and under the supervision of, the National Ethics Committee.

Source: LeRoy Walters, Unpublished Paper Presented at the Annual Meeting, American Fertility Society, Atlanta, Georgia. October 11, 1988.

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Figure 1 (cont)

ISSUES IN HUMAN EMBRYO RESEARCH

3. Permissible duration of embryo culture in vitro

A HEW Ethics Advisory Board 1979	B Waller I-IV, Victoria 1982-84	C South Australia 1984	D Demack, Queensland 1984	E Council for Science and Society U.K. 1984	F Warnock, United Kingdom 1984	G Tasmania I-II 1984-85	H Ontario Law Reform Commission 1985
14 days	14 days	---	---	2 wks.	14 days	---	14 days[1]

I Australia, Family Law Council I-II 1984-85	J Benda, German Federal Republic 1985	K Spain, Special Commission 1986	L American Fertility Society 1986	M Western Australia I-II 1984-86	N Dutch Health Council I-II 1984-86	O National Ethics Committee, France I-II 1984-86	P New South Wales I-II 1987
---	Thru the first cleavages	14 days	14 days	14 days	14 days	7 days	14 days

[1]Subject to alteration if circumstances warrant.

Source: LeRoy Walters, Unpublished Paper Presented at the Annual Meeting, American Fertility Society, Atlanta, Georgia. October 11, 1988.

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Figure 1 (continued)

THE SIXTEEN EXTENDED COMMITTEE STATEMENTS ON THE NEW REPRODUCTIVE TECHNOLOGIES: 1979-1987

A. U.S., Department of Health, Education, and Welfare (HEW), Ethics Advisory Board, HEW Support of Research Involving Human In Vitro Fertilization and Embryo Transfer (May 4, 1979)

B. Victoria, Australia, Committee to Consider the Social, Ethical and Legal Issues Arising from In Vitro Fertilization

1. Interim Report (= Waller I) (September 1982)
2. Issues Paper on Donor Gametes (= Waller II) (April 1983)
3. Report on Donor Gametes and In Vitro Fertilization (= Waller III) (August 1983)
4. Report on the Disposition of Embryo Produced by In Vitro Fertilization (= Waller IV) (August 1984)

C. South Australia, Report of the working Party on In Vitro Fertilization and Artificial Insemination by Donor (January 1984

D. Queensland, Australia, Report of the Special Committee Appointed by the Queensland Government to Enquire into the Laws Relating to Artificial Insemination, In Vitro Fertilization and Other Related Matters (= Demack Queensland, report) (March 1, 1984)

E. Council for Science and Society (United Kingdom), Working Party, Human Procreation: Ethical Aspects of the New Techniques (May 1984)

F. United Kingdom, Department of Health and Social Security, Report of the Committee of Inquiry into Human Fertilization and Embryology (= Warnock, United Kingdom) (July 1984)

G. Tasmania, Australia, Committee to Investigate Artificial Conception and Related Matters

1. **Interim Report (= Tasmania I) (December 1984)**
2. **Final Report (= Tasmania II) (June 1985)**

H. Ontario, Law Reform Commission, Report on Human Artificial Reproduction and Related Matters (tabled June 13, 1985)

I. Australia, Family Law Council

1. Interim Report (= Family Law Council I) (July 1984)
2. Creating Children: A Uniform Approach to the Law and Practice of Reproductive Technology in Australia (= Family Law Council II) (July 1985)

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http://www.nap.edu/catalog.php?record_id=18586

J. Federal Republic of Germany, Minister for Research and Technology and Justice Minister, Working Group, In Vitro Fertilization, Genome Analysis, and Gene Therapy (= Benda, German Federal Republic) (November 1985)

K. Spain, Congress of Deputies, General Secretariat, Special Commission for the Study of Human In Vitro Fertilization and Artificial Insemination, Report (April 10, 1986)

L. American Fertility Society, Ethics Committee, Ethical Considerations of the New Reproductive Technologies (September 1986)

M. Western Australia, Committee to Enquire into the Social, Legal and Ethical Issues Relating to In Vitro Fertilization and Its Supervision

1. Interim Report (= Western Australia I) (August 1984)
2. Report (= Western Australia II) (October 1986)

N. Netherlands, Health Council, Committee on In Vitro Fertilization and Artificial Insemination by Donor

1. Interim Report on In Vitro Fertilization (= Dutch Health Council I) (October 10, 1984)
2. Report on Artificial Reproduction, with Special Reference to In Vitro Fertilization, Artificial Insemination with Donor Sperm, and Surrogate Motherhood (= Dutch Health Council II) (October 16, 1986)

O. France, National Consultative Committee on Ethics

1. Report on Ethical Problems Related to Techniques of Artificial Reproduction (= National Ethics Committee I) (October 23, 1984)
2. Report on Research Involving Human Embryos In Vitro and Their Use for Medical and Scientific Purposes (= National Ethics Committee II) (December 15, 1986)

P. New South Wales, Australia, Law Reform Commission

1. Surrogate Motherhood: Australian Public Opinion (= New South Wales I) (May 1987)
2. Artificial Conception, Discussion Paper 2: In Vitro Fertilization (= New South Wales II) (July 1987)

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Other Barriers to Scientific Progress

The research agenda developed by the Institute of Medicine, Board on Agriculture committee (see Chapter 4) identifies many areas in which further research would make major contributions to improvements in medically assisted conception in humans and animals. As noted in Chapter 1, improving the application of medically assisted conception would benefit society in several ways, including making possible the preservation of some endangered species, as well as providing some relief from infertility and making production of meat and milk more economical. Progress, however, has been delayed by a number of factors, most of which stem from the generally controversial nature of concepts surrounding issues in reproduction, and specific controversies related to elements important to reproductive research -- zygotes and embryos.

As a prerequisite to developing recommendations to advance the science base of medically assisted conception, the committee first examined the impediments. In addition to the ethical considerations referred to earlier, the following barriers deserve particular emphasis: 1) deficiencies in the scientific base of this area of reproductive biology; 2) the resources available for research in this area of science; 3) lack of mechanisms for communication within the reproductive research community among basic scientists, clinicians, and animal husbandry scientists; 4) fear of abuses of reproductive technologies; 5) a relative lack of sympathy and understanding of the problems being experienced by infertile couples; 6) lack of a cohesive public interest group favoring such research, in contrast to well organized opposition; 7) limited health insurance coverage of IVFET services; 8) limited sources of research materials for experiments relating to human beings and to animals; and 9) the present dilemma of our society concerning how to handle our ethical disagreements.

Almost none of these factors is independent of the others; rather a causal relationship is often found. Some of the barriers identified may be amenable to policy intervention. The following sections briefly review the impact of the major barriers to progress and how they have come to be in existence.

Deficiencies in the Science Base

The papers presented at the committee's workshop and the research agenda developed from that workshop (see Chapters 2 and 4) indicate deficiencies in the scientific underpinnings of reproductive biology, and identify many areas in which further research efforts would make major contributions to improvements in medically assisted conception. The deficiencies are on three levels: basic science knowledge; knowledge needed to improve the technologies being used for medically assisted conception, such as cryobiology; and knowledge needed to improve both human and animal clinical practice of IVFET. As explained in detail in

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Chapter 2, deficiencies in all three levels limit both the quality and efficiency of the practice of IVFET.

Research directed toward improving the reproduction of food-producing animals such as cattle is often ahead of research in the area of human reproduction. As a result, the use of in vitro sperm capacitation, artificial insemination with frozen semen, transfer of frozen embryos, oocyte harvesting from dead animals (the Lazarus effect), and the splitting of the dividing cells of the early embryo into more than one individual animal, are routinely used in animal husbandry. A number of factors have allowed medically assisted conception to proceed at a faster pace in animals than in humans: research using food-producing animals has been stimulated by an expected economic return on the research investment, a larger volume of materials is available for study, and research has not been subject to as many ethical constraints. The latter two factors noted — greater volume of material available for study and fewer ethical constraints — derive from a difference in the esteem accorded to animals and humans by many members of society. The economic value of IVFET, which acts as a spur to progress in the animal area, is to some, although lesser extent, paralleled in human IVFET. That there is an economic value to IVFET for humans is indicated by the fact that clinicians can charge, and people are willing to pay, quite substantial fees for the procedure. This has enabled a small amount of research to be conducted in the absence of federal help.

Research Funding

Approximately $115 million annually is spent on research in human reproductive processes. The major sources of private funding (the Ford, Rockefeller, and A.W. Mellon foundations, and the Population Council) in 1985 together contributed $2.8 million to research related to reproductive processes. This includes grants to investigate male and female infertility, fertilization, zygote transport, preimplantation development and implantation, and reproductive endocrinology (National Institutes of Health, 1988). Federal agencies are the principal support for research. In 1986 they provided $109 million for research in reproductive processes (National Institutes of Health, undated).

The National Institute of Child Health and Human Development (NICHD) of the National Institutes of Health provides the major portion of federal support of human reproductive research. Approximately $100 million per year, one third of the budget of NICHD, is spent in the reproductive sciences branch of NICHD on contracts and grants to academic centers and to the NIH centers for reproductive biology. In addition, the Contraceptive Development Branch, which uses mainly contract mechanisms to support contraceptive technologies, contributes to basic reproductive research. Federal funds for research relating to agricultural animal reproduction are available from the U.S. Department of Agriculture.

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Funding for basic research in human reproductive biology is undoubtedly constrained by the lack of vocal and focused advocate groups that have been formed for some diseases such as cancer and heart disease. Lacking such a constituency, a major increase in federal support is unlikely. Yet, as the research agenda in Chapter 4 suggests, additional investment in research in reproductive biology can expect to be repaid in improvements in the reproductive health of the nation.

Lack of Communication Among Researchers

Discussion with the scientists and clinicians at the committee's workshop revealed an underuse of available mechanisms for communications among the individuals involved with various aspects of research in reproductive biology — basic, clinical, animal sciences, etc. Also revealed was a desire for greater communication to allow cross-fertilization of ideas and development of ongoing relationships among investigators pursuing similar approaches to problems.

Difficulty in establishing communications is sometimes caused by the locale of investigators and the way science is organized. Basic scientists are frequently Ph.D.s in basic science departments such as anatomy or physiology. They often use animal models for their studies and have little or no contact with patients. Clinicians have an M.D. degree with subsequent residency training and are generally housed in clinical departments such as obstetrics and gynecology. They are frequently heavily involved with patient care. S2me researchers working in animal sciences have veterinarian degrees, others have basic science degrees, and frequently work in departments related to animal sciences or agricultural practices and deal with animals of economic importance.

The excitement and stimulation experienced by the individuals attending this IOM workshop, which encouraged interaction with individuals from differing backgrounds, underscored how infrequently meaningful interactions among these communities occur, and how useful this interaction could be.

Societal Concerns

The fear of abuses of reproductive technologies, the seeming lack of societal sympathy and understanding of the problems experienced by infertile couples, and the lack of a cohesive public interest support group are to some extent related phenomena.

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Fears of abuses of the new reproductive technologies are legitimate concerns in a society that has not consistently shown an ability to monitor the ethical implications of scientific progress. Nor has this society established deliberative bodies that might reassure the public that applications of reproductive science will be controlled in ways sensitive to the delicate balance between preserving ethical standards and improving the human condition.

This is not to imply that concerns about the uses of new reproductive technologies can be easily quieted by the creation of deliberative or regulatory bodies. Rather, that to date the U.S. has not grappled with the issues as some other nations have done. However, if renewed efforts to put in place the needed mechanisms are successful in dispelling some of the worst fears about research abuses and the new technologies, scientific progress may be furthered.

It is clear that concerns are deeply felt. The strength of opposition to legal abortion demonstrates concerns that result from religious conviction and concerns about social values. The strength of support for legal abortions demonstrates concern for the freedom of individuals and the social impact of restrictive reproductive policies. Advances in science are making available technologies that open up new opportunities. Some view these technologies as opportunities to enhance the human condition. Others view them as potentially damaging to the social fabric. Indeed, there already exist technologies that are used in animal husbandry that members of society will not wish to have applied to human beings. Animals can presently be cloned, and genetic alterations have been made to animal germ cell lines. These are some of the issues that deserve careful scrutiny and public input. It is therefore important that society has mechanisms to consider and to debate the application of new technological capabilities and to ultimately provide safeguards against their misuse.

Reinforcing a sense of discomfort with some of the possible uses and abuses of new reproductive technologies, and also constraining efforts to advance in this area, may be a sense that infertility is a less vital concern than, for example, life threatening illness, disability, or more generally accepted signs of ill health. Although private third-party payers reimbursed roughly 70 percent of total non-IVFET infertility expenditures in 1980, the services are usually covered only if "they are associated with medical conditions or diseases requiring diagnosis and/or treatment and not solely related to infertility and fertilization" (Office of Technology Assessment, 1988). Insurance coverage for IVFET is still limited, in part because the procedure is considered experimental. However, coverage is available for many parts of the IVFET workup (Office of Technology Assessment, 1988). If concerns about the allocation of constrained health care resources continue to be high on the nation's agenda, it seems likely that less "acute" conditions such as infertility will lose out to more generally accepted forms of medical care, and will be available only to those who can pay the price. Also, a high cost

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procedure such as IVFET, likely to benefit a very limited number of people, will be further compromised in a cost-conscious health services environment.

That there is no single cohesive public interest group pressing for heightened research in the area of reproductive biology might be partially explained by the complex religious and ethical issues involved with certain types of research, by the lack of a cohesive research community, by fear of abuses in the area of reproductive technology, by a low public awareness of the size of the nation's infertility problem and the complex effect of infertility on individuals and couples, or a combination of these and other factors.

Sources of Research Material

The committee's workshop provided many excellent examples of instances in which information about reproductive physiology derived from animal models has been useful in understanding human physiology. Instances, however, were also presented in which the human processes have marked differences from those elucidated by animal models, and to move forward in understanding the human physiology requires the use of human tissue. An example of this is investigation of reasons for the developmental failure of human embryos, and cryopreservation of human eggs and zygotes. Preservation of human tissue by freezing allows excess zygotes to be thawed for use at a later time. At present, we do not know all the possible negative consequences of freezing and thawing of the human tissue, nor do we know the optimum conditions under which these procedures should be done. However, cryopreservation has crept into clinical practice despite these uncertainties. This has happened in part because of the dilemma caused by excess embryos. Discarding them is unacceptable to some. Therefore cryopreservation has become an acceptable option.

In addition, although specific primates are good models for some aspects of human reproductive physiology, there are only a limited number of monkeys of desirable species in captivity and many of them are presently being used for AIDS research. To optimize the use of each primate, it would be helpful to develop noninvasive procedures for steps such as oocyte harvesting. Such a procedure would also be of great importance to patients.

The committee believes that restrictions on the amount of material available for research use is slowing the rate of progress in developing a scientific base for IVFET and the technological advances that would make it more efficient. These limitations are the result of a concern that human material be used with proper respect, and that the use of animals for research purposes be controlled to ensure that they are not abused.

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There exist ways, however, of enlarging the amount of available research material while preserving a proper consideration for these issues. Making available material from organs that have been surgically removed would increase the supply of human tissue. For instance, when an organ such as the ovary is removed for medical reasons, it might be made available for harvesting oocytes and eggs.

In sum, as discussed in many places in this report, ethical, and social concerns underlie many of the barriers to progress in reproductive research and the clinical application of new technologies. Lack of mechanisms for resolving such disagreements has inhibited progress in the necessary debate that must precede the development of policies. It is encouraging that Congress has established the Biomedical Ethics Boards, and that the executive branch of government is taking steps to reactivate the Ethics Advisory Board. If these bodies become functional the nation will have taken steps to establish entities to handle difficult issues in reproductive biology.

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CHAPTER 4

RESEARCH AGENDA AND RECOMMENDATIONS

This chapter lists the areas of research that, in the view of the committee and workshop participants, hold particular promise in advancing the knowledge base and efficacy of medically assisted conception for humans and other animals. In addition, this chapter outlines the conclusions and specific recommendations that were made by the committee following the workshop. The research agenda items are organized into three main categories. The first and largest category includes promising areas of basic scientific investigation. The second category encompasses areas in which improvements in technology are needed. The third category includes questions that can be approached by cooperative agreements among clinical centers involved in IVFET. In addition to organizing the research agenda into common areas, these categories also generally reflect the different funding mechanisms that might be required to support such investigations. Research grants to support basic science projects could be obtained through competitive grant programs at the National Institutes of Health or the National Science Foundation. Formula funds and competitive grants can be obtained from the United States Department of Agriculture. Technological development, however, could be supported by contracts from projects already supported by the NIH or other agencies. Coordination of information from IVFET clinics could be accomplished by any one of a number of agencies or professional societies.

Research Agenda

The topics listed below are areas in which further research was recommended by workshop participants and committee members. It therefore reflects the areas of investigation considered to be promising by the committee. Work in these areas is expected to increase understanding of the biology of reproduction with the hope that increased knowledge will eventually lead to improvements in practice of IVFET in humans or other animals, or to advances in the area of contraception. Research areas are listed here in summary form and apply both to lower animals and human beings unless specifically noted. The reader should refer to Chapter 2 for detailed discussion of these areas.

Basic Science

Male Gametogenesis

- Definition of the role of cell adhesion molecules in interactions between Sertoli cells and developing sperm cells.

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- Understanding the function of differential protein synthesis in different stages of sperm development.

- Determination of the role of paracrine factors including fibroblast growth factor, somatomedin C, epidermal growth factor, and interleukin-1 on the development and differentiation of male gametes.

- Structural analysis to identify normal and abnormal sperm and the development of markers for abnormal sperm.

- Understanding of the biochemistry of sperm capacitation.

Female Gametogenesis

- Analysis of the effects of superovulation or hormonal stimulation protocols on oocyte development and maturation. This work should also examine differences among species.

- Development of ways to matura oocytes in vitro.

- Investigation of ways to naturally stimulate oocyte and follicular development.

- Investigation into the biochemistry of meiotic arrest and the factors, such as cyclic AMP, purines, calcium, and maturation-promoting factor, that may mediate this process.

- Development of ways to produce or synthesize hormones from non-human primates to be used in ovarian stimulation.

- Definition of the role of ovarian estrogen in oocyte maturation and ovulation and the interactions between estrogen and paracrine factors including fibroblast and epidermal growth factors, insulin-like growth factor, transforming growth factor, and inhibin.

- Definition of the point at which oocytes become sensitive to factors that influence their development.

- Elucidation of the processes that underlie oocyte depletion, to determine why oocytes are lost at a predictable rate throughout life.

- Investigation into ways to augment natural hormone release.

- Investigation into the biochemistry of protein synthesis and modification in ovarian cells.

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Fertilization

- Investigation into the biophysics of cell membranes as it relates to sperm and egg interactions at fertilization.

- Continued investigation to identify the genes for zona proteins in various species, especially humans.

- Further delineation of the role of zona proteins, especially ZP2 and ZP3, in sperm binding.

- Understanding of the biochemistry of the modification of zona proteins in preventing polyspermy.

- Elucidation of the molecular determinants of antibody formation to zona proteins and their possible role in contraceptive strategies.

- Definition of the biochemical mechanisms of the cortical reaction in the egg and the effects of this reaction on zona proteins.

- Determination of the physiological significance of germinal vesicle breakdown and the biochemistry of sperm chromatin decondensation.

- Definition of the molecular events associated with formation of the male and female pronuclei.

- Definition of the molecular events during zygote formation and the first cleavage.

Preimplantation Development

- Definition of the metabolic requirements of early embryos at different stages.

- Determination of embryonic gene expression.

- Assessing the potential of individual embryonic cells and defining the point at which embryonic cells are committed to particular fates.

- Identification of substances produced by early embryos that signal changes in the uterus prior to implantation.

- Improvements in embryo multiplication and embryo splitting, especially for food-producing animals.

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Implantation

- Definition of the biochemical events that make the uterus permissive to implantation.
- Definition of the factors released by embryos that cause endometrial changes at the site of implanation.
- Identification of the role of embryo-released factors in suppressing the immune responses of the mother.
- Isolation and analysis of substances released by endometrial cells and their effects on embryos.
- Continued work with in vitro models of human implantation to study the biochemistry and mechanisms of embryo-endometrial interactions, especially the role of extracellular matrix proteins and the biochemistry of trophoblast invasion of the endometrium.

Technological Advances

- Improved cryopreservation techniques, including freezing and thawing protocols for eggs and embryos.
- Improved resolution of ultrasonography for localization and noninvasive harvest of oocytes, eggs, and embryos—would have particular usefulness for non-human primates and food-producing animals.
- Development of new culture media and methods for in vitro maturation of oocytes.
- Development of safe methods of biopsy of early embryos for preimplantation diagnosis of genetic diseases.

Clinical Research Opportunities

The following areas are those in which a coordinated data collection effort across IVFET clinical centers would improve the quality and success rates of IVFET nationally and, possibly, internationally.

- Evaluation of hormonal stimulation protocols in terms of number of oocytes harvested, quality of oocytes, and rate of fertilization success.
- Documentation on the incidence of abnormal implantation rates in IVFET practice and correlation of incidence with particular stimulation protocol used.

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- Collection of information regarding the incidence of abnormal eggs and embryos, failed fertilization, and developmental arrest of embryos.

- Analysis of data pertaining to synchronization of embryonic stage with endometrial stage and development of methods to improve synchronization.

- Collection of information on sharing of spare oocytes and arrested embryos for research purposes.

Conclusions and Recommendations

Developing Research Policy

The lack of a mechanism for dealing with ethical disagreement over the use of embryos in research has slowed the rate of progress in research by, in effect, placing a moratorium on the use of federal funds for eight years. This has had undesirable results: the human clinical practice of IVFET is less effective than it might have been had research progressed at a faster pace; other socially desirable goals such as improved contraception, better techniques to preserve endangered species, and more cost-effective methods of producing food have developed at a pace slower than is optimal.

The recent appointment of the Biomedical Advisory Committee by the Biomedical Ethics Board, to report to Congress by November 1990 on embryo research issues, could be a step toward a solution. The committee also applauds the intention to revive the Ethics Advisory Board of the Department of Health and Human Services to rule on the ethical acceptability of research relating to human embryos, which is required before federal funding of such a research grant can be considered. However, until these groups become fully functional and show evidence of progress, their impact must remain in question.

If these groups can assume leadership roles in resolving the difficult issues of reproductive research, and develop guidelines for research that are based on information provided by science, as well as on concepts that are ethically acceptable to society, research in reproduction will be able to move forward. But if these groups become paralyzed because of political considerations or an inability to develop a framework for the resolution of differences of opinion, another organization should take over the role. The committee recommends that, if the groups currently being formed fail to come to conclusions concerning embryo and fetal research, a non-governmental organization should be established to develop guidelines for embryo and fetal research based on the most advanced knowledge that science can muster, and with serious consideration of the expressed values of society. The group should be

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composed of individuals with expertise in the relevant scientific disciplines, representatives of the lay public, and experts in the legal, ethical and social issues. The organization should be housed in an institution that would allow it to conduct its deliberations free from undue pressures from political and special interest groups. A model for such activities can be found in the Voluntary Licensing Authority of Great Britain. This group was established after a governmental committee recommended a statutory licensing authority. Recognizing that it would be some time before legislation would be completed, the Medical Research Council and the Royal College of Obstetricians jointly sponsored the voluntary body. Five of its 13 members are lay people. The group has a mandate to undertake five major activities:

- to approve a code of practice on research related to human fertilization and embryology;
- to invite all centers, clinicians and scientists engaged in research on IVF to submit their work for approval and licensing;
- to visit each center before it is granted a license;
- to report to the sponsoring organizations; and
- to make known publicly the details of both approved and unapproved work.

Basic Science Foundations

The number and range of topics included in this chapter's research agenda indicate the exciting potential for productive scientific exploration. Funding that would allow investigation of the areas targeted in the research agenda would allow significant advances to be made in understanding reproductive processes. The committee believes that fundamental research to enhance the basic science foundations of reproductive biology should be stimulated and supported. This includes studies of human beings, laboratory animal models, and food-producing animals. The knowledge that would be generated is fundamental to an understanding of how to reverse infertility, to new approaches in the area of contraception, and to increasing the world's food supply.

This report attempts to define not only the state of knowledge in reproductive science relating to IVFET, but to assess and highlight some research opportunities. It is important that aspects of male as well as female reproductive biology be studied. It is also important that investigators make use of some opportunities that are largely ignored today. These opportunities occur as a result of clinical activities as

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well as research activities. For example, eggs that have failed to become fertilized can become material for studies seeking chromosomal abnormalities. Fertilized eggs that fail to develop may be used to investigate the reasons for developmental failure, and to answer questions about the natural wastage that occurs in pregnancy.

Research of this type would generally be funded by the grant mechanisms of the National Institutes of Health and by the United States Department of Agriculture. For adequate attention to a research agenda, however, administrators of NIH need mechanisms to insure that studies on a variety of these topics are being funded. For this an RFP or a contract mechanism might be necessary to insure adequate coverage of the various aspects of reproductive biology. Foundations are also encouraged to consider increased support for basic studies in reproduction. A stable funding base for reproductive research will encourage young, well-trained scientists to pursue research in reproductive biology.

The committee recommends that a vigorous program for funding of a basic science agenda in reproductive biology be maintained in a coordinated fashion by an appropriate office in the National Institutes of Health.

Applied Research

Research needs to be stimulated concerning technologies used in medically assisted conception in food producing animals and in human beings. Lack of support in these areas is leading to inadequate scientific underpinnings for safe and effective clinical practice. An example of a technique used, but not carefully evaluated for possible detrimental effects, is freezing eggs or zygotes. Further experiments should be conducted to assess the effects on safety and viability of this technology which is standard practice in many IVFET clinics. Other areas of technology that need to be developed include less invasive ways to retrieve oocytes, ways to mature oocytes in vitro, and ways to assess the quality of spermatozoa or oocytes to be used for fertilization.

Since these studies would be technology driven, it is unlikely that the research will be funded by a grant mechanism, therefore a contract mechanism should be used.

The committee recommends that applied research into technologies used in medically assisted conception be undertaken to provide a firm foundation for the safe and effective practice of in vitro fertilization and embryo transfer. Such applied research should be coordinated by the appropriate office at the National Institutes of Health.

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Clinical Research Opportunities

Perhaps the most obvious missed opportunity is the failure to learn from the diverse experiences of the approximately 160 clinical programs that provide human IVFET. In addition to scientific questions, there are questions to do with the organization of clinics and the outcomes of procedures. The answers to these questions would enable practitioners to work more effectively, and enable policy makers to make decisions on the basis of the best available information. Such questions include: Who provides the quality assurance for facilities and procedures used (including the training of those providing services)? How are protocols developed? How standard are they? What are the outcomes for each protocol? What is the role of Institutional Review Boards in the establishment and maintenance of clinical facilities?

Some specific questions to be answered from data that could be collected from clinics include: What ovarian stimulation protocols are the most successful? Is chromosomal damage associated with any of the procedures used? What indicates whether a zygote will implant successfully? Much data relating to these questions already exist in the centers. Other questions may require a cooperative prospective study to be undertaken.

Clinical IVFET centers can also provide unique opportunities for important studies. For example, human eggs that fail to fertilize in vitro are material that could be used to investigate the phenomenon of failed fertilization. Improved understanding of reasons for failure has implications for reversing infertility and ensuring conception. Another area for investigation for which the IVFET centers are particularly suited is arrested zygotic development. In producing embryos by in vitro fertilization, it has been noted that certain zygotes stop dividing. The reasons for such arrested zygotic development are unknown. Have these cells died? Can ons tell from studying the newly dividing zygotes which ones will be most likely to initiate a successful pregnancy?

Failed fertilization in the peri-implantation period in both the natural situation and during the procedures of IVFET is an important area to be understood. Developmental failure in the early stages of embryonic development appears to be a normal event for the human species. Whether this loss results from lethal genetic defects, chromosomal anomalies, biochemical or structural abnormalities, or technical difficulties needs further elucidation. Research that seeks to understand the basis of reproductive failure, and its relationship to hyperstimulation should be encouraged. Coordinated studies utilizing the mass of material and experience from IVFET centers could begin to answer these and other questions. In addition, the experience of centers performing IVFET for farm animals could provide valuable information.

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The committee applauds the activities of the various professional societies that have issued non-binding statements about the quality of practice of IVF. The American Fertility Society has also provided a voluntary registry for centers. On the animal side, The American Embryo Transfer Society has started to establish some quality measures for commercial bovine embryo transfer. States in general get involved by licensing physicians, hospitals, and clinics, and as yet have not played a dominant role in assuring quality of care.

The committee believes that a mechanism is needed to monitor and evaluate clinical practice so that existing information that is relatively easy and inexpensive to collect can be disseminated. This would enable clinicians to build on the broadly based experience of the community and help ensure that patients have access to information about developments in IVFET and to well-informed physicians. The committee recommends that a mechanism for multi-centered data collection be established to monitor and evaluate human and veterinary practices of medically assisted conception in order to improve the safety, effectiveness, and quality of clinical practice. A cooperative group composed of the relevant professional societies should be established to fund and initiate data collection under the direction of an inter-society council composed of representatives of each participating organization.

Improving Communications

The IOM Workshop on Medically Assisted Conception brought together researchers from basic science, clinical practice, and animal sciences. The resulting interaction was viewed as extremely helpful by investigators from each of these communities. The committee recommends that a mechanism (or multiple mechanisms) be found for fostering continued communication between researchers in diverse areas of reproductive science. The initiative should come both from NIH research administrators who could sponsor additional workshop opportunities and from the professional societies, either individually or through an intersociety council.

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