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Embryo culture, stem cells and experimental modification of the embryonic genome

An interview with Professor Ralph Brinster

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The factors that determine an individual's professional orientation and research direction are often largely fortuitous. This was certainly the case for Ralph Brinster. Born in 1932, he spent his childhood on a small farm in Cedar Grove, New Jersey, a rural area forty miles west of New York City. This background established his early interest in animals and served as the basis for a decision to enter the School of Agriculture at Rutgers University, the State University of New Jersey. During these undergraduate years, he became fascinated by the process of animal development and the intricacies involved in regulating animal physiology, a fascination that ultimately led to his decision to continue into postgraduate school. However, this was the time of the Korean Conflict, and his educational plans were interrupted by military service, including a year of duty in Korea. When Ralph returned to his studies in 1956, he entered the School of Veterinary Medicine at the University of Pennsylvania and financed this part of his education with savings made during service in the Air Force and benefits provided to military veterans. During his veterinary training, Ralph began to focus his attention on the earliest stages of animal development, in part because this is the time of significant embryonic death, but also because of a growing interest in germ cells and their importance in species maintenance and medicine. To continue studies on these embryos and cells, Ralph entered graduate school at the University of Pennsylvania in 1960 and began studying for a Ph.D. in physiology. As a graduate student, Ralph was influenced by John Brobeck, Chairman of Physiology, who placed considerable importance on student initiative and originality in selecting a thesis research problem. With the encouragement and support of Professor Brobeck, Ralph began to explore and expand his interest in the first stages of mammalian development, from the union of gametes at fertilization to implantation of the embryo in the uterus. Because of the flexibility afforded students in his department, Ralph also studied at The Jackson Laboratory in Bar Harbor, Maine, and the Marine Biological Laboratory in Woods Hole, Massachusetts, as part of his graduate training. Through this gradual process of experience and training, Ralph's early interest in animals became increasingly focused, and he identified germ cells and early embryo development as an area of fundamental importance and exciting possibilities.

At that time in 1960, when Ralph was beginning graduate training, there was a long history of work on early embryos, but Ralph felt that progress in understanding these stages of development would be greatly facilitated by a good system to maintain and observe embryos outside the body. In fact, he became convinced that such a system was essential for the types of studies and embryo manipulation that he believed were possible. Therefore, to pursue his interests, Ralph carried out Ph.D. thesis research in the laboratory of John Biggers. The studies were extremely difficult,

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and he was faced with the same experimental unreliability and complexities that had plagued others working on embryo culture during the previous fifty years. However, through determination and perseverance, Ralph discovered a system of placing embryos in small drops of medium under oil, which he published in 1963 (Brinster, 1963). This paper was fundamental to his future work and has become the foundation upon which almost all egg and embryo work of today is based. It was recognized and highlighted as a Citation Classic by the Institute for Scientific Information *(Current Contents/Life Sciences 33*:25, 1990). This is rather remarkable considering the small size of the field in the years following publication of the paper when most citations occur. Now the method is so widely used and accepted that no one would consider citing such an essential but common experimental process. Ralph used this method to study in detail the culture, metabolism, and biology of preimplantation embryos, particularly of the mouse, and published more than 60 papers on these topics during the next ten years. A major finding, one that stands as a second pillar underlying current work on mammalian embryos, was the identification of pyruvate rather than glucose as the major energy source of mouse embryos (Brinster, 1965a). This allowed the development of support medium for eggs and embryos of many species, and the formulations first established by Ralph became the basis for all subsequent derivations. These two contributions by Ralph, the culture technique and importance of pyruvate, were quickly adopted by all workers in the field and were the foundation for a great surge of interest and activity in research on early embryos. Many feel that his demonstration of a reliable egg culture method is the most important breakthrough in mammalian embryo research in the past 40 years, because it underlies and makes possible all the other magnificent accomplishments with embryo manipulation which now play such a prominent role in biological and medical research. An interesting comparison might be made to other areas where great advances followed the development or dramatic improvement of a technique. For example, while many will immediately recognize the names of Jonas Salk and Albert Sabin as great scientific contributors in the quest to eradicate polio, a much smaller group will be able to describe the efforts of John Enders, Thomas Weller, and Frederick Robbins who made possible this eradication by their detailed studies on cell culture that allowed the poliomyelitis virus to be grown reliably *in vitro*. It has often been said that biology is "method driven"; the improvement or development of a method will allow an explosion of new experiments and unique approaches.

Ralph now capitalized on his "new method" in studies to try and modify the embryo and its development in a unique manner. The work of Leroy Stevens and Barry Pierce caught the attention of this young scientist with an interest in germ cells and embryos. Thus began a second movement or phase in the scientific biography of Ralph Brinster. In an extremely creative process, Ralph decided that the work of these two scientists, who had described the origin and development of teratocarcinomas, suggested the existence of multipotent cells in older embryos which he believed might be useful in altering and studying normal differentiation. Ralph realized he could test his hypothesis by using a system developed independently by Andrzej Tarkowski and Beatriz Mintz and extended by Richard Gardner. This system allowed cells from preimplantation embryos of the same age to be mixed and reintroduced into a recipient uterus where they would produce a chimaeric animal. Brinster decided to test his hypothesis by transferring cells of different ages and types into mouse blastocysts to determine if the foreign cells would colonize the embryo and contribute to tissues of the adult. Between 1967 and 1969, he planned several types of experiments and began these studies in 1969. In the first experiments, he transplanted older embryo cells into blastocysts and produced a few chimaeric fetuses (Moustafa and Brinster, 1972a, b). This was important because it supported his ideas, but the system was inefficient. In the second experiments, he transplanted adult bone marrow cells to the blastocyst in an attempt to produce an adult animal containing bone marrow cells arising from the transplanted cells (Brinster, 1974). Evidence of embryo colonization by transplanted bone marrow cells was obtained, but no chimaeric adults. The difficulty of obtaining bone marrow stem cells and the poor cell marker system in those years presented formidable obstacles. However, the clarity and originality of Ralph's vision have recently been documented by scientists in Germany. Using modern techniques for bone marrow stem cell enrichment and a transgene cell marker, it has been demonstrated that the hypothesis was true. Donor bone marrow cells introduced into the blastocyst will colonize the fetus and occupy as well as function in the bone marrow of the chimaeric adult (Geiger *et al.,* 1998). In the third series of experiments, Ralph transplanted teratocarcinoma cells to the blastocyst cavity and generated a chimaeric adult animal with small areas of the coat containing agouti hair, like the mice from which the donor teratocarcinoma cells originated (Brinster, 1974). This was a fascinating and an important result for several reasons. First, it confirmed Ralph's hypothesis that the blastocyst could be colonized by cells that were not the same age as the blastocyst and, furthermore, were not even from the embryo, and these cells would contribute to the adult animal. Second, these transplanted donor cells were cells with cancer phenotype that would kill an animal in three weeks if injected into the peritoneal cavity. Therefore, the embryo could control the cells and prevent the development of a malignancy under certain conditions. Third, the donor cells were able to be cultured *in vitro* under appropriate conditions, which could allow genetic changes to be made. And fourth, the results suggested that the germ line of the chimaeric adult might also be subject to colonization by teratocarcinoma cells or other donor stem cells. These experiments by Ralph, between 1969 and 1974, produced a dramatic change in thinking, and the results were quickly confirmed by others. The experiments were clearly unique and innovative. Although Ralph used reagents available and known to many, these reagents were combined in a novel way to prove a hypothesis that introduced a new method in biology. If teratocarcinoma cells could be grown *in vitro*, genetically altered, and made to become part of a chimaeric adult, even the germ line, then a powerful tool to study development would be available. This objective proved to be difficult but attainable, and 12 years after the publication of Ralph's 1974 paper several laboratories reported germ line transmission of genetic changes that were introduced using the embryonic stem cell approach to transgenesis. Many contributed to the ultimate success, but belief in the concept first arose in scientists' minds and the quest began when it was demonstrated that teratocarcinoma cells could colonize a blastocyst and produce an adult chimaera. Using his culture system, a unique hypothesis, and a type of perseverance that has marked his entire scientific career, Ralph Brinster introduced an approach which ultimately subjected germ cell genes to experimental manipulation.

Ralph Brinster working in the laboratory at the Lippincott Building, School of Veterinary Medicine, University of Pennsylvania c. 1963.

While developing the system of stem cell transplantation to the blastocyst, Ralph also began to examine other possible ways to alter embryos or germ cells in order to learn about the process of differentiation. He initiated studies on germ cell fusion, nuclear transplantation, and the introduction of new chromosomes into fertilized eggs by microinjection. These approaches were made possible by the ease with which embryos could now be manipulated using the culture methods and media he had pioneered in the first years of his research. His studies soon led to the realization that recombinant DNA and not chromosomes could provide a way to introduce new genes into an egg. Thus, in 1979 he began experiments injecting nucleic acids into mouse eggs. These experiments were dramatically successful and were published in *Nature* and *Science* (Brinster *et al.*, 1980, 1981a). Nonetheless, they did not accomplish his original objective of introducing new genes permanently into the egg and resulting animal. Ralph continued to focus on this objective, and for one of his studies contacted Richard Palmiter to obtain a supply of messenger RNA. This turned out to be an important phone call, an event that gives rise to great stories in science, for these two scientists with immense talent in their respective areas began to collaborate in 1981. Almost immediately they produced transgenic mice from eggs injected with new genes and went on to publish more than 120 papers together over the course of the next ten years. Among these publications were ground breaking revelations about techniques, principles, and approaches for developing transgenic animals and elucidating mechanisms of gene regulation. Many of these contributions are referenced or described in the Interview and articles of this Special Issue. Particularly remarkable was the production of "giant mice" following the injection of growth hormone genes into eggs. The paper appeared in *Nature* (Palmiter *et al.*, 1982), and a picture of the mice captured the cover of the journal. Many textbooks now feature the picture. It represents the culmination of a dream of biologists to change experimentally individual genes of an animal and heralds the beginning of a new era in experimental biology and medicine. In a single picture, a great success is documented and a great potential exposed. The work over the previous 20 years leading up to this feat will surely stand as a landmark in biology. The ability to change selectively and permanently individual genes of a life form is a remarkable achievement. The fundamental and decisive position of the earlier work in this field is reflected by the almost simultaneous reporting of transgenic mice by six laboratories during a twelve month period beginning in December 1980. Such a great breakthrough accomplished by six groups independently surely indicates that the stage was set and the techniques ready. The ability to culture and manipulate eggs *in vitro* without loss of viability made the production of transgenic animals possible and facilitated the rapid spread of the technique to many laboratories throughout the world. Although Ralph Brinster is most often recognized for his tremendous accomplishments in the development of transgenic animals between 1980 and 1990, it is his pioneering work on egg culture that enabled such experiments to proceed so well for him and his collaborators as well as many others in science. These culture experiments were crucial and made possible the manipulations necessary to achieve stem cell transplantation, gene microinjection, and nuclear transfer or cloning.

In his most recent work, Ralph Brinster has again provided a novel approach to germ cell manipulation. He transplanted male germ line stem cells, spermatogonia, from a fertile to an infertile male where the donor stem cells produce spermatogenesis (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994). Ralph has once more made use of stem cell transplantation to gain access to germ cell genes. These imaginative experiments will allow a wide range of studies on male germ cells. It should eventually be possible to culture these stem cells and to modify their genes, thereby creating a new method for transgenesis. These latest experiments of Ralph Brinster represent another example of a vision and creativity that have marked his scientific career from its earliest days. Through his studies on germ cells and early embryos, he has made enormous contributions to biology, and these contributions have been valuable in their application to medical problems. Perhaps the most interesting and provocative aspect of this body of work is the rapidity with which his innovative approaches have been adopted by other scientists. It is remarkable to look back and realize how much the exciting new techniques in germ line modification depend on the contributions of this individual.

While this overview has been brief, it is readily apparent that Ralph Brinster has had a brilliant career. The uniqueness and great value of his innovative work are widely known. He has published, with his students and collaborators, more than 320 articles in

journals representing diverse fields. Many of these papers were in prominent journals held in high esteem; for example, more than 80 appeared in *Cell* (19), *Nature* (30), *Proceedings of the National Academy of Sciences* (22) and *Science* (11). The quality of Brinster's publications reflects the fundamental nature and wide applicability of the research from his laboratory. As a result of these distinguished contributions, Professor Brinster has received many honors. He is a Member of the Institute of Medicine, National Academy of Sciences (1986); Fellow of the American Academy of Arts and Sciences (1986); Member of the National Academy of Sciences (1987); Fellow of the American Association for the Advancement of Science (1989); Fellow of the American Academy of Microbiology (1992); and Doctor Honoris Causa in Medicine, University of the Basque Country, Spain (1994). Professors Brinster and Palmiter were jointly awarded the Charles-Léopold Mayer Prize (1994), the highest award given by the French Academy of Sciences; and Professors Brinster and Beatriz Mintz received jointly the first March of Dimes Prize (1996). Recently Professor Brinster's contributions in laying the foundation for transgenic animals have been recognized by the Carl G. Hartman Award of the Society for the Study of Reproduction (1997); the John Scott Award of the City of Philadelphia (1997); and the prestigious Bower Award and Prize for Achievement in Science from the Franklin Institute (1997).

In addition to his distinguished accomplishments in research, Professor Brinster has been an active teacher at the School of Veterinary Medicine of the University of Pennsylvania, rising from the rank of Instructor in 1964 to Professor in 1970. He was appointed the Richard King Mellon Professor of Reproductive Physiology in 1975, a position in which he still continues. During this period, he has instructed professional and graduate students in reproductive physiology and developmental biology. He also established a program to train students simultaneously for a doctorate degree in veterinary medicine and a Ph.D. in graduate school. This program was established in 1969 and directed by Professor Brinster for 15 years. It continues today and more than 50 outstanding clinician-scientists have been trained in many biomedical disciplines. In this respect, it is appropriate to remember a comment of the great Spanish Nobel Laureate, Severo Ochoa, "University crisis is always linked to the crisis in the existence of good mentors". The University of Pennsylvania should be proud to have Professor Ralph Brinster, one of the most prestigious personalities in science of our time, as a member of its academic faculty.

The following interview was held in Ralph Brinster's laboratory at the School of Veterinary Medicine of the University of Pennsylvania in the summer of 1995, and was recently updated.

How did you decide to go into scientific research?

While I was studying veterinary medicine at the University of Pennsylvania, I became interested in the problem of infertility. This is a major clinical problem in veterinary medicine, especially in farm animals. Reproductive problems, of course, exist in all animals including humans, and in veterinary medicine they involve considerable loss. In farm animals, a high rate of embryonic loss occurs, generally between 30 and 40 per cent. It is actually higher in humans, over 50 per cent. Much of this embryonic loss in farm animals occurs near the time of implantation. Following completion of veterinary school in 1960, I decided to investigate this problem in more detail and started Ph.D. training in physiology at the University of Pennsylvania. As a thesis problem, research on early embryo development seemed an ideal way to understand the requirements of these early stages. One approach was to study embryos outside the body with an *in vitro* culture system. Large animal eggs were difficult to obtain; consequently, I decided to use mouse eggs as an experimental model system. At that time John Biggers was a professor at the School of Veterinary Medicine, and although his work was on *in vitro* organ culture of chick tibia bones, he had an interest in early mouse embryos. Therefore, I decided to do my Ph.D. thesis research in the laboratory of John Biggers.

In the recent history of mammalian experimental biology there is definitely a «before» and «after» your Ph.D. thesis work. Could you briefly describe the biology scene at the time you began this project?

Attempts to culture preimplantation stages of mammalian embryos had been made in several species before 1960, beginning just after the turn of the century, with most success in the rabbit. In many studies the culture media were poorly defined and success was limited. Because of its small size and economy of maintenance, I believed the mouse was a good choice as an experimental model. Furthermore, the genetics of the mouse, at that time, was better described than for any other mammalian species. There had been several reports on mouse egg culture, and Wesley Whitten had described an experiment in which lactate was used in the culture medium and some advanced 2-cell mouse eggs continued to develop (Whitten, 1957). This seemed like a good starting point. However, the culture methods being used at that time were very cumbersome, and I believed a good culture technique that was simple to use and very reproducible was essential for extensive experimental work on embryos. Therefore, my first objective was to develop and standardize a technique to culture embryos.

Your 1963 paper has recently been identified as a "Citation Classic" by the Institute for Scientific Information. The mammalian egg culture technique described in this paper is now universally used by scientists all over the world. What types of assays/tests did you have to perform, and what do you think are the reasons for the success and rapid spread of your original culture technique?

The method described in the 1963 paper was the result of a long-term comparison of many different approaches. Over twenty techniques were evaluated and modified (reviewed in Brinster, 1972). A small volume of culture medium to minimize loss of nutrients from the eggs seemed to be an important parameter. However, evaporation is a problem in such a system. To achieve my objective and overcome the problem, microdrops of medium were placed in a culture dish under mineral oil to prevent loss of water (Brinster, 1963). Actually, while the oil prevents the loss of most water, there is still a small amount of evaporation. Consequently, it is necessary to equilibrate with a humidified gas phase. I was fortunate in that oil appears to have other beneficial effects, because culture techniques, initially unsuccessful, were much better when an oil layer was placed over the culture media. One possible explanation is that gas exchange is altered by the oil layer,

A photograph taken during an international symposium «The Mammalian Oviduct», held at Washington State University, July 31 to August 4, 1967 when Ralph Brinster presented his extensive studies on mouse egg culture. *Brinster is shown, front row, sixth from the left. Others present are John Biggers (third row, first on the left), Anne McLaren (front row, first on the left) and M.C. Chang (fourth row, third from left), and other distinguished scientists working on embryos and reproduction.*

which may improve the oxidation-reduction potential within the egg. The technique published in 1963 made eggs easy to handle, easy to observe, and easy to place in predetermined groups of equal size, all of which greatly facilitated evaluation of results and made small differences in treatment effects readily determined. The culture dish with microdrops of medium under oil provided many advantages, and as a result it has allowed a wide range of egg manipulations to be performed in an efficient manner. Most of these complicated manipulative procedures would be nearly impossible without a simple and reliable culture technique for the eggs. I know this has been true for many of the experiments we undertook during the past 35 years. Nonetheless, I was surprised several years ago to learn the 1963 paper was so highly cited. Perhaps this reflects the explosive growth of interest in egg manipulation as a way to alter genes and modify animals. In transgenic techniques, in nuclear transplantation and in chimera formation, short- or long-term embryo culture is an integral and essential requirement of the experimental approach. Consequently, many scientists rely on this technique for their studies.

During the next decade you undertook extensive studies on mammalian embryo metabolism *in vitro* with the aim of defining the most significant biochemical mechanisms characterizing the first days of development. The immediate outcome of your investigations was the formulation of a chemically defined culture medium –Brinster's Medium– which has been the basis for all embryonic culture media developed to date. Among the most outstanding results of your research are the identification of pyruvate as the main energy source and the high level of lactic dehydrogenase (LDH) in the mouse egg. How do you assess these results, which are characteristic of the first stages of mammalian development?

After the culture technique was developed in 1963, I began to investigate the metabolic requirements needed to support egg development. In a long sequence of experiments that were essential to optimize egg development *in vitro*, energy sources, protein sources, pH, osmolarity, and a variety of nutrients were examined. These findings were published in a series of papers and reviews during the 1960s and 1970s (reviewed in Brinster, 1972,1973). One of the early and important findings was the central role of pyruvate in development of mouse eggs (Brinster, 1965a). It is the main energy source for newly ovulated mouse eggs up until the 8-cell stage (day 3). The necessity of pyruvate for survival is fundamental to all mammalian eggs, and a basic metabolic requirement regarding these early stages of life. The identification of pyruvate was a key factor for future studies on mouse eggs, because it allowed survival of the egg during various experimental manipulations. Unusual metabolism is involved in many aspects of early cleavage, but the requirement for pyruvate as an energy source as well as the very high level of lactate dehydrogenase (LDH), with a unique isozyme pattern, are characteristics that are laid down very early in oogenesis (Brinster, 1965b; Auerbach and Brinster, 1967). In the 1970s, we found that germ cells in the mid-gestation mouse embryo have the same general characteristics of metabolism as the newly ovulated egg, high use of pyruvate, high LDH activity, and the prominence of the B subunit of LDH (Brinster and Harstad, 1977). This is very interesting, because there are only a few days between when the cleavage stage embryo relies on pyruvate and mid-gestation in the mouse. Therefore, in the blastocyst there may be cells that maintain the characteristic reliance on pyruvate, and these cells may represent the lineage from which germ cells arise (Brinster, 1979). Alternatively, there may be a brief period when the cells actually lose their dependence on pyruvate and can utilize glucose and then revert to a dependence on pyruvate as they are

converted to germ cells. This is an issue that may now be able to be addressed with modern biochemical and molecular biology techniques. It provides an interesting question in developmental biology.

During your investigations you demonstrated a significant increase in protein turnover in preimplantation embryos, i.e., a decrease in protein content and a simultaneous increase in the rate of protein synthesis. How do you explain this in the light of present knowledge, and in particular, what do you think would be the role of the fertilization process in this regard?

I became interested in protein metabolism in the egg when we were studying the need for amino acids in culture. Early cleavage stages had a very simple requirement for protein and amino acids; in fact, much of the requirement for a protein macromolecule could be met by synthetic compounds that were not metabolized (Brinster, 1965c). It was also known that the size of the embryo diminished as it cleaved. On the basis of work with amino acids in the culture media and other protein substitutes, I decided to measure the actual protein content of the embryo. This information was important to understand protein metabolism in quantitative terms and to determine metabolic rate. Measurement of protein content in a small embryo (about 70 μm in diameter) in the early 1960s was extremely difficult, but after many months of effort and thousands of eggs we were successful (Brinster, 1967). It has been rewarding to see that these values, determined with not very sophisticated techniques, have been confirmed using more modern methods. The results of these experiments demonstrated that protein content of the embryo decreased significantly during cleavage, and led to our interest in protein synthesis and degradation in the embryo. Protein content decreased from the 1-cell to morula stage but, simultaneously, an increase in the rate of protein synthesis and a considerable turnover of protein occurred (Brinster *et al.*, 1976,1979). These findings, along with our studies on energy metabolism, indicated a dramatic increase in the metabolic activity of the embryo, particularly around the 8-cell stage (Brinster, 1972, 1973). Related to this work was the finding that embryo development was easily blocked by α-amanitin and other inhibitors of nucleic acid synthesis, suggesting an early and essential role of the embryonic genome in development (Levey *et al.*, 1977). Soon, other studies demonstrated that genes for specific proteins were activated very early in mouse embryo cleavage stages. A gradual accumulation of data indicated that there is an early activation of the embryonic genome in mammalian eggs (Levey *et al.*, 1978). This is significant when taken in the context of the 1960s and 1970s, when it was known that the embryonic genome in sea urchin and amphibian eggs were turned-on later in development. Therefore, the findings regarding energy metabolism, protein metabolism, nucleic acid metabolism, and gene activation set the mammalian egg apart from sea urchin and amphibian eggs and justified detailed studies in mammalian eggs that were necessary to understand how development proceeds.

In the late 1960s and early 1970s your scientific interest centered, however, on experiments aimed at artificially modifying the embryo genotype *in vitro*. You put together a series of experiments including nuclear transplantation, blastomere fusion and totipotent stem cell injection into blastocysts. Of these, the production of chimeras with embryonic carcinoma cells is one of the milestones in experimental biology and medicine of our time. What scientists helped prepare the ground for this idea to suddenly sprout in your mind and what was its later impact on other technologies arising from this experiment?

In the mid 1960s, while culture studies and metabolic studies were underway, I became increasingly interested in developing a method to modify the embryo to affect later development. My interest arose from a desire to understand the process by which a single cell, the egg, eventually produces a mature animal with diverse tissues and structures. Having developed a system that allowed one to manipulate eggs *in vitro* in an easy manner, I began to explore ways of modifying the genotype of the egg to influence future development. We tried a number of different experiments including, nuclear transplantation into the egg, fusion of embryo blastomeres using Sendai virus, and fusion of teratocarcinoma cells with eggs using Sendai virus. One of the most interesting observations of these experiments was the extraordinary control that the egg and oocyte cytoplasm exercised on the donor nucleus. Others also were pursuing this line of investigation (Graham, 1969), but inclusion of the foreign nucleus in a resulting animal could not be demonstrated. While our experiments in this area were partially successful, results were never sufficient for publication of a paper. However, another approach to modify the embryo seemed possible.

During the 1950s and 1960s, Leroy Stevens had described the development of mouse teratocarcinomas, tumors of the testis that contain nests of undifferentiated cells which are capable of producing many types of tissue (Stevens, 1967). While these tumors normally arise from male germ cells in the seminiferous tubules of the fetal testis, Stevens had shown that identical teratocarcinomas could be produced by transplanting preimplantation (3-day old) and early postimplantation (6-day old) embryos to the adult testis (Stevens, 1970). Individual cells of these teratocarcinomas had been shown by Barry Pierce to be multipotent and capable of developing into a variety of tissues (Kleinsmith and Pierce, 1964). Therefore, I thought that these multipotent teratocarcinoma cells (also called embryonal carcinoma cells), which can arise from embryos, might be able to colonize a mouse embryo and participate in subsequent development following microinjection of the cells into a blastocyst. If this occurred, then genetic characteristics of the cells might be altered by treatment (e.g., radiation or mutagenic agents) before transfer and thereby provide a mechanism for experimental manipulation of fetal development and the germ line. I knew these cells could be grown in culture (Rosenthal *et al.*, 1970), which would allow them to be altered genetically. Coincidentally, in the early 1960s, Andrzej Tarkowski and Beatrice Mintz had individually demonstrated that fusing two 8-cell mouse embryos (3-day old) would produce chimeric adults containing cells from each original embryo (Tarkowski, 1961,1963; Mintz, 1962,1964). This work was extended by Richard Gardner to show that transfer of cells from one blastocyst (4-day old) to another would also result in chimeric mice (Gardner, 1968). These studies producing chimeras had all involved mixing of cells from preimplantation embryos of the same developmental age (3- or 4-days old). However, I believed that there were multipotent cells in

older postimplantation embryos (e.g., 6- to 8-days old) as well as cells from teratocarcinomas that would colonize a blastocyst, thereby influencing differentiation of an embryo in a predictable way, and perhaps enter the germ line. I decided to test this hypothesis by transplanting cells from postimplantation embryos and teratocarcinomas into blastocysts to determine the ability of the transferred cells to incorporate into the developing embryo and contribute to various tissues, including germ cells.

To accomplish this objective, Laila Moustafa, a postdoctoral fellow in my laboratory, dispersed 4-day blastocysts, 8-day embryos and 12-day embryos, and injected the cells from each into recipient blastocysts which were transplanted back to pseudopregnant recipient mothers (Moustafa and Brinster, 1972a,b). We generated hundreds of fetuses and live animals from these injected blastocysts. Cells from blastocysts that were the same age as the recipient blastocyst often produced a chimera. Twelve-day old embryo cells never produced chimeras. However, 7- to 8-day embryos produced several fetuses with unequivocal dark eyes, which reflected the contribution of injected cells (Moustafa and Brinster, 1972b). These experiments suggested that multipotent and perhaps totipotent cells were present in the 7- to 8-day embryo. The results were encouraging, but the efficiency of generating chimeras was low. Furthermore, at that time there was no way to culture these embryo cells and introduce genetic changes. However, these studies did support my idea that the blastocyst could be colonized by nonsynchronous cells, and therefore multipotent teratocarcinoma cells might work.

Leroy Stevens and Ralph Brinster at the Lake Placid Symposium in 1987. *This symposium was held in their honor.*

Leroy Stevens provided me with a teratocarcinoma, OTT 6050, which was being maintained as an ascites tumor (Stevens, 1970). Much of the teratocarcinoma field at that time and in the 1970s was supplied with starting material by Leroy Stevens from The Jackson Laboratory. It is noteworthy that the majority of embryonic stem cell lines that are used today arise from mouse lines that Stevens developed during the late 1950s and 1960s. Soon after receiving the OTT 6050 teratocarcinoma, we began to inject teratocarcinoma cells into blastocysts, trying to use the inner cells of the embryoid bodies in this specialized ascites tumor. These studies were probably the most difficult I have ever undertaken for several reasons. First, the experiments were technically demanding and labor intensive; second, they were very inefficient. However, the most difficult aspect of the experiments was that there was very little indication of any progress along the way. The only thing that allowed the continuation of the work was my conviction that these teratocarcinoma cells that had come from an embryo and still maintained primitive multipotent capability would be able to colonize the embryo. After the production of several hundred offspring from these injections over several years, we generated a male chimeric mouse with agouti stripes on an albino background (Brinster, 1974). The stripes were an unequivocal indication of success, because the teratocarcinoma was from an agouti pigmented animal and the blastocyst from a random-bred albino mouse. This was the result I had been looking for since beginning this experimental approach in 1969.

Once knowing that a teratocarcinoma cell could colonize a blastocyst, it opened up the possibility of colonizing the blastocyst with other types of stem cells or with a pure population of teratocarcinoma stem cells. If this were now possible, then modifications could be made in the genotype of these cells that might influence the development of the chimeric animal or its progeny. Teratocarcinoma cells appeared to be a way to get at the genes in an animal and make a change. We were not able to demonstrate transmission of the teratocarcinoma genotype from the chimeric animal, but I was sure this would be possible by making more animals and using better cell preparations. The results of this study were published in 1974 and generated considerable excitement; however, the success was known in the research community before that time because I was in communication with Leroy Stevens and others who were very interested in the outcome of these unusual experiments. In 1975, Beatrice Mintz and Karl Illmensee confirmed and extended our observations (Mintz and Illmensee, 1975). Using the same OTT 6050 teratocarcinoma, they produced chimeras and demonstrated that germ line transmission of the teratocarcinoma genotype could be obtained. In Richard Gardner's laboratory, Virginia Papaioannou and Michael McBurney with Martin Evans injected cultured teratocarcinoma cells into blastocysts and reported that chimeras were produced (Papaioannou *et al.*, 1975). Thus, there were two confirmations of our studies the following year. Each of these papers demonstrated an important extension of our work, one indicating germ line contribution and the other chimera formation from cultured cells. It was clear that transplantation of teratocarcinoma stem cells to the early embryo was a method to modify development and that genetic alterations made in teratocarcinoma cells in culture could likely be found in germ cells of a resulting chimeric animal. This was an exciting direction

for developmental biology.

Our laboratory and several other laboratories began work on modifying the genes in these teratocarcinoma cells and injecting the cells into blastocysts. In the mid 1970s, only primitive methods by today's standards were available to make changes or mutate genes, e.g., X-ray or mutagenic agents. However, chimeric mice carrying genetically altered teratocarcinoma cells deficient in hypoxanthine phosphoribosyltransferase (HPRT) were produced in Mintz's laboratory, but the genetic change was not transmitted through the germ line because of a chromosomal defect in the cultured cells (Dewey *et al.*, 1977). Although a variety of studies were done using the teratocarcinoma cell approach, it became apparent that these cells were not efficient in making chimeras and often had chromosomal abnormalities, which prevented them from entering the germ line. Many of us began trying to develop a better cell line, either from teratocarcinomas or preimplantation embryos (from which it was known that teratocarcinomas could be produced). In 1981, Martin Evans with Matt Kaufman, and, independently, Gail Martin reported the production of embryonic stem (ES) cells from mouse blastocysts in culture (Evans and Kaufman, 1981; Martin, 1981). Allan Bradley, Elizabeth Robertson and others demonstrated that these cells were efficient in making chimeras and in entering the germ line (Bradley *et al.*, 1984; Robertson, 1986). During the 1980s, the ES cell was used to accomplish our original objective from the early 1970s, but recombinant DNA techniques made the experiments much better than I could have imagined might be possible at the beginning. Introduction of new genes into an animal using transfected ES cells injected into blastocysts was achieved by several laboratories (see for example Gossler *et al.*, 1986; Robertson *et al.*, 1986).

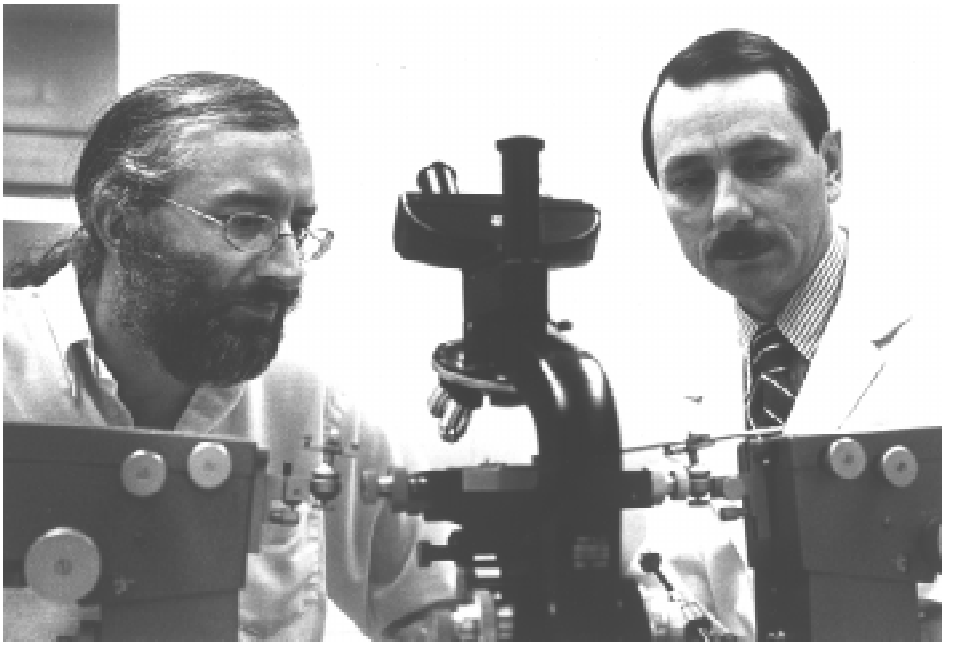
From 1977 on, you began to concentrate on introducing genetic material directly into mouse eggs, first in the form of mRNA and, later, certain specific genes that were beginning to be purified. What people, ideas or collaborations were most important to you in this period and why?

I first became interested in introducing new genetic material into the egg as a result of a paper published by McBride and Ozer (1973) that demonstrated the genotype and phenotype of cultured cells could be changed by exposing them to chromosome preparations. It seemed possible to do similar experiments with eggs, since techniques to obtain, handle and manipulate *in vitro* large numbers of mouse eggs were available. However, my plan was actually to inject chromosomes into the egg. During my investigation of the approach, it became apparent that chromosomal preparations of sufficient purity with biological activity would be difficult to obtain. However, in the mid 1970s, recombinant DNA techniques were developing, and pure preparations of genes could be produced. To pilot these experiments and develop the necessary techniques, we injected messenger RNA into eggs and assayed for specific proteins. In the approach to these experiments, we were influenced by studies of John Gurdon with amphibian eggs (Gurdon, 1977). Although the amphibian egg was much larger, similar techniques could be adapted to the mouse egg. The plan I had developed for mouse eggs consisted of three main steps: first, to demonstrate translation by the egg of an injected mRNA; second, to show that a gene injected into the nucleus was faithfully transcribed; and third, to inject the egg nucleus with a gene and demonstrate the presence of the gene in an animal resulting from the egg. To accomplish the first step, we microinjected into mouse eggs the messenger RNA for rabbit β-globin, which Jerry Lingrel generously provided. Techniques to inject mRNA and analyze the resulting translated protein were developed, and the results were published in *Nature* (Brinster *et al.*, 1980). The second step in this series was done with the *Xenopus 5S* RNA gene, which was a gift from Donald Brown of the Carnegie Institute in Baltimore. In these studies we demonstrated precise transcription of an injected gene by the mouse egg (Brinster *et al.*, 1981a). As part of this second step, we also injected a gene into the egg nucleus and measured the production of a functional protein from the gene (Brinster *et al.*, 1981b). This was accomplished using a thymidine kinase gene provided by Carlo Croce of the Wistar Institute. The third and final step was to inject a gene into the egg, transfer the egg back to a pseudopregnant recipient mouse and look for the presence of the gene or its activity in the animal that developed from the egg. Although these steps were sequential in planning, we actually pursued them in an overlapping, simultaneous manner. The studies with mRNA were initiated early in 1979, and shortly thereafter we began to introduce genes into eggs and transplant the eggs back into pseudopregnant recipients. The studies appear simple but were actually quite labor intensive. Mary Avarbock, Howard Chen and Myrna Trumbauer in my laboratory played an important role in developing these experiments, which laid the foundation for our work later in the 1980s. One of the underlying difficulties in developing a new approach is the long lag-time. Culturing eggs and preimplantation embryos, injecting blastocysts with teratocarcinoma cells, and injecting genes into eggs all involved a long series of experiments before success was achieved. In each of these areas, at least three years were spent in developing techniques and procedures before data were ready for publication. These experiments were performed with my research assistant, because students and postdoctoral fellows cannot invest that much time in a project that may fail, and many new ideas do fail.

Your *in vitro* translation and transcription assays of injected nucleic acids were the initiation of a tremendous leap forward in science; the production of transgenic animals. How did the experiments introducing DNA into animals begin?

Although my first studies involved the injection of messenger RNA and DNA into oocytes or fertilized eggs and the analysis of the resulting protein or message, the objective from the beginning was to introduce new DNA into the egg with the hope that it would become part of the genome and influence subsequent development. These latter experiments were begun in the summer of 1979. In May, 1979, I attended a macromolecule injection symposium at Temple University and talked to Carlo Croce about obtaining a rabbit β-globin gene to initiate the idea I had been developing for several years. In July of that year, we began injecting this gene into eggs and transferring the eggs to pseudopregnant recipient mothers. Almost simultaneously, an article by Paul Berg was published describing a β-globin SV40 plasmid he had constructed, and I wrote to him in July, 1979 inquiring about the possibility of obtaining the plasmid he had constructed. A few weeks later, an enthusiastic letter of support arrived indicating his interest in the studies and their possible outcome. He sent SV40 DNA in September, 1979, and it was injected into eggs, which were transferred to recipient

mothers for subsequent analysis. In the fall of that year, Donald Brown from Carnegie visited the Wistar Institute for a seminar, and we discussed the possibility of using the *5S* RNA gene from *Xenopus*. He was quite willing to provide that gene and was very helpful. By the winter of 1979, we had three genes with which to work, the rabbit globin, *SV40* and *5S* RNA genes. Through 1980, we worked on experiments producing animals that might contain these three genes as well as on experiments to demonstrate the production of messenger RNA or protein in the egg following injection of genes. There was a large array of experiments underway in our laboratory during 1979 and 1980 involving the microinjection of nucleic acids. During that period, we also began analyzing mice resulting from eggs that had been injected with foreign genes. We were not able to identify the presence of β-globin DNA, *SV40* DNA, or the *5S* RNA gene in animals resulting from eggs injected with this DNA. However, using a plasmid provided by Carlo Croce containing pBR 322, polyoma early region and herpes simplex virus thymidine kinase (HSV-TK), we did produce mice that showed an interesting pattern of DNA when analyzed by Southern blot. The DNA bands on the blot were the size of the Bam H1 restriction fragments within the injected construct. We had expected to see junction fragments generated from the site at which the foreign DNA integrated into the mouse chromosome, and junction fragments were not visible. However, the data did suggest that DNA from the plasmid existed within the resulting mice. Thus, near the end of 1980, there was evidence that suggested the presence in mice of foreign DNA injected into the egg. These were tantalizing and encouraging results but certainly did not warrant publication. They were mentioned only as a note "added in proof" in the 1981 *Science* article describing the *5S* RNA gene injections.



Ralph Brinster and Richard Palmiter. *Photograph taken in 1981 on occasion of R. Palmiter' s visit to Brinster laboratory.*

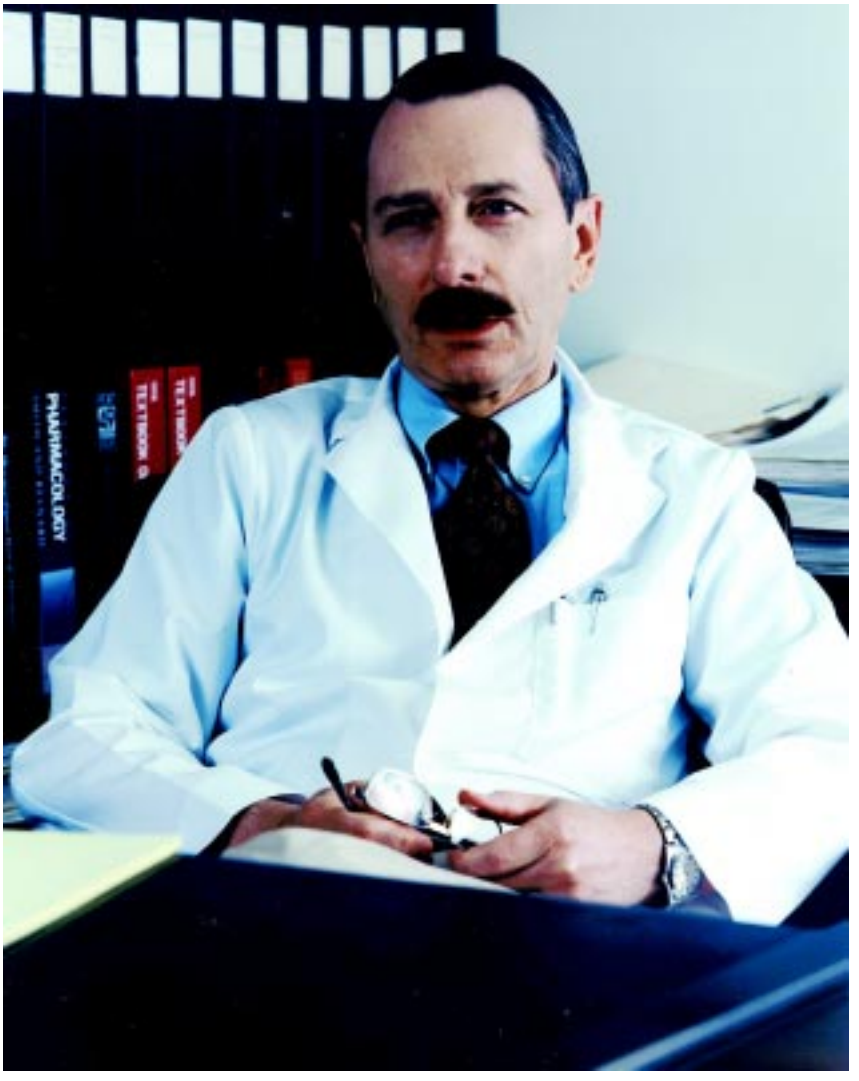
It was in the fall of 1979, after we had begun injecting genes into eggs and transplanting eggs back to pseudopregnant recipients, that I first contacted Richard Palmiter. My telephone call was to obtain chicken ovalbumin messenger RNA for studies to determine if foreign protein could be secreted by the one-cell egg following injection of an appropriate message. In early 1980, Richard sent the mRNA, and we began experiments. I had several conversations with Richard in the fall of 1980 about progress I had made with the ovalbumin messenger RNA, and he described his work with the mouse metallothionein-1 *(MT)* gene. Although I was very interested in trying new genes, the *MT* gene did not seem useful because the assay for expression of the mouse mRNA and protein product would be difficult. However, late in the fall, Richard indicated that he was going to fuse the 5' end of the *MT* gene to the *HSV-TK* structural gene. I thought this fusion gene would be useful in our experiments to introduce new genes into mice, because during the course of our experiments with Carlo Croce, we had developed assays to detect expression of *HSV-TK* in mouse liver. A potentially valuable characteristic of the fusion gene was that expression of *MT* could be induced or increased by exposure to heavy metals. In the first week of January 1981, the *MT*-TK fusion gene construct arrived from Richard Palmiter. We injected the construct into fertilized 1-cell eggs and 24 h later assayed the eggs for production of viral thymidine kinase. We also injected the gene into fertilized eggs and transplanted the surviving eggs into pseudopregnant recipient mothers, with the expectation that the animals developed from these eggs would be analyzed for expression in the spring of that year. The use of the fertilized mouse egg as a transient assay system proved to be a very powerful tool in delineating the 5' regulatory elements of the metallothionein gene. The dissection of the *MT* promoter was an endeavor in which Richard had a great deal of interest and had been pursuing using stably transfected cells as an assay system. This later approach required up to one month to obtain information regarding expression of a gene construct. Transient or acute assays in cultured cells had not yet been developed. Most analyses of gene regulatory elements were done on stably transfected cell lines and involved considerable work. Consequently, the possibility of injecting eggs with test constructs and obtaining definitive expression data in 24 h was very appealing, and we devoted considerable work in subsequent months and years defining the regulatory elements of the *MT* gene. In April 1981, Howard Chen, a postdoctoral fellow in my laboratory, began analyzing mice that had developed from eggs injected with the *MT-TK* fusion gene. In the hope of potentially increasing expression of the *MT-TK* gene, we injected mice with cadmium, sacrificed the animals the next day, and assayed the liver for viral thymidine kinase activity. One of the first twelve animals born from injected eggs had a very high thymidine kinase activity. The level of hepatic TK enzyme activity was about 200-times greater than normal, and more than 95% of this activity was blocked by an HSV-TK specific antibody. This was a phenomenal

result, which generated enormous excitement in the laboratory. In May, Richard finished analyzing the DNA of the mice and confirmed that the animal with high hepatic viral TK activity contained the *MT-TK* gene. Several other animals carried the *MT-TK* gene but did not have significant viral thymidine kinase activity. In July, we identified two additional animals that contained the *MT-TK* gene and had high levels of viral thymidine kinase in their liver. By this time, we were not sacrificing animals for analysis of hepatic viral TK and the presence of the gene, but were taking small pieces of the tail to determine integration of injected DNA. These two *MT-TK* mice appeared on the cover of *Cell* later that year (Brinster *et al.*, 1981c).

Between December, 1980 and November, 1981, six laboratories demonstrated new genes in mice that developed from eggs injected with DNA. Jon Gordon and colleagues in Frank Ruddle's group were the first to report the introduction of new DNA into mice (Gordon *et al.*, 1980). Within a few months other reports began to appear: Erwin Wagner with colleagues in Beatrice Mintz's group (Wagner *et al.*, 1981); Tom Wagner with Peter Hoppe and colleagues (Wagner *et al.*, 1981); Klaus Harber with Rudolf Jaenisch and colleagues (Harber *et al.*, 1981); Frank Costantini and Elizabeth Lacy (1981); and the paper from our work with Richard Palmiter (Brinster *et al.*, 1981c) were in that first group of publications reporting transgenic mice. Following this initial wave of papers, other laboratories became interested in the technique, and work with transgenics began to intensify, particularly after 1984.

Between 1981 and 1983, your laboratory pioneered the three stages that marked the beginning of this prodigious new technology: verification of the integration into the host chromosome of a gene experimentally introduced, phenotypic expression of the injected gene, and tissue specific expression of the foreign gene. What are your recollections of this time and in particular what international impact did your experiments have in the scientific community and for the general public?

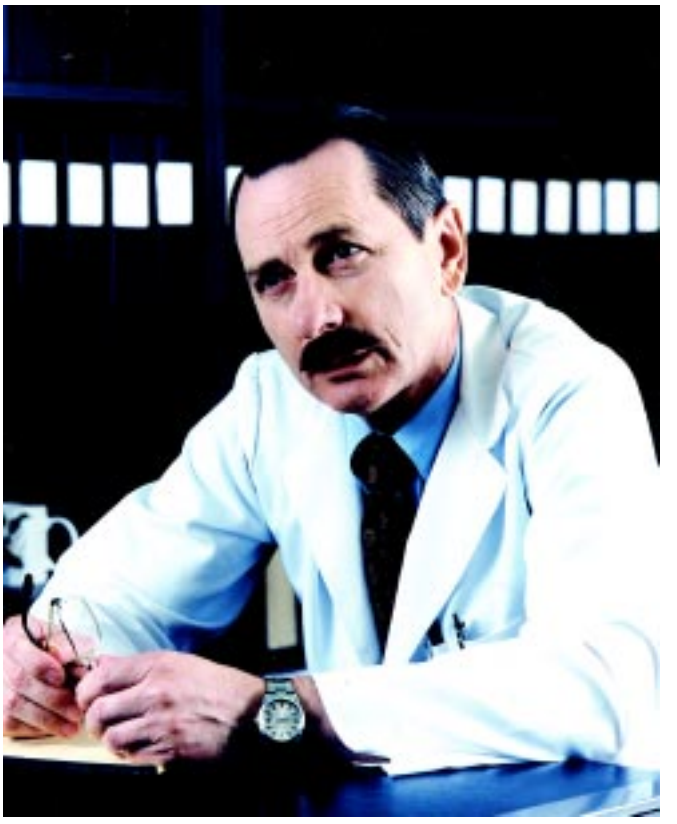
My collaboration with Richard Palmiter began that first week of January, 1981 and continued until the early 1990s. We realized in April, 1981 that there were many exciting possibilities for utilizing transgenic animals. Richard was interested in understanding the regulatory elements that control gene expression. We began these investigations by using defined pieces of the metallothionein promoter region fused to the TK reporter gene and analyzing expression in the acute assay with eggs and also in the transgenic mouse approach (Brinster *et al.*, 1982). Richard and I were particularly interested in several other avenues of investigation in which we thought transgenic animals would be valuable, including the correction of a genetic defect, the possibility of introducing a cancer causing gene into mice, and, because of my background in agriculture and veterinary medicine, applying the techniques to farm animals. These projects, of course, would be difficult to develop and results were quite unpredictable, yet the potential biological significance made them worthwhile.



We began our studies into the possibility of correcting a genetic disease with the β-globin gene, because a great deal of information was available about the gene. In addition, a mutant mouse model existed. Richard and I, in collaboration with Y.W. Kan, began studies using a metallothionein-β-globin construct in an attempt to obtain expression of a normal β-globin gene with a heterologous promoter, similar to our studies with *MT-TK*. However, these experiments were not successful. In the middle of November 1981, Richard was traveling to the East coast to give a seminar and came by my laboratory to visit and discuss experiments. We had collaborated extensively during the year and published the paper in *Cell,* but had never met. Richard arrived at my laboratory around the end of November, and someone at his seminar had mentioned the dwarf mouse known as *little*. Richard and I decided to try and correct the growth defect in *little* mice by providing them with exogenous growth hormone from a metallothionein-growth hormone fusion gene con-

structed in a manner similar to the *MT-TK* gene. We did not have experience with growth hormone and decided to contact Ron Evans at the Salk Institute who was working on growth hormone genes. We invited him to join us in this project and together began designing a suitable transgene for injection. In subsequent months, the transgene construct was engineered, and I purchased a few *little* mice, which were difficult to obtain. Bob Hammer arrived in the laboratory in January, 1982 as a postdoctoral fellow, the same week the *little* mice arrived from The Jackson Laboratory, and Bob became the lead person on the project to correct the genetic defect. The logistics and scientific expertise necessary to accomplish this goal were formidable, and success probably never would have been achieved without the drive and scientific ability of Bob Hammer. In early 1982, a metallothionein-rat growth hormone construct was generated, and during the same period preliminary observations suggested that a linearized DNA construct might integrate more efficiently than circular DNA. Therefore, we decided to use a linearized construct and injected about 170 eggs. Twenty-one mice developed from these eggs, six of which grew more rapidly and were larger than their siblings. It was obvious by the time the pups were weaned that the experiment had been successful. Subsequent DNA analysis of these 21 mice indicated that there was another animal in the group which had not grown larger than normal but had integrated the gene. The results of this experiment were published in *Nature,* December, 1982, and received considerable attention both from the scientific community and the general public (Palmiter *et al.*, 1982). The demonstration that the growth of animals could be altered by such an approach captured the interest of the scientific community and alerted the public to this emerging technology. The genetic defect resulting in decreased growth in *little* mice was then corrected by germ line incorporation of a metallothionein-rat growth hormone transgene (Hammer *et al.*, 1984). The dramatic growth of mice reported in 1982 was a clear indication of transgene expression and indicated this technique could be used to study gene function.

While each of these steps, foreign gene integration and transgene expression, was important and extremely valuable, one key question that needed to be addressed was whether transgenes would be expressed in a tissue-specific manner. Would they contain sufficient regulatory information to direct expression to the correct cell-type at the correct developmental time? The first documentation that tissue-specific expression could be obtained was demonstrated in collaborative experiments with Ursula Storb. In these studies the mouse kappa immunoglobin gene was introduced into mice, and it was expressed specifically in lymphoid tissue (Brinster *et al.*, 1983; Storb *et al.*, 1984). This was an exciting and important result. It indicated that a gene integrated randomly into the genome of a mouse could be expressed in the correct spatial and temporal pattern. Some wondered whether this kappa gene result might be a special case because of the rearrangement that occurs within immunoglobulin genes. However, within a few months tissue-specific regulation of a transgene was confirmed using the rat elastase gene in collaboration with Ray MacDonald from Texas (Swift *et al.*, 1984). Developmental timing of transgene expression was then definitely demonstrated with the α-fetoprotein gene (Krumlauf *et al.*, 1985a; Hammer *et al.*, 1986). By this series of experiments primarily between 1981 and 1984, the three important steps in development of transgenic methodology (DNA integration, gene activity, and tissue-specific expression) were established.



The fourth and perhaps final step was targeting of foreign DNA to a specific chromosomal location. While the transgenic technique was a powerful advance in biology, scientists working with the method in the early 1980s recognized that targeting of foreign DNA was an important objective (Brinster and Palmiter, 1986). It was generally accepted that homology between the injected sequence and the chromosomal target area was the way this could be accomplished. However, studies in other mammalian cells indicated that the frequency of homologous recombination was very low, and it would be difficult to achieve targeted integration of the transgene by direct microinjection of eggs. Fortunately, the embryonic stem cell approach to transgenesis was developing, following the derivation of embryonic stem cells in 1981 that had high potential to contribute to the germ line. In the mid 1980s it was known that transgenes could be introduced into the germ line of mice by transfecting ES cells before injecting them into a recipient blastocyst; therefore, selection of an ES cell that had integrated the foreign DNA at a specific location by homologous recombination was a possible way of generating targeted gene changes. Gerald Fink's group, in an elegant series of experiments, had demonstrated DNA integration in yeast by homologous recombination, and these experiments stimulated scientists and paved the way for similar work in mammalian cells (Hennen *et al.*, 1978). In an important paper, Oliver Smithies with Raju Kucherlapati and colleagues in 1985, were the first to demonstrate targeting of DNA in mammalian cells (Smithies *et al.*, 1985), and Mario Capecchi and colleagues confirmed this approach (Thomas *et al.*, 1986). It seemed reasonable that these techniques could be applied to ES cells and used to mutate genes in the chromosomes of mice. In the late 1980s, Capecchi and Smithies were leaders in developing this

approach (Doetschman *et al.*, 1987; Thomas and Capecchi, 1987), and in 1989 the first targeted genes from ES cell experiments were passed through the germ line of mice (Koller *et al.*, 1989; Schwartzberg *et al.*, 1989; Thompson *et al.*, 1989; Zijlstra *et al.*, 1989). This fourth step in the development of the transgenic technique represents the merging of several important areas, particularly work on teratocarcinoma and embryo stem cell transplantation with sophisticated recombinant DNA techniques. In 1989, Richard and I were successful in introducing a targeted change in a mouse gene by direct injection of DNA into the fertilized egg (Brinster *et al.*, 1989). However, about 10,000 eggs were injected and 500 transgenic animals produced to make one targeted change. Currently, the embryonic stem cell approach is the most efficient method for achieving targeted DNA integration (reviewed in Capecchi, 1989; Smithies and Koller, 1989). Recently, a technique (the Cre-*loxP* system) has been developed to alter genes in individual tissues. In this system a transgenic mouse expressing the Cre enzyme in a selected tissue is mated to a transgenic mouse that carries a targeted gene flanked by *loxP* sequences. In the offspring, only cells that express Cre will delete the targeted gene. It is important to realize that currently the mouse is the only species in which this level of sophisticated gene manipulation can be achieved. A gain-of-function transgenic by direct egg injection can be generated in a number of animal species. However, targeted mutation can only be achieved in mice. Appropriate ES cells or alternative strategies have not yet been developed for other species.

In developing this new technology you were interested in several approaches to use transgenic animal models to understand biology as well as address medical problems. In this context, the «supermouse» you obtained had quite an impact on both the scientific world and on the general public. Simultaneously you were looking for transgenic models for the experimental study of cancer. How did you get the idea of injecting oncogenes into the mouse egg, what were the main results obtained and what are the future prospects for this experimental approach to oncology?

To determine the usefulness of the transgenic model, our studies with Richard Palmiter were far ranging and extensive. One of the areas in which we were particularly interested was the expression of oncogenic genes and the possibility of developing cancer models. Richard and I tried to produce a transgenic cancer model by introducing the *Src* gene, but it was not expressed. However, as often happens in new areas of science, developments are unpredictable. We had noted that the *MT-TK* fusion gene produced very low levels of messenger RNA in liver (Brinster *et al.*, 1981c). However, the metallothionein-growth hormone fusion gene produced high levels of messenger RNA, similar to those obtained from the endogenous metallothionein gene in liver (Palmiter *et al.*, 1982). This was perhaps the first indication that viral and bacterial genes may suppress the production of message from a mammalian promoter, which has now been observed with a number of genes. Richard and I were curious about why the TK mRNA was lower than growth hormone mRNA. About that time DNA sequences that enhanced gene activity had been described, and one of the enhancers identified was a sequence upstream of the T-antigens in SV40. In order to determine if this enhancer might increase the expression of the *MT-TK* gene, Richard and I decided to insert the sequences upstream of the gene. A construct was generated by joining the enhancer plus the T-antigen genes head-to-head with the *MT-TK* fusion gene. We introduced the construct into mice with the intent of determining whether TK mRNA was increased by the presence of the SV40 T-antigen enhancer. When TK message was analyzed in livers from these transgenic animals, an increase in TK message was not found. The experiment had not been successful; the enhancers did not affect expression of the MT-TK fusion gene. However, in a short time several mice died. In examining the animals, Howard Chen and I found several with an enlarged cranium. When the brain of one of these animals was examined, there was a small, pink mass about 3 mm in diameter within the ventricle. On the basis of my recollection from veterinary pathology, the mass appeared to be an ependymoma. However, for a definitive diagnosis we sent the tumor to Albee Messing, who was a veterinary pathologist training for a Ph.D. at the University of Pennsylvania. He identified the mass as a choroid plexus tumor. Subsequent studies clearly established that the tumors were caused by the SV40 T-antigen. This work was reported in June of 1984 in *Cell* and was the first publication describing production of cancer from injection of an oncogenic transgene (Brinster *et al.*, 1984). Later that year, Phil Leder's group described a breast tumor in mice caused by a transgene; the transgenic mouse was patented and became famous as the onco-mouse (Stewart *et al.*, 1984). Following the report of the SV40 transgenic cancer mouse, we and others used this approach to create a large variety of cancer models. Many promoters fused to the SV40 large T-antigen gene will generate cancer with a very precise tissue distribution dictated by the promoter/enhancer. Furthermore, the etiology and pathogenesis of the cancer is very repeatable. These transgenic models of cancer provide a rich source of material for study of malignancy in the whole animal. A wide range of modifications in this experimental approach to the study of oncology in transgenic mice has developed in the last 12 years, and the models will continue to be a valuable resource as the versatility of transgenics evolves (Brinster and Palmiter, 1986; Palmiter and Brinster, 1986; Jaenisch, 1988; Hanahan, 1989).

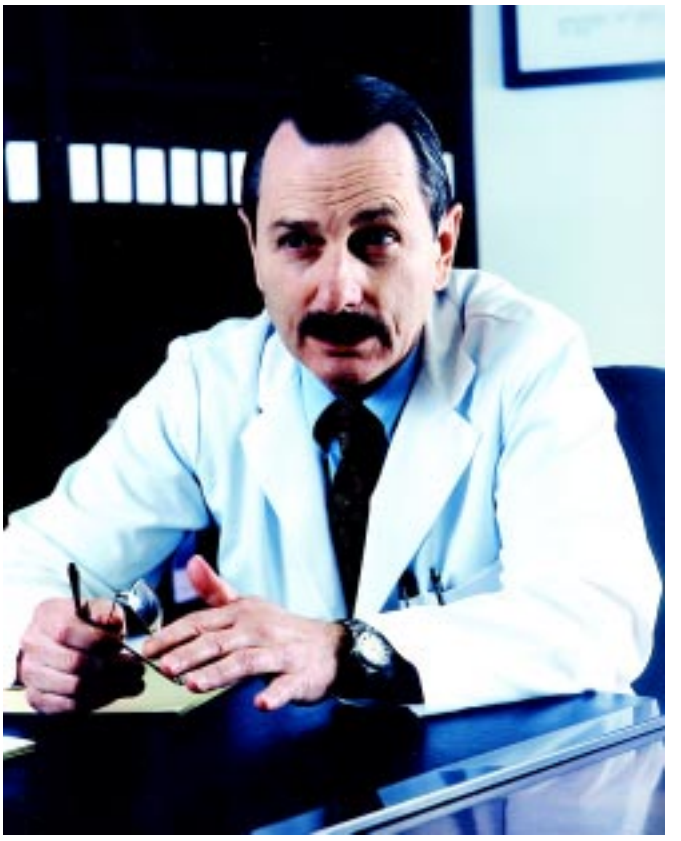
In the early 1990s you and your collaborators reported another newsworthy result: the production of transgenic mice expressing the human sickle hemoglobin and carrying in their blood the pathological erythrocytes characteristic of that disease. What benefits may be drawn from the existence of transgenic animal models for the study of other human diseases?

Many of the models that have evolved from transgenic animals, certainly the early ones, have an unusual or interesting history. The model for sickle cell anemia that developed in our laboratory arose from early globin experiments. While the β-globin gene was the first gene I tried to introduce in 1979 to generate transgenic mice, the globin constructs always proved to generate low levels of mRNA in transgenic mice. Our subsequent studies were begun with Jerry Lingrel and his postdoctoral fellow Tim Townes. Globin genes could be introduced into mice, but expression was poor. However, in trying to increase expression using various constructs, we introduced a globin gene without bacterial vector sequences attached. Surprisingly, this β-globin gene was expressed well. In

the months before finding expression of the β-globin gene which lacked vector sequences, three other experiments had suggested this possibility. First, in our studies with Richard we had used a growth hormone gene without vector sequences and better expression was obtained (Hammer *et al.*, 1985a). Second, in a series of experiments with Ursula Storb, we noticed that the immunoglobulin gene expression seemed to be decreased by plasmid sequences. Third, experiments to examine developmental timing of α-fetoprotein expression showed clearly that prokaryotic vector sequences severely depressed gene activity (Krumlauf *et al.*, 1985b). Thus, four experiments in our laboratory suggested a suppressing effect by bacterial plasmid DNA. On further analysis, it became clear that plasmid sequences in transgenic constructs were undesirable (Townes *et al.*, 1985). Simultaneously, Costantini and co-workers published similar findings (Chada *et al.*, 1985). Following this revelation about the inhibitory effects of plasmid DNA, the work on globin moved ahead quite rapidly. Richard Behringer, an excellent postdoctoral fellow in my laboratory, played a key role in the development of a series of experiments in which we worked with the laboratories of Tim Townes, Richard Palmiter, and Toshio Asakura to develop a mouse in which human sickle hemoglobin was produced (Ryan *et al.*, 1990). These animals and the general approach should be valuable in studying sickle cell anemia of humans, and excellent models have now been described (Pászty *et al.*, 1997; Ryan *et al.*, 1997).

How did you begin studies in animals other than the mouse?

My interest in creating transgenics in other animals arises from my background in agriculture. I grew up on a farm, went to agriculture school, then to veterinary school and had an interest in improving the health of animals throughout most of my life. Therefore, when it was clear that new genes could be introduced into mice, I felt it would be desirable to generate transgenic farm animals in an attempt to improve their resistance to disease. This would allow them to be used effectively in countries throughout the world. Initially, oocytes were obtained from animals being sacrificed at meat packing facilities, but the number and usefulness of these eggs was limited. Therefore, a collaboration was established with Caird Rexroad at the Beltsville Experimental Station (USDA) who was working with sheep. After preliminary experiments with these eggs, a collaboration with Verne Pursel, also at Beltsville, was begun to microinject pig eggs. The eggs were transported by car from Beltsville to Philadelphia, a three hour trip, microinjected with genes, and then taken back to Beltsville to be placed in pseudopregnant recipient mothers. This work was extremely laborious and was led in my laboratory by Bob Hammer; Karl Ebert also played a role in these early studies and, later, Carl Pinkert joined the effort. Richard Palmiter was, again, an essential partner in the development and execution of these studies. One of the particularly difficult aspects of large animal transgenics initially was the identification of the pronucleus. In sheep eggs, we were able to visualize the pronucleus by interference contrast microscopy. However, pig eggs are very opaque. To overcome this problem, we used an old technique from sea urchin experiments in which the eggs are centrifuged. Bob Hammer in my laboratory and Bob Wall in Verne Pursel's laboratory developed techniques for centrifugation of pig eggs, which resulted in stratification of the cytoplasm and allowed identification of the pronucleus. Routine injection of the



pronucleus with genes was then possible. Because of the large number of eggs ovulated by pigs and the continuous breeding season, work on pig transgenics proceeded more rapidly than studies with sheep transgenics. After several years work, production of rabbit, pig and sheep transgenic animals was described in 1985 in *Nature* (Hammer *et al.*, 1985b). The cover of that issue of *Nature* is a dramatic photograph, taken by Bob Hammer, showing the microinjection of a centrifuged pig egg. Studies on farm animal transgenics have continued since that time and have proceeded well considering the difficulty obtaining eggs, the long gestation period, and the large size of the animals (Pursel *et al.*, 1989). One particular valuable approach with transgenic farm animals will be the generation of foreign proteins in serum or milk and harvesting these products for human or animal health use. Because of the great potential of these transgenic farm animals, it would be very advantageous to increase the efficiency of generating gain-of-function genetic alterations. Equally important would be to generate loss-of-function transgenic large animals. Unfortunately, the approach with embryonic stem cells has not been successful in species other than the mouse. However, recent experiments using nuclear transplantation into cultured oocytes of farm animals suggest that it may soon be possible to generate transgenic animals of species other than the mouse efficiently by this technique (Schnieke *et al.*, 1997; Wilmut *et al.*, 1997; Cibelli *et al.*, 1998).

The most recent phase of your studies is characterized by a combined approach using stem cells and transgenesis. Your first studies focused on hepatic regeneration with trans-

Ralph Brinster using his micro-manipulation system for cell injection in seminiferous tubuli.

planted hepatocytes. How can this model help us gain a better understanding of liver biology, and what therapeutic implications could it have in the future?

The animal model for these studies arises fortuitously from a transgenic experiment, and the element of stem cell transplantation arises from my long-term interest in germ cells and their close relatives, tissue stem cells. The germ cells are the most complete of all stem cells, since they can ultimately generate various types of stem cells, which in turn produce specific differentiated tissues. The studies involving hepatic regeneration arise from experiments begun more than ten years ago. Eric Sandgren, a veterinary student and postdoctoral fellow in my laboratory, was interested in the possibility of extending the cancer models described above to shed light on metastasis of cancers. In general, the original cancer does not kill an animal, it is the metastases throughout the body that results in death. Many metastatic cancers and the metastases they produce are characterized by high expression of several growth factors and proteases. Eric developed experiments in which transgenic animals expressing a primary hepatic cancer were mated with other transgenic animals expressing a growth factor or protease. One of the proteases often associated with metastatic lesions is urokinase-type plasminogen activator (uPA). When Eric generated these transgenics with plasminogen activator expressed in the liver, he noted that about half of the neonates carrying the transgene died within a few days of birth from internal hemorrhaging. Clotting abnormalities in humans and many mammals are often characterized by internal hemorrhaging. The death of these neonates resulted from low fibrinogen as a result of uPA activity in the liver from the transgene (Heckel *et al.*, 1990). Eric decided to study a few of the surviving animals in greater detail. It was because of his good scientific judgement that these animals were kept alive, and subsequent experiments on liver replacement were possible. Eric found that half the progeny from these surviving founder transgenic animals, which carry the plasminogen activator gene, had pale or white livers when they were born, and nodules of normal red hepatic tissue developed in these white livers and gradually replaced the liver. The studies were done in collaboration with Jay Degen's laboratory and with Richard Palmiter. Examination of this model indicated that the liver in the plasminogen activator mice was abnormal at birth; the transgene poisoned the hepatocytes. The normal red liver nodules arose as clonal expansions of single hepatocytes that lost the functional transgene; an occasional hepatocyte would delete the transgene and then be able to regenerate (Sandgren *et al.*, 1991). Eric, Jay, and others involved in the project reasoned that any normal hepatocytes introduced into the liver would have a very strong stimulus to also grow. Jonathan Rhim arrived in our laboratory about that time and pursued this project with Eric Sandgren. They demonstrated that transplanted mouse hepatocytes would indeed colonize the transgenic liver and replace the entire liver (Rhim *et al.*, 1994). Jonathan continued these studies to demonstrate that rat hepatocytes would replace 95 to 100 percent of the mouse liver when transplanted to immunodeficient transgenic uPA mice; thus, generating a mouse with a rat liver (Rhim *et al.*, 1995). This is an exciting model because it allows the study of hepatic regeneration and replacement in a unique manner. Liver is often damaged either as the primary organ from the effect of a toxic agent, or secondarily, as a result of diseases affecting other sites in the body. Consequently, information about the ability of liver to regenerate is extremely important. An exciting possibility of this model is that human hepatocytes can be transplanted to the immunodeficient uPA transgenic mouse and that these hepatocytes will colonize the transgenic liver and establish a human liver model within the mouse. This would allow a variety of studies regarding human liver regeneration and liver disease that cannot be performed in patients.

Finally, in recent months research from your laboratory appeared on the front page of the New York Times and other major international newspapers describing the successful transplantation of spermatogonia from a fertile testis to an infertile testis where they generate spermatogenesis. Apart from the controversy aroused by the ethical implications of its use in humans, what possibilities does your technique open up for the advancement of developmental biology, human and veterinary medicine?

These studies have a very old history. I first became interested in spermatogonia when I was working with teratocarcinomas in the early 1970s. Teratocarcinomas arise from male germ cells as well as from transplanted early embryos. From embryos the likely origin is totipotent epiblast cells; however, teratocarcinomas within the gonad arise from germ cells. Thus, when transplantation of teratocarcinoma cells to the blastocyst was successful, I thought a

possible extension would be to transplant primordial germ cells from the fetus and then spermatogonia from the testis. Although I was not able to progress to those steps in the 1970s, the possibility of using spermatogonia as stem cells remained in my mind. Consequently in 1990, I began to re-evaluate the potential use of spermatogonial stem cells. There are several aspects to this potential. First, spermatogenesis is one of the most complicated, highly organized, and efficient processes in the body. If spermatogonial stem cells could be manipulated, it would provide an opportunity to study this important process in unique ways. Second, the spermatogonial stem cell might act as a source for stem cells of other tissue types under proper conditions. Spermatogonial stem cells are only a few differentiation steps removed from male primordial germ cells, and the primordial germ cell will convert in culture to an embryonic stem cell. Third, the spermatogonial stem cell could provide a means to introduce a genetic change into the germ line. This cell is indeed fascinating; it is the only cell in the adult body that continues to divide throughout life and contributes genes to subsequent generations. No other cell in the body has these two characteristics. All female germ cells in mammals have stopped dividing by the time of birth.

In order to study this interesting population of spermatogonial cells, I felt that it was essential to be able to remove them from the testis and then reintroduce them into the seminiferous tubules in the testis of another animal for subsequent development. Once this window of opportunity was open, where the cells were removed from the body, one could expand the procedure to make modifications in the genetic composition of the cell before reintroduction into another testis. In this sense, it is very much like the development of the egg culture system in the 1960s. One had to develop a system to culture, handle, and manipulate eggs *in vitro*, and then a variety of studies could be done before putting them back into the body. I reasoned that the spermatogonial stem cell transplantation procedure would have to be made simple, because it was likely to be inefficient initially, much like the transfer of stem cells into the blastocyst in the early 1970s. Therefore, we collected testis cells from fertile mice, using the techniques that Anthony Bellvé and others had developed (Bellvé *et al.*, 1977), and injected the cells into testes of infertile males. It was an experiment simple in concept, but difficult to accomplish. The rete testis, the collecting area to which all seminiferous tubules lead, is very small in the mouse and proved impossible to enter efficiently with an injection pipette. Therefore, we injected the testis cell suspension directly into seminiferous tubules of mice in the hope that the cells would be forced under pressure into many of the tubules in the testis by passing through the rete testis. To provide the best opportunity for the injected cells to colonize the testis it was desirable to eliminate endogenous spermatogenesis. Destruction of endogenous spermatogenesis can be accomplished in several ways, but we eventually established treatment with busulfan, a chemotherapeutic agent with known cytotoxic action, as an effective technique. These studies were undertaken with my long-time research assistant Mary Avarbock, and a graduate student in the laboratory, Jim Zimmermann, who participated in the early stages of the work. After much effort, we were able to demonstrate the presence of spermatogenesis from the injected donor testis cells in recipient seminiferous tubules (Brinster and Zimmermann, 1994). While this was encouraging, I felt it was necessary to produce mice from spermatozoa generated by donor cells in a recipient testis, which required a great deal of effort but was successfully accomplished (Brinster and Avarbock, 1994). There is little doubt that the technique can be applied to other species.

The ethical implications you mention regarding the use of spermatogonial transplantation in humans need to be evaluated and discussed. My general feeling is that scientists provide information which has the potential to make human and animal life better. It is then the decision of society how that information should be used. Any possible application of the spermatogonial transplantation system in humans must be discussed carefully, and there is adequate history and example for this discourse from discussions regarding human fetal tissue and certain reproductive technologies. Appropriate regulations and legislation can then be developed.

In biology, agriculture and medicine, spermatogonial transplantation will be important. As research continues, it should be possible to culture and expand in number the spermatogonial stem cell population, which would allow genetic modifications to be made. A more efficient system to produce gain-of-function transgenic changes in animals other than mice is needed, and a method to generate loss-of-function mutations in all species other than the mouse is currently not available. Spermatogonial stem cell modification may provide an opportunity to accomplish these goals. In addition, of considerable interest is the possibility to use the transplantation technique to understand the complicated and important process of spermatogenesis. A long-term development might be culture of spermatogonial stem cells with the intent to redirect their lineage to generate stem cells for other tissues (Brinster, 1993).

I was lead to study this population of cells because of their unique characteristics and because of the potential they have in many areas of experimentation. Since I entered research in 1960, I have focused on germ line cells and how they could be used to study development. The process by which a single cell, the fertilized egg, develops into a complete animal is probably the most complex, fascinating and important process that man will ever address. Studies on germ cells allow one to modify and thereby understand this developmental process in unique ways. In terms of the immediate future for the use of spermatogonial stem cells, there are several exciting areas to investigate. The first is to increase the efficiency of the system. It is essential for the transplantation procedure to work easily, then many scientists can use the system to address diverse questions in biology. Second, it is important to culture these cells outside the body and to expand the cell population. Initial experiments indicate that spermatogonial stem cells survive in culture for more than three months (Nagano and Brinster, 1998; Nagano *et al.*, 1998). Thus, future studies need to be directed to expanding the number of stem cells *in vitro*. Standard transfection techniques can then be used to generate either gain-of-function or loss-of-function genetic changes in the stem cell. Since it is likely that the spermatogonial transplantation technique can be used in many species, extension of stem cell culture techniques to other animals will greatly expand the range of possible experiments, particularly regarding generation of transgenic animals. Third, it would be desirable to develop a system for xenogeneic spermatogenesis. Generation of spermatogenesis of large or rare species in smaller or more easily maintained species would have great benefit. We have already demonstrated that transplantation of rat donor testis cells to the

seminiferous tubules of a recipient immunodeficient mouse will generate normal rat spermatogenesis and morphologically normal rat spermatozoa (Clouthier *et al*., 1996; Russell and Brinster, 1996). Extension of xenogeneic spermatogenesis to other species should be feasible. Furthermore, successful cryopreservation of spermatogonial stem cells of mice and rats suggests that the germ line of individual males of many species can be preserved indefinitely (Avarbock *et al*., 1996). The ability to both cryopreserve and transplant spermatogonial stem cells of many species provides countless opportunities for research (Nagano and Brinster, 1998). A great deal of work remains to be done to improve the system, but spermatogonial transplantation has enormous potential in biology, medicine and agriculture (Ogawa *et al*., 1997; Brinster and Nagano, 1998).

Do you have comments in any area we have not covered?

I would like to end by thanking my colleagues, collaborators, students and laboratory co-workers who have contributed enormously to our studies over many years. I have been privileged to work with many excellent individuals during my career, and it has been possible to mention only a few during our conversation. I am especially grateful to Mary Avarbock, my long-time research assistant, and Carolyn Pope, who has been my secretary and laboratory manager for many years. I also thank Clarence Freeman and Rose Naroznowski for their excellent maintenance of our research animals.

My friend and colleague, Richard Palmiter, is an extraordinarily talented scientist, and my interaction with Richard has been particularly enjoyable. The years we worked together left me with many great memories of challenges and successes shared during a fantastic period in our research careers and lives.

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