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Egg culture: the foundation

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Prologue

By the time Ralph Brinster entered veterinary school in 1956, he had developed a strong interest in animal reproduction. His early years growing up on a farm had taught him the practical importance of animal fertility in the economics of farming. Later, as an undergraduate student in the College of Agriculture at Rutgers University, he learned the scientific aspects and biology of animal reproduction. Thus, it is not surprising that this long history of experience with animal breeding and reproduction should influence Ralph's interest while at veterinary school. It became apparent during these years that a large part of animal infertility was associated with embryo loss near the time of implantation, and Ralph determined to investigate these early developmental stages and identify factors resulting in embryo loss. He believed that *in vitro* studies were an excellent way to identify requirements of developmental stages between ovulation and implantation, and the information generated could be the basis to enhance animal fertility. To perform such studies it was necessary to remove the embryos from the mother, culture the embryos to study their biology, and then return them to a recipient foster mother. However, a review of the literature indicated that, although egg transfer between females was possible, a reliable culture system was not available to permit a critical *in vitro* evaluation of preimplantation embryo stages.

Transplantation of eggs and embryos from one female to another has a long experimental history, and many details necessary for effective recovery of ova and transfer to another reproductive tract have been investigated. In a famous paper read before the Royal Society of London in 1890, Walter Heape began:

"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 foetal development of fertilized ova of another variety of rabbit."

In this extraordinary paper, Heape (1891) demonstrated a technique that captured the imagination of reproductive physiologists, and experiments on egg transplantation were systematically extended to other species (reviewed in Betteridge, 1981; Adams, 1982). By 1956, the transfer of embryos between mothers had been demonstrated in at least seven species, including rabbit (Heape, 1891), goat (Warwick *et al.*, 1935; Warwick and Berry, 1949), rat (Nicholas, 1933), sheep (Warwick and Berry, 1949), mouse (Fekete and Little, 1942), cow (Willett *et al.*, 1951), and pig (Kvasnickii, 1951). A variety of modifications and permutations were added to the procedure following these initial publications. Some of the most interesting were interspecies transplantation, particularly between sheep and goat, short periods of culture during the transplantation procedure, and an examination of the

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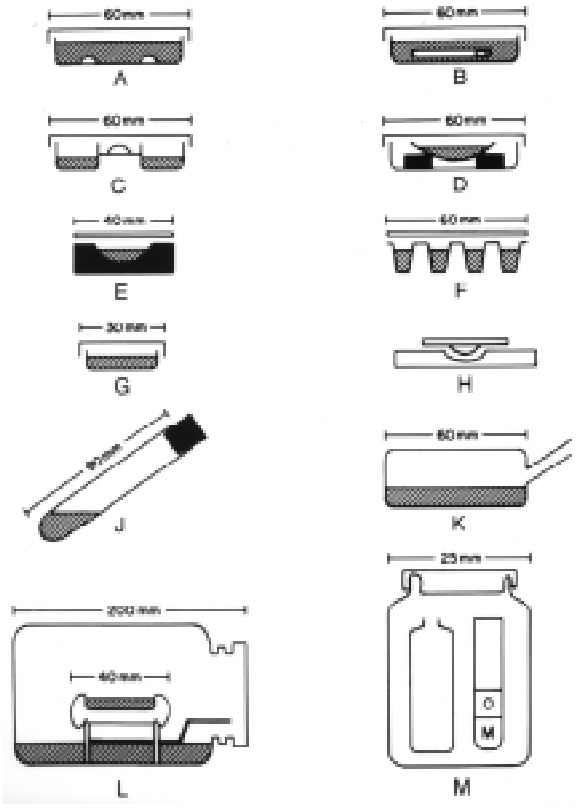

Fig. 1. Culture methods studied by Ralph Brinster for their ability to support mouse eggs *in vitro.* *Through these experiments he developed the method in which eggs are cultured in microdrops of medium under paraffin oil in a Petri dish* **(A)** *that subsequently has been widely used for in vitro studies and manipulation of eggs from all mammalian species. (See Brinster, 1963,1964,1969a, for details and review).*

importance of egg "age" and uterus "age" (reviewed in Austin, 1961; Betteridge, 1981). Four talented scientists who contributed extensively to experimentation on egg transfer were Walter Heape, John Hammond, Min Chueh Chang, and Lionel Rowson (Adams, 1982). Their research laid the foundation for subsequent work in this field and established the feasibility of removing eggs from donor females, maintaining the eggs *in vitro*, and reintroducing the eggs into a recipient female for further development. By 1956, when Ralph entered veterinary school, more than 75 separate experimental studies (36 in the rabbit, 17 in the mouse, and the remainder in other species) on egg transplantation had been published, and the feasibility of egg recovery for culture and reintroduction of the cultured eggs into a recipient was well established (Austin, 1961; Adams, 1982).

Studies on culture of mammalian eggs during the first half of this century had not been as successful. Although eggs of many species had been used in a variety of *in vitro* studies, the rabbit had received most attention. Schenk (1880) indicated that he had inseminated eggs from rabbit and guinea pig *in vitro* and could identify the first cleavage division. Between 1900 and 1920, Brachet also performed experiments with rabbit eggs (Brachet, 1912,1913). However, after 1930, investigation of egg culture increased considerably, and Gregory Pincus and co-workers cultivated one-cell fertilized rabbit ova to late morula in a variety of media (Pincus, 1934,1937; Pincus and Werthessen, 1938). At first they used plasma and embryo extract, but in later studies they used more defined media. These workers demonstrated that eggs could be collected and cultured *in vitro* for at least two days and then transferred to mothers where the cultivated eggs produced normal young. Subsequently, Adams (1956) demonstrated that Krebs-Ringer bicarbonate with 0.2 percent (2 mg/ml) bovine plasma albumin fraction V could be used to cultivate rabbit eggs for two days, suggesting that simple solutions were adequate. Simple salt solutions such as Ringer's, Tyrode's, and Gey's were also frequently used in cultivation of the eggs of other species (Austin, 1961; Brinster, 1964).

Mark and Long (1912) were the first to study mouse ova cultured *in vitro*. They employed Ringer's physiological saline or serum for periods of about 12 h. Later, Lewis and Wright (1935) observed cell division of mouse eggs *in vitro* and photographed these cleavages. Hammond (1949) was the first investigator to report successful cultivation of mouse eggs through several cleavages. For a culture medium, a simple salt solution was used containing NaCl, KCl, $CaCl_2$ and $MgCl_2$, with glucose at a concentration of 1 mg/ml, to which was added about 5% egg white. Most eight-cell eggs developed into blastocysts and even some four-cell eggs developed into blastocysts. These were extraordinary results, particularly considering the simple solution he had used. Together with the work of Adams and others, they indicated that a simple salt solution with protein and glucose was sufficient to support cleavage of mouse eggs for several days. Whitten (1956) extended these early studies and confirmed that eight-cell mouse ova could develop into blastocysts in a simple salt solution; (Krebs-Ringer bicarbonate containing 1 mg/ml of glucose and bovine serum albumin). The eggs were cultured in small test tubes. The following year, Whitten (1957a) reported that some late two-cell eggs would develop into blastocysts when calcium lactate replaced calcium chloride in the media or when isotonic sodium lactate was added to the media.

TABLE 1

BRINSTER'S MEDIUM FOR OVUM CULTURE

Component	g/l	mM
NaCl	5.546	94.88
Sodium Lactate	2.253	25.00
Sodium Pyruvate	0.028	0.25
KCl	0.356	4.78
$CaCl_2$	0.189	1.71
KH_2PO_4	0.162	1.19
$MgSO_4 .7H_20$	0.294	1.19
$NaHCO_3$	2.106	25.00
Glucose	1.0	5.56
Bovine Serum Albumin	1.0	—

Used in an atmosphere of 5% carbon dioxide with balance air, pH=7.4. An early formulation by Brinster from which most subsequent egg culture media have been derived. The inclusion of pyruvate and the low calcium level are two ubiquitous characteristics of later recipes. Pyruvate is the essential energy source for early stages of mammalian eggs. (Brinster, 1965b,e,1969a,1972)

There was no further report about two-cell egg development, but Whitten employed this culture method with eight-cell eggs *in vitro* to examine the effect of progesterone on development (Whitten, 1957b).

The foundation

When Ralph began his research in 1960, it was clear that eggs could be recovered and transferred to another female in a reliable manner, but a dependable culture system had not been developed. He began a series of experiments to establish such a system in order to study the biology of these early developmental stages. The findings of Hammond, Adams, Whitten and others suggested a simple salt solution such as Krebs-Ringer bicarbonate was a good starting point from which to begin formulating the components of a medium, but there was little information about the influence of culture vessel characteristics, solution volume, gas phase or other factors that could have profound effects on egg viability and cell division (see Brinster, 1964). Therefore, Ralph systematically examined many culture vessels and methods (Fig. 1) for their ability to support mouse embryo development (see Brinster, 1969a,1972 for review). Cultivation of eggs in microdrops of medium under a layer of paraffin oil in a Petri dish (Fig. 1A) proved superior to all other methods (Brinster, 1963). The identification of this simple, reliable system was enormously important to Ralph's subsequent work.

He first began an orderly assessment of culture media characteristics and determined the effect on egg development of changes in osmolarity, pH, amino acid composition, and energy sources. Results from these studies were published in a series of papers that established the foundation of all egg culture media that have subsequently been developed (Brinster, 1965a,b,c,d,e). The formula of a specific egg culture medium that has been widely used or slightly modified is shown in Table 1 (Brinster, 1965d,e,1969a). Two important contributions from this early work were the identification of the microdrop culture system (Brinster, 1963) and the essential nature of pyruvate to survival of mouse eggs (Brinster, 1965b). Later studies identified pyruvate as an essential energy source for cleavage stage eggs of most mammalian species (Brinster, 1969a,1970a,1972). An early modification Ralph made in Krebs-Ringer bicarbonate used as a basic salt solution to develop egg culture medium was the reduction in the concentration of calcium from 2.54 mM to 1.71 mM (Brinster, 1964,1969a). The purpose of this reduction was to prevent precipitation of the media in microdrops, a finding Ralph noted in his Ph.D. thesis (Brinster, 1964). Egg culture media continue to contain this lower calcium concentration, although it is likely that few are aware of the underlying experiments or know the purpose. The presence of 1.71 mM calcium and 0.25 to 0.5 mM pyruvate are two of many characteristics that identify Ralph's work as the foundation for many current formulations of egg culture media (Brinster, 1972; Biggers, 1998).

In a series of experiments between 1960 and 1970, Ralph used the culture technique to study the biology of egg development. A fundamental question about any tissue is its overall capability to produce energy for use in metabolic processes. To answer this question, Ralph undertook two very difficult experiments to measure oxygen consumption and to determine protein content of the embryo throughout preimplantation development (Brinster, 1967a; Mills and Brinster, 1967). Following several years effort and the use of thousands of embryos, the studies were completed and provided quantitative data about the ability of the embryo to perform metabolic work (oxygen consumption) as well as the amount of embryo

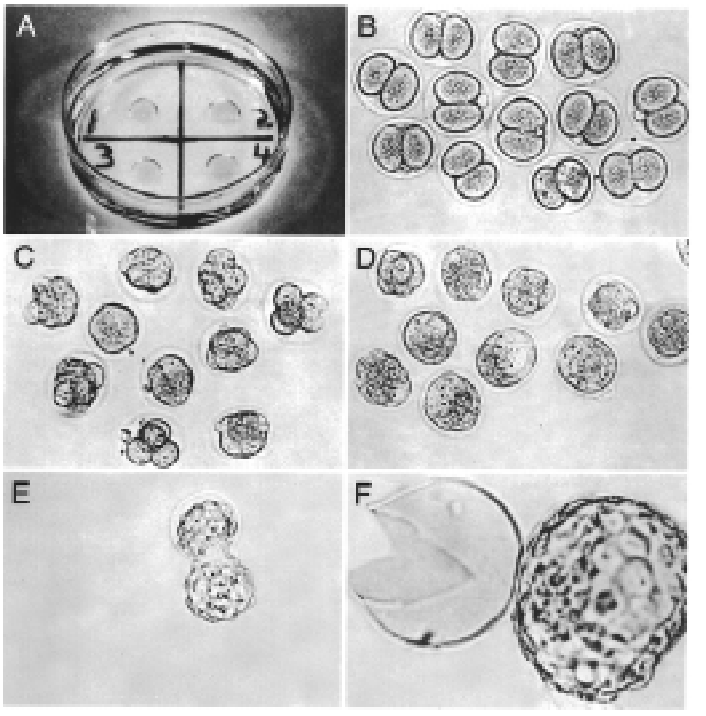

Fig. 2. Culture of mouse eggs demonstrating developmental progression. (A) *Culture dish with four microdrops of medium under paraffin oil.* **(B)** *Two-cell mouse eggs at beginning of culture.* **(C)** *Eight-cell stages.* **(D)** *Morulae and early blastocysts.* **(E)** *Late blastocyst escaping from zona pellucida.* **(F)** *Late blastocyst free of zona. (Brinster, 1963). This series of photographs and the publication describe a simple and reliable method for culturing and maintaining mouse eggs.*

TABLE 2

RESPIRATORY ACTIVITY AND METABOLIC CAPABILITY OF PREIMPLANTATION MOUSE EMBRYOS

Stage of Development	O2 Uptake µl x 106/embryo/hour	Protein ng/embryo	Qo2 µl/mg Dry Weight
Unfertilized	155	27.8	3.73
Fertilized	156	-	3.77
Two-cell	150	26.1	3.84
Eight-cell	191	23.4	5.44
Morula	351	20.6	11.39
Blastocyst	460	23.9	12.88
Late blastocyst	534	20.1	16.32

Dry weight of the stages was calculated from values for the protein content of each stage assuming protein = 0.66 x dry weight. These were the first studies to provide quantitative information about metabolic parameters necessary to understand the biology of early mammalian embryos (Brinster, 1967a, 1969a, 1971b; Mills and Brinster, 1967).

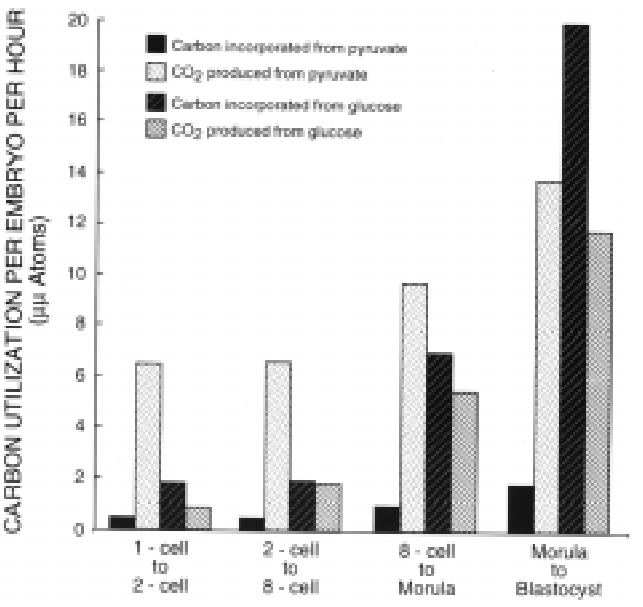

Fig. 3. Glucose and pyruvate converted to carbon dioxide or incorporated by the mouse embryo during the preimplantation period. *A dramatic increase in metabolic processes occurs beginning approximately at the eight-cell stage of development. (Based on data from Brinster, 1967b,c,1969b). These studies and similar experiments by Brinster resulted in an understanding of early developmental metabolic processes and led to widespread use of microanalytic techniques to study mammalian embryos.*

tissue (protein content) at each developmental stage (Table 2). These values made it possible to relate results of subsequent experiments on energy substrate utilization, amino acid incorporation and other metabolic parameters to different tissues of the body and among embryonic stages in a definitive manner. Surprisingly, fertilization was not associated with an increase in oxygen consumption. The major increase in metabolism occurred from the third to the fourth day as the eight-cell egg differentiates to form a blastocyst (Brinster, 1967b,c,1969b,1970b,1971a). The early stages of development are relatively inactive, comparable to skin or bone, with a Qo_2 of 3 to 4; whereas, later stage embryos have a Qo_2 comparable to whole brain (Table 2). The results of these experiments provided a base line for analysis of many subsequent studies on embryo metabolism and biology (Brinster, 1970b,1971a,1972).

Ralph continued his investigation of early development using novel adaptations of microanalysis to measure substrate use and enzyme activity in the embryo. A versatile microchamber (Fig. 1M), was devised that allowed measurement of substrate oxidation and incorporation by embryos (Fig. 3). In concert with the rise in Qo_2 of the egg, a dramatic increase in metabolism of pyruvate and glucose occurred (Brinster, 1967b,c,1969b). These metabolic studies with radioactive substrates confirmed earlier culture studies and demonstrated the importance of pyruvate as the central source of energy. Subsequent experiments demonstrated that carbon from glucose and amino acids was incorporated into embryo protein at high levels, particularly at the eight-cell stage and later (Brinster, 1969b,1970b,1971a,c). These experiments employing microanalysis of energy metabolism and amino acid use were pioneering investigations and demonstrated the feasibility of studying the preimplantation embryo with quantitative techniques.

To examine regulation of metabolic processes, particularly the unusual reliance of the embryo on pyruvate rather than glucose, Ralph adapted enzymatic assay procedures to identify regulatory points in metabolic pathways during development (Brinster, 1965f,1966,1968a,1971b). A remarkable result of these early studies was that the activity of lactate dehydrogenase (LDH) was very high in the eggs of many species (Brinster, 1965f; 1967d,e,f), which may be essential to assure rapid conversion of lactate from extracellular origin to pyruvate for oxidation (Brinster, 1965e). The enzyme LDH is approximately 5% of the protein in the newly ovulated mouse egg (Brinster, 1965f,1967a). The activity of enzymes important for glucose metabolism was also determined (Brinster, 1966,1968a). Hexokinase, the first enzyme involved in the metabolism of glucose, had a very low activity in the zygote and early stages of egg development (Brinster, 1968a). Ralph demonstrated that hexokinase was the key regulator of glucose utilization during the first five days of mouse embryo development (Brinster, 1968a,1970b,1971a). The rise in hexokinase activity during preimplantation embryo differentiation is the basis for the increasing ability to use glucose (Brinster, 1970b). As a result of these culture, metabolic, and enzyme experiments, an understanding of early embryo substrate utilization was established (Brinster, 1971a,1973).

Simultaneously with these studies on energy metabolism described above, Ralph and his students were also investigating protein and nucleic acid metabolism (Brinster, 1965c,1968b,1971c). The microassay techniques developed for energy metabolism were modified and adapted to study protein changes in the egg (Brinster, 1971c; Brinster *et al.*, 1976,1979). During the first five days of development in the mouse, the rate of protein synthesis increases even though protein content is decreasing. This surprising finding was then shown to result from a dramatic increase in the rate of protein turnover during this period (Brinster *et al.*, 1976). In subsequent studies, it was demonstrated that both the rate of synthesis and decay vary dramatically for individual embryo proteins (Brinster *et al.*, 1979). Ralph and his students also contributed extensively to our understanding of nucleic acid metabolism in the mouse embryo. They demonstrated that α-amanitin, an inhibitor of RNA polymerase II, blocked embryo development as early as the one-cell stage (Levey *et al.*, 1977). In addition, they were the first to document an increase in poly A containing RNA from the two-cell to blastocyst stage of development (Levey *et al.*, 1978). These two studies, as well as other experiments, indicated that transcription in mouse eggs began soon after fertilization and was critical for egg development, which has been confirmed in subsequent investigations. It is not possible to review here completely the extensive contributions Ralph made to culture, energy metabolism, and embryo development between 1960 and 1980, but excellent reviews of this work have been published (Brinster, 1972,1973; Hammer, 1988).

Epilogue

The development of a simple and reliable egg culture method by Ralph in 1963 has had a significant impact on biology over the last 35 years. Initially, Ralph used this method to investigate the

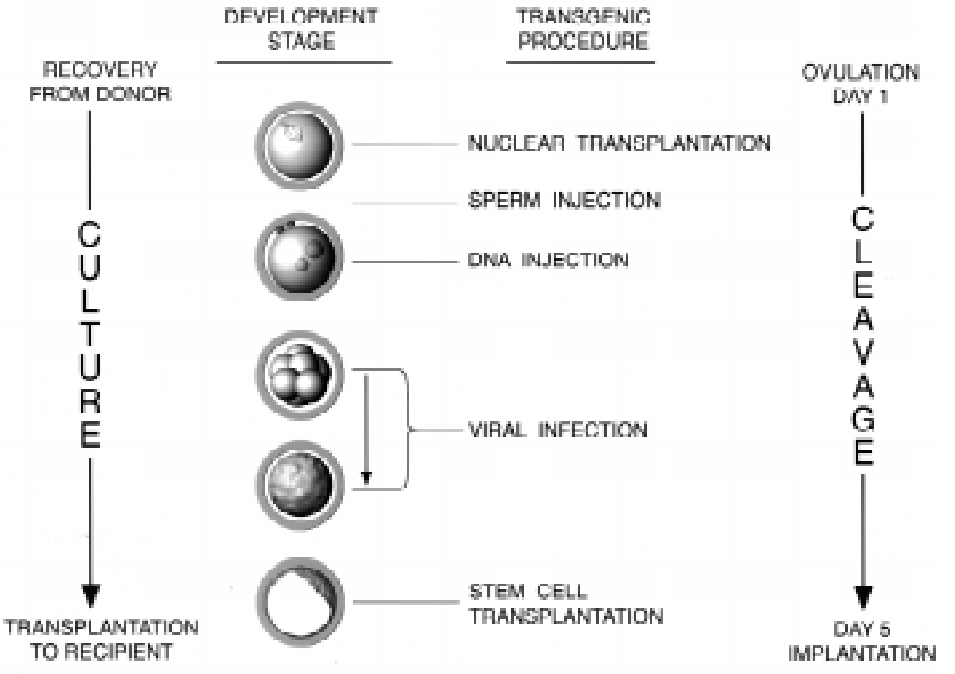

Fig. 4. Fundamental role of egg culture in production of transgenic animals. *On the left, the period during which the embryo can be readily cultured and manipulated is indicated. In the center, the development stage of the embryo and the transgenic procedure generally used to modify the genome at each stage are shown. On the right, the approximate time interval following ovulation when the embryos can be removed from the reproductive tract for manipulation is indicated. Implantation occurs later than day 5 in many species, but manipulation is more difficult after that time. Details of the various procedures necessary to produce transgenesis during the in vitro culture period are described in many articles of this issue.*

metabolism and regulation of early egg development. However, by 1970, he had already begun using culture techniques to investigate stem cell transplantation as an approach to modify the embryo and potentially to introduce genetic alterations. Others also found the culture system valuable for their studies, and the results of Ralph's work paved the way for a wide range of experiments. John Biggers, in whose laboratory Ralph did his Ph.D. research, had been primarily working for many years on chick tibia organ culture (Biggers *et al.*, 1961; Biggers, 1965, and references therein). However, the development of an efficient egg culture system was a significant breakthrough and, following Ralph's characterization of egg energy metabolism, John and his students demonstrated that pyruvate was also essential for mouse oocytes and fertilized one-cell eggs (Biggers *et al.*, 1977). These findings extended Ralph's studies with pyruvate and suggested an early origin to this energy requirement. It was later shown that primordial germ cells in the fetus already have the unusual metabolism characteristic of the ovulated egg (Brinster and Harstad, 1977). By 1970, the culture system was widely adopted by most laboratories studying embryo development and became a mainstay for work in this field. Many reviews on this topic now exist (see for example Biggers, 1998; Conaghan *et al.*, 1998, in this issue).

The most far-reaching impact of Ralph's early work on egg culture and metabolism has been to allow extensive and diverse studies on early developmental stages of the embryo that have revolutionized biology. Ralph was one of the first to recognize the enormous potential of manipulating the genotype of embryos in culture before transferring them back to foster females, and his studies beginning in 1970 are described elsewhere in this issue. However, others quickly adopted the culture system, and it has played a fundamental and an enabling role in development of many experimental approaches to study embryos. The most significant of these is in germ line modification. For all the major techniques used to introduce genes into the germ line of animals, egg culture is essential to the approach (Fig. 4). The simplicity and reliability of the culture technique have enabled these difficult but profound experiments, and Ralph's fundamental work between 1960 and 1970 played a significant role in laying the foundation for development of transgenesis and stem cell biology.

Summary

Ralph Brinster began his classic work on egg culture more than 35 years ago. His interest in mammalian egg culture had developed, in part, as a consequence of his experiences with animal breeding and reproduction that he gained while growing up on a farm. Ralph decided early in his career that an *in vitro* approach to culturing eggs would provide a powerful tool with which to study the development of these cells. Beginning at the close of the 19th century, a number of investigators had performed *in vitro* studies on egg culture and the related area of egg transfer; however, the ability to recover and transplant eggs had reached a much higher level of perfection than had culture. Eggs of many species could be successfully transferred, but there was no reliable technique for egg culture. In 1963, Ralph reported a method for culturing eggs in microdrops of medium under oil (Brinster, 1963), which has become universally used. Two years later, he identified pyruvate as the central and essential energy source for early stages of mouse eggs (Brinster, 1965b). These two developments revolutionized *in vitro* studies of mammalian eggs and issued in an era of intense research activity concerning egg culture and egg manipulation. Effective formulations of culture media could now be developed to allow routine *in vitro* maintenance of eggs, and important parameters for these recipes were soon determined. It was quickly established that the requirement for pyruvate as an energy source exists at ovulation in many species and is already present in germ cells of the mouse fetus. The metabolic activity of the fertilized mouse egg was shown to be low and comparable to bone; however, four days later, at the blastocyst stage of development, the metabolic activity was comparable to brain. Thus, a foundation

of understanding about the biology of early mammalian eggs was established between 1960 and 1970, and subsequent studies have broadened this understanding. However, the greatest impact of a simple, reliable egg culture method has been to provide the ability to perform complicated manipulative procedures on preimplantation stages of mammalian embryos. In no area has this been more important than in development of transgenic animals. All methods for generating germ line genetic modifications rely on the ability to maintain and manipulate eggs and early developmental stages *in vitro* without loss of developmental competence. The importance of efficient egg culture to manipulation and transgenesis is fundamental and enabling.

KEY WORDS: *egg culture, mouse, embryo, germ cell*

Acknowledgments

This chapter is based primarily on information contained in the cited references. For historical aspects of the account, three extensive reviews of early literature in the field were particularly helpful (Austin, 1961; Brinster, 1964; Betteridge, 1981). In addition, several summaries of Ralph Brinster's contributions to germ cell research have been compiled recently in conjunction with awards he has received (see, e.g., Hammer, 1988; Citation for the Pioneer Award from the International Embryo Transfer Society, Theriogenology 37:1-3, 1992; Citation for the Carl G. Hartman Award from the Society for the Study of Reproduction, Biol. Reprod. 58:312-313, 1998). These citations were especially useful in providing perspective to the role of Ralph's egg culture work in establishing a foundation for subsequent studies on embryos and germ line modification. Insight into Ralph's early interest in reproductive biology arises from conversations with his students and my time training in his laboratory. I apologize to those whose work could not be included because of space limitations. The reviews cited, as well as other chapters in this issue, provide a more extensive account of the field.

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