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Special Review

Stem cells and transgenic mice in the study of development[1]

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[1]*This review is based on a lecture given in December, 1991 at the Nobel Symposium entitled «Genetic Control of Embryonic Development».*

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During the 1960s, Barry Pierce described a series of experiments regarding the developmental potential of teratocarcinoma cells. Because of my long-time interest in germ cells, the most versatile of all stem cells, it was natural that Barry's experiments with teratocarcinoma cells would play a role in the evolution of my work. It is a pleasure to write a review of the work in my laboratory describing how Barry's contributions influenced its development.

Cell transfer into blastocysts

During training in veterinary school, I became aware of the high level of infertility that was characteristic of reproduction in farm animals and in man. Since a sizable portion of this infertility resulted from embryonic mortality near the time of implantation, it seemed logical to examine the preimplantation stages of embryo development and look for reasons to account for this failure. Therefore, following graduation in 1960, I decided to study the early stages of development and reasoned that much could be learned from *in vitro* experiments. Unfortunately, there was no reliable method by which to grow these embryos. Therefore, the first step was to develop a culture system that was simple, efficient and dependable. After many experiments between 1960 and 1963, an effective technique was identified consisting of placing embryos in small droplets of culture medium under mineral oil (Brinster, 1963). In the subsequent few years we used this technique to establish optimum characteristics of a culture medium for mouse eggs. The most important finding was that early stages of egg development required pyruvate rather than glucose as an essential energy source (Brinster 1965a,b). This simple culture technique and the identification of pyruvate as the essential energy source formed the basis of subsequent culture systems that allowed us and others to study development of the mouse embryo as well as to manipulate it *in vitro*. These studies also presaged work on the early embryos of other species, including culture of farm animal eggs and *in vitro* fertilization of human eggs.

Initially, I began using the culture system to study energy, protein, and nucleic acid metabolism in eggs (reviewed by Brinster 1969, 1973), but by the late 1960s my interest had turned to the possibility of modifying the embryo genotype in order to learn about the control of subsequent development. One approach that seemed very promising to me at that time was the addition of older embryo cells to the mouse blastocyst. Basically there were two types of cells that could be used.

1. Pluripotent cells, which can be defined as stem cells from a differentiated tissue that have the potential to participate in development of a circumscribed set of cells of a particular lineage.
2. Totipotent cells, which can be defined as stem cells that have the potential to participate in development of all cell types including germ cells.

The genotype of either a pluripotent or totipotent cell also might be modified experimentally prior to transfer, thus altering its ability to influence subsequent patterns of development.

Supporting the feasibility of this approach, Tarkowski (1961) and Mintz (1962) had demonstrated that fusing genetically distinct 8-cell embryos resulted in the formation of adult chimeras. Gardner (1968) had extended this approach and shown that moving the inner cell mass from one embryo to another would also result in the development of a chimera. It therefore seemed possible that there might be older cells in the embryo that could participate in development if introduced into the blastocyst. However, results from our experiments to test this hypothesis indicated that while synchronous cells from blastocysts were successful in producing chimeras, older embryo cells appeared to have very little ability to colonize the blastocyst (Moustafa and Brinster, 1972a,b).

Although the latter experiments had not been particularly successful, I remained convinced that the approach of transferring cells into the blastocyst was potentially a very useful method of modifying an embryo. During the 1960s and coincident with our embryo studies, the characteristics of the teratocarcinoma cell were becoming apparent. Stevens (1959, 1964) had identified and described teratocarcinomas in mice and shown that many cell types were present in these tumors. In a series of critical experiments Barry Pierce and his colleagues demonstrated that some individual teratocarcinoma cells were multipotential (Pierce *et al.*, 1960; Kleinsmith and Pierce, 1964; Pierce *et al.*, 1967). On the basis of cellular characteristics and developmental potential, the most primitive of these teratocarcinoma cells have been designated embryonal carcinoma (EC) cells (Pierce, 1975; Stevens and Pierce, 1975). Thus, the primitive teratocarcinoma or embryonal carcinoma cell appeared to be an excellent candidate for a totipotent cell to use in blastocyst transfer experiments, and the bone marrow stem cell was chosen as a candidate pluripotent cell.

Embryonal carcinoma and bone marrow stem cells as donors

In 1969 we began studies in which these cells were transferred into mouse blastocysts (Fig. 1). There are several mechanisms by which to assess the effect on development of transferred cells. If the cells persist and participate in development, one might expect that skin grafts from the cell donor strain of mice would be retained

Abbreviations used in this paper: EC, embryonal carcinoma; ES, embryonic stem; MT, metallothionein; HSV, herpes simplex virus; TK, thymidine kinase; uPA, urokinase-type plasminogen activator; Alb, albumin; SCID, severe combined immune deficiency.

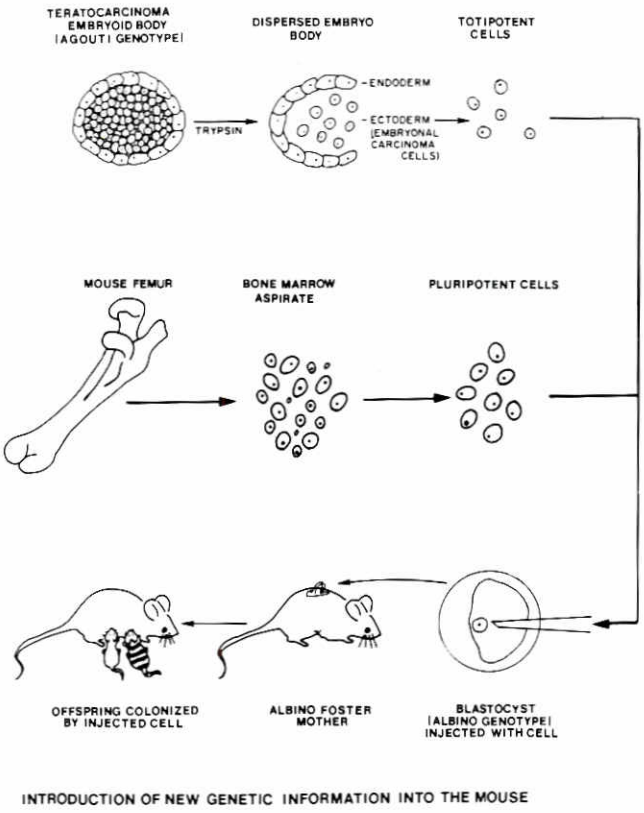

Fig. 1. Diagram of technique used to introduce either totipotent or pluripotent stem cells into the mouse blastocyst following which they may participate in development (Brinster, 1974). *Totipotent stem cells, such as embryonal carcinoma (EC) or embryonic stem (ES) cells, may contribute to all tissues including germ cells and hair follicle cells. Therefore, their derivatives can be transmitted to progeny and are able to generate coat color chimeras as illustrated. Pluripotent stem cells, such as bone marrow cells, may contribute only to myelogenous or hematopoietic cells. Their derivatives are unlikely to be transmitted to progeny or create coat color chimeras. The genotype of either totipotent or pluripotent cells may be experimentally modified prior to the in vitro transfer procedure.*

for a longer period of time on animals that developed from injected blastocysts than on control animals. Indeed, a significant increase in retention of such skin grafts was observed (Fig. 2) on mice arising from blastocysts into which putative bone marrow stem cells were placed (Brinster, 1974). Following the transfer of EC cells into blastocysts, a similar prolongation of skin graft survival was observed. However, even more dramatic was the appearance of thin stripes of agouti hair on a mouse developing from a blastocyst with albino genotype into which EC cells had been injected (Fig. 3). Since the EC cells were of agouti genotype, the stripes were incontrovertible evidence that the transferred cells had participated in development. This was an exciting result, because it clearly established a mechanism by which one could modify development following experimental manipulation of cells and embryos in culture.

These experiments were repeated and extended by two laboratories in the following year (Mintz and Illmensee, 1975; Papaioannou *et al.*, 1975). However, it soon became apparent that the EC cell did not colonize the developing embryo efficiently, and the cells infrequently entered the germ line of resulting mice (Dewey *et al.*, 1977; Russell, 1978). Because of this deficiency, our laboratory and others began searching for a better cell line. In 1981, three groups published reports describing cell lines that were more efficient in colonizing the germ line of mice derived from injected blastocysts. One cell line arose from embryonal carcinoma cells (Stewart and Mintz, 1981), and the other two arose from cultures of blastocysts (Evans and Kaufman, 1981; Martin, 1981). These latter cells have received the designation of embryonic stem (ES) cells. Embryonal carcinoma and embryonic stem cells are very similar in characteristics. In particular, both will colonize the blastocyst and both will form teratocarcinomas when injected into syngeneic hosts (Damjanov and Solter, 1974; Evans and Kaufmann, 1981). Like EC cells, some clones of ES cells participate poorly in embryonic development. Fig. 4 shows an animal resulting from transfer of ES cells that has a degree of coat color chimerism similar to the original mouse colonized by EC cells (Fig. 3). Only one offspring in 100 from the animal in Fig. 4 is agouti (the donor cell gene type); the other 99 are albino. However, clones of ES cells can be developed that colonize 100 per cent of germ line cells (McMahon and Bradley, 1990; Soriano *et al.*, 1991).

Since 1981 the embryonic stem cell has evolved into an important means of modifying the mouse genome. During the early 1980s it was demonstrated that homologous integration of foreign DNA could be achieved in cultured somatic cells (reviewed by

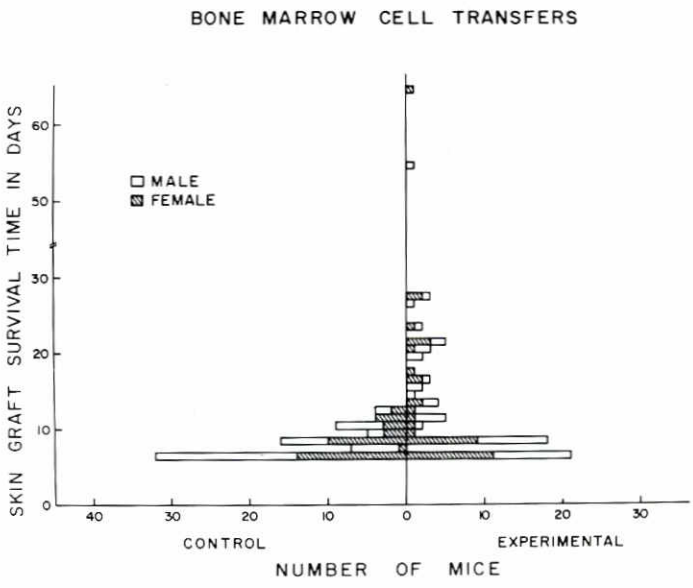

Fig. 2. Distribution of skin graft survival times on male and female mice *that developed from blastocysts receiving bone marrow cells (experimental), and survival times on comparable animals that arose from blastocysts that were not injected with foreign cells (control). The increase in skin graft survival time for the experimental animals was significant for both males and females (Brinster, 1974).*

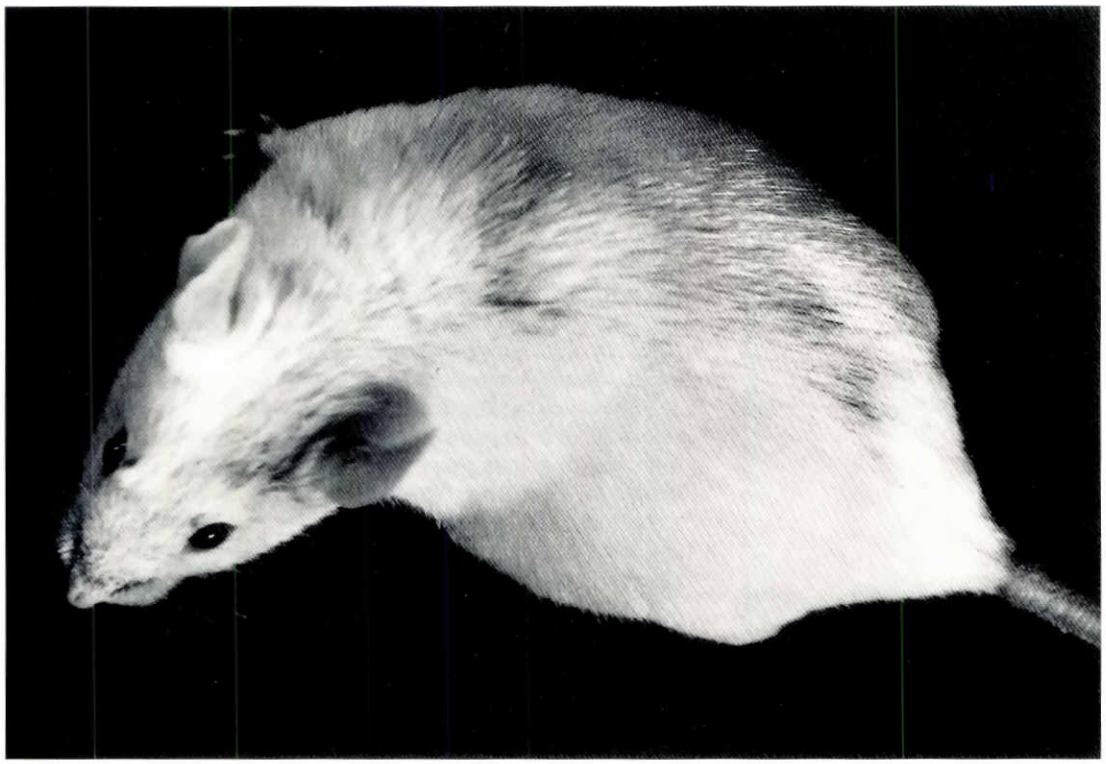

Fig. 3. A random-bred Swiss albino mouse with several stripes of agouti hair extending from the midline over the right flank and thorax. *An additional group of agouti hairs is present at the anterior base of the left ear. The animal developed from a blastocyst of Swiss albino genotype that received strain 129 embryonal carcinoma (EC) cells of agouti genotype (Brinster, 1974).*

Kucherlapati and Moore, 1988; Subramani and Seaton, 1988). Subsequently these results were extended to embryonic stem cells, and it was demonstrated that ES cells with these targeted modifications could be introduced into blastocysts to produce chimeras (reviewed by Capecchi, 1989; Frohman and Martin, 1989). Transmission of a targeted modification through the germ line was then reported (Thompson *et al.*, 1989). Thus, the EC cells identified and first described by Stevens and subsequently shown by Pierce to possess multipotential characteristics led to the isolation of the closely related ES cell that can efficiently colonize the blastocyst and carry with it modifications of the genome which influence development.

Development of transgenic mice

The value of the stem cell approach was greatly enhanced by the development of transgenic techniques during the 1980s. The ability to introduce new genes into animals and plants represents a dramatic advance in biology. Throughout the history of biology, the experimentalist has been primarily restricted to observation, dissection, and description which lead to hypotheses regarding function. For the first time humans have the capability of producing a new life form, one that has a genotype uniquely modified by experimental choice. Surprisingly, the ability to achieve this remarkable feat was first demonstrated in mice (reviewed by Hammer, 1988); frequently such innovative accomplishments are initially perfected in less complex animals such as flies, worms or sea urchins (Table 1). The precocious achievement of transgenesis in the mouse was facilitated by the development of excellent mammalian somatic cell culture techniques, the early success with mouse embryo culture and the ability to transfect cultured animal cells (Graham and van der Eb, 1973; Wigler *et al.*, 1977).

I first became interested in the possibility of transferring genes

TABLE 1

PRODUCTION OF TRANSGENIC ORGANISMS

Organism	Gene	References
Mice	TK, SV40	Gordon *et al.*, 1980
	hß-globin, TK	E. Wagner *et al.*, 1981
	M-MuLV	Harbers *et al.*, 1981
	rß-globin	T. Wagner *et al.*, 1981
	rß-globin	Costantini and Lacy, 1981
	mMT-TK	Brinster *et al.*, 1981b
Xenopus	rß-globin	Rusconi and Shaffner, 1981
Drosophila	P element	Spradling and Rubin, 1982
	XDH	Rubin and Spradling, 1982
Plants	NOS-NEO	DeBlock *et al.*, 1984
	NOS-NEO	Horsch *et al.*, 1984
C. elegans	col-ß-gluc	Stinchcomb *et al.*, 1985
Sea urchin	actin-NEO	McMahon *et al.*, 1985
Farm animals	mMT-hGH	Hammer *et al.*, 1985

Abbreviations: Species: h, human; r, rabbit; m, mouse. Genes: SV40, simian virus 40 DNA; TK, herpes simplex viral thymidine kinase; M-MuLV, Moloney murine leukemia virus; MT, metallothionein-1; XDH, xanthine dehydrogenase; NOS, nopaline synthase; NEO, neomycin phosphotransferase; col, collagen; ß-gluc, ß-glucuronidase; GH, growth hormone. (modified from Hammer 1988)

into the mouse egg as a result of studies reported by McBride and Ozer (1973) demonstrating that chromosome preparations could alter the genotype and phenotype of somatic cells in culture. The use of this approach to modify eggs seemed complimentary to our stem cell studies. While investigating the feasibility of these experiments, the advantages of using pure DNA preparations became obvious. Graham and van der Eb (1973) were successful in transfecting cells in culture with viral genes and in the following few years recombinant DNA techniques made possible the isolation and production of genes in high concentration and great purity. Efficient transfection of genes into mammalian somatic cells was then accomplished (Wigler *et al.*, 1977). The development of these recombinant DNA and somatic cell transfection techniques made the use of chromosomes unnecessary, and we began a series of experiments in the late 1970s to introduce DNA into mouse embryos by microinjecting macromolecules into the fertilized one-cell egg. These experiments were piloted by first demonstrating in cultured eggs that injected messenger RNA was faithfully translated (Brinster *et al.*, 1979; 1980a) and then showing that injected genes could be transcribed accurately (Brinster *et al.*, 1980b; 1981a). With this work as a background, we began attempts to achieve gene integration by injecting the DNA into the pronucleus of fertilized mouse eggs and transferring the eggs to foster mothers. From 1979 to 1981, in collaboration with several other research groups, five different genes were injected: rabbit beta globin, sea urchin histone, Simian Virus 40 (SV40), mouse polyoma virus and herpes simplex virus (HSV) thymidine kinase (TK). In one case there was evidence for gene integration (Brinster *et al.*, 1981a). However, the interpretation of the Southern blot analysis was puzzling, because the insertion of injected DNA in a tandem array was not understood. While we were microinjecting genes into eggs and analyzing for their presence in adults, the experiments with mRNA continued. Early in 1980 I had contacted Richard Palmiter for ovalbumin mRNA to be used in microinjection experiments and learned that he was studying the regulatory regions of the mouse metallothionein (MT) gene. Late in 1980 he fused these regions to the herpes simplex virus thymidine kinase structural gene. Since I had used the HSVTK gene in previous experiments and had good assay procedures, microinjecting this fusion gene into eggs appeared promising. Richard Palmiter sent the MT-TK gene, which was injected into eggs in January, 1981. The experiment was successful and transgenic mice resulted (Brinster *et al.*, 1981b), establishing the start of our subsequent productive collaboration.

Microinjection of genes into the pronucleus is not the only way that foreign DNA can be introduced into mouse eggs. The ability of viruses to penetrate the zona pellucida and infect mouse eggs had been demonstrated almost 20 years earlier (Gwatkin, 1963; Gwatkin and Auerbach, 1966), and SV40 virus replication in blastocysts was subsequently described (Biczysko *et al.*, 1973). It was then shown that SV40 DNA injected into the blastocyst cavity could be found in the adults that developed from these blastocysts (Jaenisch and Mintz, 1974); recent studies suggest that most of the DNA was in episome form (Willison *et al.*, 1983). However, about 60 per cent of cleavage stage embryos can be infected with Molony murine leukemia retrovirus, and some cells in the embryo will integrate the virus in one or more chromosomal locations (Jaenisch, 1976; Harbers *et al.*, 1981). The resulting animals will be mosaic for the presence of the viral genome in somatic and germ line tissue, but subsequent breeding can isolate the individual germ line integrations to specific progeny. Approximately one-half of the integrated

Fig. 4. A random-bred Swiss albino mouse with several stripes of agouti hair extending from the midline over the back and left flank. *Additional patches of agouti hair are present between the eyes and behind the left nostril. The edges of both ears also have agouti hair. The animal developed from a blastocyst of Swiss albino genotype that received strain 129 embryonic stem (ES) cells of agouti genotype. The level of chimerism is similar to that present in the animal in Fig. 3, and approximately one per cent of the germ cells are of agouti genotype. (Avarbock, Zimmermann, Masters and Brinster, unpublished)*

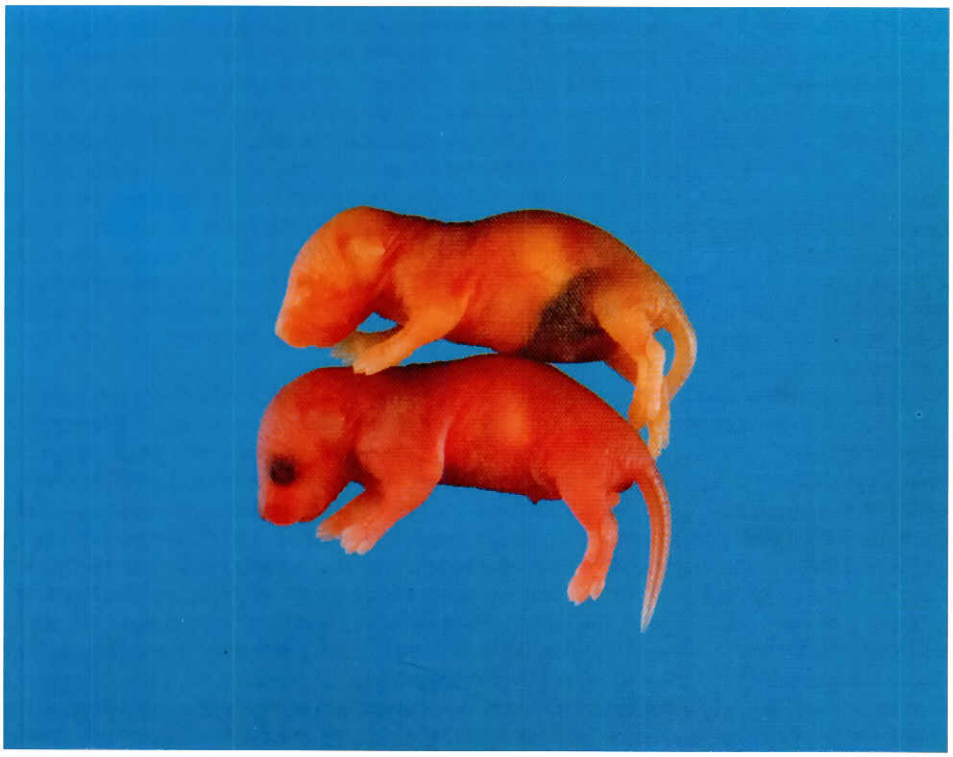

Fig. 5. Gross appearance of a live Alb-uPA transgenic pup showing the bleeding phenotype that results from excess production of uPA by the liver. *The transgenic mouse (top) is icteric and contains blood in its intestine (dark patch in the caudal abdomen). Milk is present in the stomach of both mice. The transgenic mouse is an albino, and thus lacks the retinal pigment seen in the lower control pup (Heckel et al., 1990).*

viral genomes are not expressed, and the remainder generally exhibit different but characteristic expression patterns that are influenced by chromosomal position (Jaenisch *et al.*, 1985). By including a predetermined gene construct within the virus, this technique can be used to introduce foreign DNA other than the viral sequences into germ cells of mice (van der Putten *et al.*, 1985). Because viruses have evolved over many millions of years and can escape or manipulate regulatory mechanisms within cells, control of nonviral genes included in viral sequences is often impaired or modified. As a result of the integration and expression characteristics of viruses, their use to introduce foreign genes into the germ line of animals has so far been limited. The advantages, disadvantages and future potential of retrovirus vectors have recently been reviewed (Temin, 1989).

Although considerable work occurred in the 1960s and 1970s to provide a foundation for the development of transgenic animals, the critical experiments to establish an effective method were performed during the 1980s and relied primarily on the technique of microinjecting fertilized eggs (reviewed by Brinster and Palmiter, 1986; Palmiter and Brinster, 1986; Jaenisch, 1988; Hanahan, 1989). Progress was divided into four rather distinct steps.

1. Demonstrating that foreign DNA was integrated into host chromosomes. This was reported by six laboratories between December, 1980 and November, 1981 (Gordon *et al.*, 1980; Harbers *et al.*, 1981; Wagner, E. *et al.*, 1981; Wagner, T. *et al.*, 1981; Costantini and Lacy, 1981; Brinster *et al.*, 1981b).
2. Documenting that integrated DNA was expressed. Although several of the original six reports described some expression of integrated DNA, the accelerated growth of mice carrying a metallothionein-rat growth hormone fusion gene was the most dramatic demonstration of transgene expression (Palmiter *et al.*, 1982). This report catalyzed interest in the transgenic approach within the scientific community and provoked the imagination of the general public.
3. Proving that specific DNA sequences directed tissue-appropriate transgene expression. This was first demonstrated for a kappa immunoglobin gene (Brinster *et al.*, 1983; Storb *et al.*, 1984) and subsequently confirmed with the rat elastase gene (Swift *et al.*, 1984).
4. Achieving targeted integration of the introduced DNA in the germ line by homologous recombination. This was first accomplished employing the EC/ES cell approach (Thompson *et al.*, 1989). Targeted integration of DNA can also be achieved by direct injection of the appropriate DNA into fertilized one-cell eggs (Brinster *et al.*, 1989), but the efficiency is currently too low for general use.

These four steps, accomplished over a period of less than ten years, constitute a remarkable advance in our ability to study development as well as to examine a host of questions in biology and medicine.

Combined stem cell and transgenic approach

While transfer of stem cells and production of transgenic mice each provide a powerful approach to understanding development, when used together they are complimentary techniques and can facilitate studies on organ development as well as the evolution of cell lineages. This complimentarity can be illustrated by a series of recent experiments using the mouse urokinase-type plasminogen activator (uPA) gene, in which a valuable model was generated to

study liver development. In these experiments, the regulatory region of the mouse albumin (Alb) gene was fused to the uPA structural gene, and this construct (Alb-uPA) was introduced into mice (Heckel *et al.*, 1990). Many transgenic mice developed clotting dysfunction and died within four days of birth due to hemorrhage into the gut (Fig. 5). Surprisingly, in two lines of transgenic mice derived from surviving founders, about half of all newborn transgenics also developed the bleeding phenotype, but the remainder survived and grew to adulthood. These mice subsequently transmitted the transgene and phenotype to progeny. By three weeks of age the survivors displayed a recovery of clotting function (Sandgren *et al.*, 1991), which is an unexpected result for an abnormal phenotype caused by albumin-directed transgene expression, since albumin expression does not diminish with age. Correlated with this change in clotting function was a remarkable alteration in liver phenotype. At birth, transgenic livers were pale to white and the hepatocytes were abnormal, but focal red areas appeared and enlarged to nodules of normal hepatocytes as mice grew older (Fig. 6). By 6 to 8 weeks of age, the liver was entirely replaced by red nodules, and these areas no longer expressed the transgene, even though expression of other liver-specific genes was normal. Examination by Southern blot analysis of the transgene integration site in red nodules from these mice suggested that individual cells in the white, abnormal liver can lose all functional transgenes within the tandem array by chromosomal rearrangement (Fig. 7). As a result, these individual cells escape the adverse effect of transgene expression and clonally expand to form nodules of healthy liver. The red nodules then reestablish normal liver function. As few as one clonal nodule can replace up to 95 per cent of normal liver mass (approximately $2x10^8$ hepatocytes), clearly dramatizing the remarkable regenerative capability of the liver stem cell. There are several other examples in which transgene expression results in liver damage, but in these cases there is neither total loss of the original cells nor total replacement by new cells (Chisari *et al.*, 1987; Carlson *et al.*, 1989). The Alb-uPA model allows us to study the total replacement of defective hepatocytes by proliferation of liver stem cells, because there is a gradual loss of function and death of transgene-expressing hepatocytes coupled with rapid growth of functionally normal liver foci.

This unique model of spontaneous liver regeneration can be used to study the mechanism underlying the remarkable regulation of liver cell number and organ size. However, an approach of even greater interest to the developmental biologist will be the use of these mice to examine repopulation of liver with exogenous cells. In several studies, exogenous liver cells have been introduced into the portal vein or the spleen (Fuller, 1988; Ponder *et al.*, 1991; Gupta and Chowdhury, 1992), and these injected donor hepatocytes have subsequently been identified in the liver parenchyma. However, it is unclear to what extent they have become part of the functional architecture or whether they are only passenger cells, and most of the original liver remains intact. In the Alb-uPA model, the transgene-expressing cells are replaced early in life by endogenous cells that have inactivated transgene expression. In principle, this process could be mimicked by foreign cells that are introduced into the young animal. The foreign donor cells could be marked by the presence of a transgene, such as E. coli beta-galactosidase, or have their function modified by a transgene. A particularly useful recipient mouse for these experiments is the homozygous Alb-uPA mouse. The frequency with which endogenous hepatocytes inactivate uPA expression is greatly reduced in homozygotes (Fig. 6), because both transgene alleles must be inactivated (Sandgren *et al.*, 1991). In homozygotes, survival will depend critically on clonal expansion and functional development of injected donor cells, since more than 80 per cent of these animals die from severe edema by 6 weeks of age if normal liver function is not restored (Sandgren *et al.*, 1991). This provides a very powerful selective mechanism to test the functional capability of injected cells. One can then ask what type of cell will act as a liver stem cell. For example, both hepatocytes and nonhepatocyte liver cells of several developmental stages can be tested to determine their potential to regenerate liver function. Perhaps even more interesting is the question of whether cells from other organs, particularly those of endodermal origin, can repopulate the liver. Under special conditions cells lining the small pancreatic ducts in the hamster or rat take on the appearance of hepatocytes following treatment with carcinogenic agents (Scarpelli *et al.*, 1989; Makino *et al.*, 1990), and the capacity of similar cells in the mouse to clonally expand when introduced into the selective environment of the Alb-uPA mouse liver can be tested. By extension of this approach, cells from fetal or adult gut can be similarly evaluated. It should be possible to assess the developmental potential of a variety of stem cells and to uncover the relationships among cell lineages that arise from a common embryonic location. Finally, one could ask whether totipotent stem cells such as EC/ES cells could contribute to liver formation in this environment, or whether they might require some pretreatment in culture before transfer.

This model can also make valuable contributions to medicine and our understanding of organ pathology. The liver is commonly involved in human and animal diseases. It represents 5 per cent of body weight and provides many critical functions. An exciting and valuable approach will be to replace the failing liver of Alb-uPA transgenic mice with human hepatocytes. Problems associated with histocompatibility can be circumvented by introducing the Alb-uPA phenotype, through appropriate matings, into immunologically tolerant host mice such as the athymic nude mouse or mice with severe combined immune deficiency (SCID). The characteristics of the SCID mouse have recently been reviewed (Bosma and Carroll, 1991), and, in fact, human immune cells have already been shown to survive in SCID mice (McCune *et al.*, 1991). Using Alb-uPA SCID mice it may be possible to identify the human cell or cells that can act as stem cells in liver regeneration. Furthermore, models of either genetic or acquired human liver disease could be generated. For example, human liver cells with a genetic alpha-1-antitrypsin deficiency could be used to repopulate the liver. Alternatively, an acquired disease such as cirrhosis could be studied following repopulation of the Alb-uPA liver with human hepatocytes; currently no adequate models exist to examine this important liver disease. A variety of other potential applications for these animals can be imagined.

It is possible that differentiation in other organs could be studied by a similar combination of stem cell and transgenic methods. Colonizing the seminiferous tubules of the male gonad with stem cells would have great scientific potential. The germ cell is the ultimate stem cell and of enormous interest in understanding how genomes are modified during commitment and differentiation of cells. It is possible to destroy the germ cells in the seminiferous tubules by irradiation or chemotherapeutic agents (Withers *et al.*, 1974; Bucci and Meistrich, 1987), and there are several mutant mouse strains that lack germ cells (Silvers, 1979). In both these situations potential stem cells, either from normal mice or those carrying transgenes that confer modified functions, could be reintro-

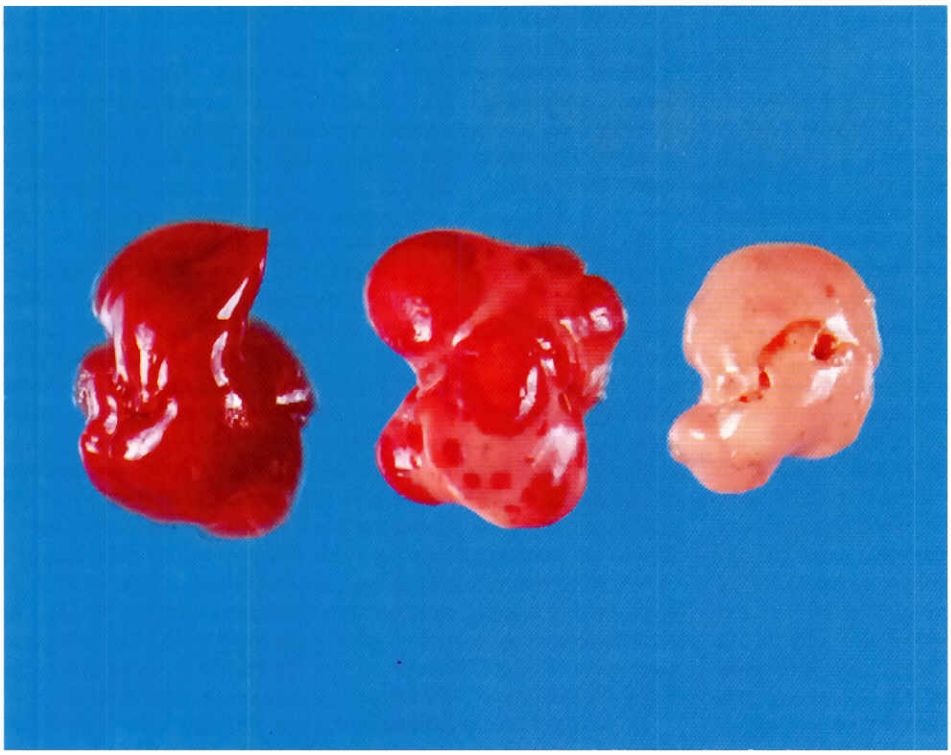

Fig. 6. Gross appearance of Alb-uPA transgenic mouse liver. *Livers were removed from 5-week-old male mice. The liver to the left is from a nontransgenic control mouse, that in the center from a hemizygous transgenic mouse, and that to the right from a homozygous transgenic mouse. Note the large red nodules in the hemizygote's liver and their absence from the homozygote's liver. The homozygote liver has a few small red foci that are barely visible (Sandgren et al., 1991).*

duced into the tubules, and the ability of the cells to differentiate into mature spermatozoa assessed. Transfer of immature spermatogonia might be expected to result in spermatogenesis. However, the potential of EC/ES cells to differentiate into spermatozoa either directly or following *in vitro* conditioning also would be a logical avenue to explore, since it is thought that the EC cell arises from male primordial germ cells (Pierce, 1967; Stevens, 1967). Likewise, one might devise methods to return partially differentiated stem cells to a totipotent state and then test their adequacy in this system.

Conclusions and future prospects

The range of opportunities to combine stem cell and transgenic methods in the study of development is enormous. Logically, it can be divided into four categories.

1. Pluripotent cells transferred into the blastocyst may modify select lineages of cells during development. For example, transferred bone marrow stem cells, as used in our 1974 experiment, might colonize only hematopoietic cell lines and influence primarily their subsequent development.
2. Totipotent cells transferred into the blastocyst may colonize the germ line and influence the genotype of progeny. This is the approach used in the transfer of EC cells in 1974 and has, by extension to ES cells, become very valuable in combination with gene targeting to study development.
3. Pluripotent cells transferred into an organ may replace endogenous tissue. This is the approach that is being utilized to study repopulation of the Alb-uPA transgenic mouse liver by hepatocytes and other potential stem cells.
4. Totipotent cells transferred into an organ may recapitulate differentiation of either somatic or germinal tissue. This type of study is represented by experiments in which EC/ES cells are transferred into the liver or another organ to evaluate their potential for regenerating a somatic tissue. If spermatogonia, primordial germ cells or EC/ES cells are introduced into the seminiferous tubule, their potential to become functional germ cells could be evaluated.

These categories represent the four major approaches to examining the relationship between totipotent or pluripotent cells and a developmentally permissive environment (such as a blastocyst) or a more restrictive environment (such as the liver). These experimental approaches have the potential to teach us a great deal about cell lineages and development in mice, and perhaps by extension in humans as well.

Stem cell and transgenic methods have become extremely valuable in the study of both normal and abnormal development. Like all advances in science, the foundation for these approaches dates back many years and includes the early work on EC cells. It is appropriate to remember this pioneering work, in which Barry Pierce played an essential role, and the reviews contained in this issue reflect the many ways Barry's contributions have influenced the research of others.

Summary

In recent years, totipotent stem cells and transgenic mice have been widely used to understand the complex changes that occur during development, and these approaches underlie much of the dynamic growth in this field. The work of Barry Pierce in defining the

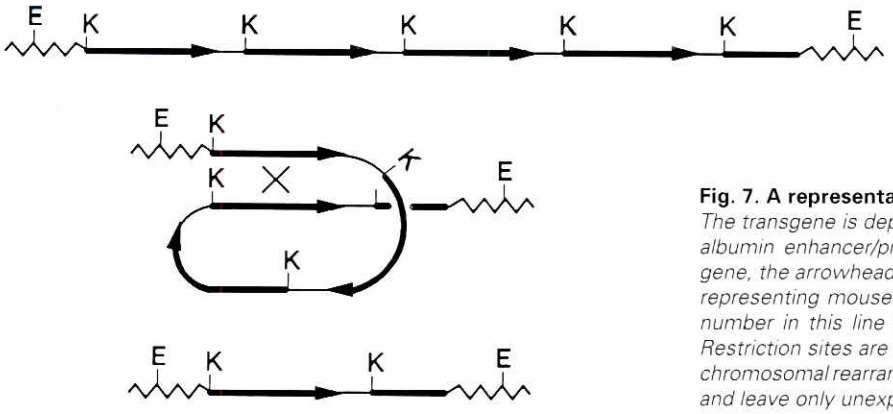

Fig. 7. A representative intrachromosomal transgene deletion event. *The transgene is depicted as an arrow, with the thin line representing the albumin enhancer/promoter, the bold line representing the mouse uPA gene, the arrowhead representing an hGH 3' sequence, and the wavy line representing mouse chromosomal DNA. Quantitation of transgene copy number in this line of mice suggests that there are about four copies. Restriction sites are indicated as follows: K for KpnI and E for EcoRV. The chromosomal rearrangement illustrated would delete the active transgenes and leave only unexpressed foreign DNA (Sandgren et al., 1991).*

multipotential characteristics of teratocarcinoma or embryonal carcinoma stem cells in the 1960s was an important milestone for the field and was instrumental in our choice of these cells for transfer into blastocysts in the first experiments designed to colonize a mouse with foreign totipotent cells. Following the development of transgenic techniques, the stem cell approach has become even more powerful, and during the past five years the combination of the two techniques has made possible the experimental creation of virtually any genetic change in mice, and ultimately in other species. In this review, the work in our laboratory over the past 30 years is summarized, and it reflects only a small part of the exciting array of experiments that have contributed to the explosive evolution of developmental biology during this period.

KEY WORDS: *stem cells, transgenic mice, development, genetics*

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