Int. J. Dev. Biol. 42: 841-846 (1998)

The coming of age of the transgenic era

VIRGINIA E. PAPAIOANNOU*

College of Physicians and Surgeons of Columbia University, New York, NY, USA

Those were heady days. For those of us entering the fields of genetics and mammalian embryology in the 60s and 70s, the excitement was palpable. As graduate students and post-docs, we saw that long-standing barriers were tumbling down before an onslaught of technological advances. And not only that, the scientists breaking those barriers were all around us as mentors and colleagues. The rapid pace of progress and the seemingly boundless possibilities hooked us into the field and we gradually became aware that we, too, were part of a revolution that was on the way to opening the mammalian genome to experimental alteration. Who could forget the thrill of seeing mice born of a foster mother, knowing that as embryos, they had spent a large part of their preimplantation life in a petri dish? (Fig. 1) (McLaren and Biggers, 1958). Or seeing a variegated mouse that was actually the product of two separate embryos, combined to make a single animal? (Fig. 2) (Tarkowski, 1961; Mintz, 1964; Gardner, 1968). Or later, seeing transgenic mice with visible and profound indications of the activity of an introduced, foreign, mammalian gene functioning within its genome? (Fig. 3) (Palmiter *et al.*, 1982,1983). As we stood on the shoulders of giants, we could peer into a new age of genetic engineering, where alteration of the genome would become a predictable and precise science.

Ralph Brinster was undoubtedly one of those giants and continues to this day on an unswerving course aimed at understanding the complexities of the control of gene expression and, ultimately, of manipulating the information transmitted from one generation to the next. The field of genetic engineering, in the broadest sense, owes a great deal to the work and innovations of Dr. Brinster. With hindsight, it is easy to see how his contributions advanced the field at key, critical moments, but it was also obvious at the time that his work was at the forefront and led the way into uncharted territory. Even more striking is that Brinster seemed at all times to know exactly where he was heading and to understand the greater significance of his work. As the contributions to this special issue of The International Journal of Developmental Biology will attest, the circles of influence of his work spread widely. What follows is a highly personal view of some of the major trends that converged to create the field of transgenic technology (Fig. 4), highlighting some of the seminal contributions made by Ralph Brinster.

A young person entering science today takes it for granted that the units of heredity can be dissected, altered, cloned, manipulated and otherwise subjected to experimental analysis in all species including mammals. In this age of genetic engineering, the entire human genome seems to be giving up its secrets in the face of technological advances. In laboratory animals, it has become standard technique to add foreign genes and to create specific mutations in endogenous genes at will. The permanent alteration of the germ line has become commonplace and is a primary tool in the elucidation of issues of gene regulation, development, the disease process, and virtually any other area of biological endeavor. With the exponential pace of progress, it is amazing to realize that a mere twenty years ago, the field of molecular biology and

Address for reprints: Department of Genetics and Development, College of Physicians and Surgeons of Columbia University, 701 W. 168th Street, New York, NY 10032, USA. FAX: 212 305-5484. e-mail: vep1@columbia.edu

0214-6282/98/$10.00

Printed in Spain

Fig. 1. The first adult mice produced by embryo transfer in which part of the preimplantation stages of development occurred *in vitro* (McLaren and Biggers, 1958). *The two albino mice were cultured from the 8-cell to the blastocyst stage (from Biggers, 1987).*

recombinant DNA technology was in its infancy. The first transgenic mouse was produced by DNA microinjection just 17 years ago, and the first mouse with a targeted gene mutation made the scene less than 12 years ago. The number of transgenics produced by DNA microinjection is vast and a glance at a current database puts the number of new mutations created by gene targeting around one thousand.

This recent history of the genetic revolution has deep roots, however, and involves the convergence and synergism of different lines of endeavor during a dynamic time for biological research (Fig. 4). One line comes from a long tradition of embryo culture and transfer (reviewed by Biggers, 1987). For the purposes of this essay, a reasonable starting point might be 1949 when Hammond cultured preimplantation mouse embryos from the 8 cell stage to the blastula stage in a medium containing egg white (Hammond, 1949). From this laconic report, it is not entirely clear what his purposes were, other than an attempt at embryo culture, but not long thereafter, concerted efforts were made to refine embryo culture methods by defining the metabolic requirements of preimplantation embryos at different stages of their development (Whitten, 1956;1957). That their requirements did change was evidenced by the *in vitro* developmental arrest exhibited by the embryos at specific stages.

The observation of continued cleavage and morphogenesis was, of course, only one means of determining the success of *in vitro* culture; determining whether embryos retained viability after culture was equally important. The most stringent test for the quality of *in vitro* culture conditions is to determine whether cultured embryos will thrive when returned to the uterus of a foster mother for continued development. Embryo transfer procedures had been attempted with success in many species since the previous century although not always with reproducible results (reviewed by Biggers, 1987). In 1958, McLaren and Biggers reported that embryos cultured from the 8 cell through the blastocyst stage could successfully complete their development when transferred to the uterus of a foster mother (McLaren and Biggers, 1958). This accomplishment opened the way for maintaining and ultimately manipulating preimplantation embryos during extended periods of their preimplantation development, while still permitting analyses to be carried out at later stages or even after birth.

The next decade brought intense activity in the development of *in vitro* culture methods for embryos. It was clear that any attempts to study early development and reproduction would be greatly enhanced by the availability of methods that could sustain mammalian embryonic development during periods of *in vitro* culture. The possibility of manipulating preimplantation embryos and replacing them in the reproductive tract of foster mothers for postimplantation development and eventual birth gave this effort extra impetus. Working for his Ph.D. in the laboratory of Dr. John Biggers, Ralph Brinster began a systematic effort to develop culture methods and to define the requirements of preimplantation mouse embryos in order to provide them with an optimal environment for development outside the mother (Brinster, 1963,1965a-d,1970). Key advances in this area, including the use of pyruvate as a metabolic substrate, opened the way for reproducible, high quality embryo culture which was a necessary basis for exploiting the rapidly increasing use of embryo manipulation, another essential contribution to the revolution. To this day, Brinster's culture methods and media, including the well-known BMOC (Brinster's Medium for Ovum Culture) are widely used.

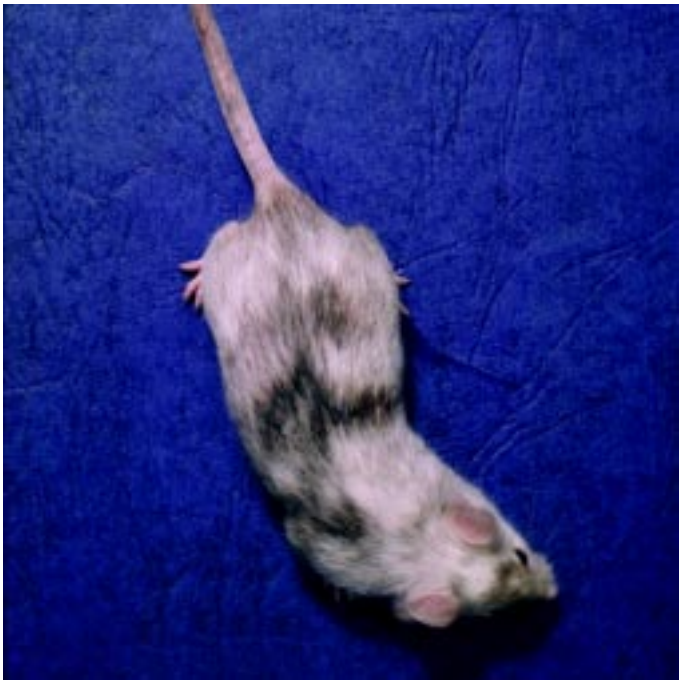

Fig. 2. Adult mouse chimera made by the aggregation *in vitro* of 8-cell embryos from a pigmented and a non-pigmented strain (Papaioannou, 1981).

Hand in hand with improvements in mammalian embryo culture came the development of an experimental approach to the study of mammalian preimplantation development, akin to the manipulative probings that had been applied to embryos of oviparous animals for centuries. During the sixties, several techniques for the production of mouse chimeras were published in which either whole preimplantation embryos were aggregated together (Tarkowski, 1961; Mintz, 1964) or cells were inserted into the blastocoelic cavity of blastula stage embryos (Gardner, 1968). The composite animals that developed from such combinations of genetically different cells were dramatic to look at (Fig. 2) but were even more impressive considering the potential they held for tracing cell lineages, testing cell potential, and eventually, as we shall see later, as vehicles for gene manipulation. The preimplantation period was rapidly opening up as a window of opportunity for experimental manipulation of mammalian development.

In the sixties and seventies, mouse chimeras were primarily used to approach a multitude of questions centering on normal development and gene expression (McLaren, 1976; Russell, 1978; Le Douarin and McLaren, 1984). Chimeras provided a means of testing ideas about stem cells and the existence of multi- or totipotent stem cells at different stages of embryogenesis (Papaioannou *et al.*, 1978). The first chimeras were made between synchronous embryonic cells, but investigators soon began pushing the limits to test the potential of more and more asynchronous cells. It was reasoned that stem cell populations in later stages of embryogenesis might maintain their stem cell properties even when placed in an asychronous environment, and could thus be persuaded to reveal their potential in a chimera. The possibility was certainly not missed that if a stem cell for the germ line could be found, there was the potential for genetic manipulation of these cells with subsequent return to the animal. For example, metabolic mutants could be selected *in vitro* prior to their reintroduction into chimeras, where, if the selected cell contributed to the germ line, the mutation would be perpetuated in the offspring.

In 1972, Moustafa and Brinster (Moustafa and Brinster, 1972a,b) succeeded in making chimeras with cells that not only were asychronous by several days, being taken from postimplantation embryos, but had also been cultured *in vitro* for 24 h. The principle was thus established that the later embryo contained multipotential stem cells that could contribute extensively to a chimera. But as those of us trying similar experiments at the time can attest, this success was the exception, and many other embryonic cell types from later embryos, including primordial germ cells, the precursors of the sperm and eggs, made little or no contribution to chimeras when placed in the preimplantation embryo (Weissman *et al.*, 1977 and unpublished).

At this point we pick up a different thread, because in a few laboratories, a different kind of stem cell, one from a tumor known as a teratocarcinoma, was under study as a potential vector for genetic information in chimeras. Teratocarcinomas are spontaneous tumors of the germ cells composed of different types of differentiated cells and also multipotential stem cells known as embryonal carcinoma (EC) cells. The discovery that teratocarcinomas could arise not only from adult germ cells but also from normal embryos transplanted to ectopic sites (Stevens, 1970), strengthened the idea that stem cells of this tumor were the hypothetical,

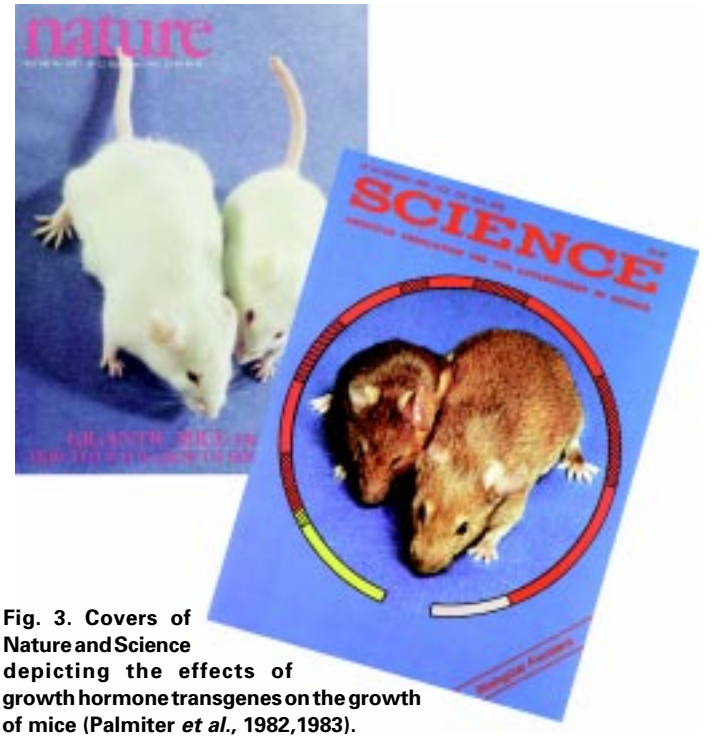

Fig. 3. Covers of Nature and Science depicting the effects of growth hormone transgenes on the growth of mice (Palmiter *et al.*, 1982,1983).

totipotent stem cells of the embryo that should be able to contribute to all cells of a chimera, including the germ cells, if only the right conditions could be found. Brinster, (1974) and others (Mintz and Illmensee, 1975; Papaioannou *et al.*, 1975) showed that EC cells could indeed contribute to chimeras when introduced into the preimplantation embryo, even following extended periods of growth *in vitro* (Papaioannou *et al.*, 1975). Furthermore, EC cells could be selected *in vitro* for specific metabolic mutations (Slack *et al.*, 1978). Unfortunately, however, these stem cells never fulfilled their promise as the elusive, totipotent embryonic stem cells, and in spite of many attempts, no mutation was ever propagated through the germline of an EC cell chimera (reviewed by Papaioannou, 1979; Papaioannou and Rossant, 1983).

The setback was only temporary. Not long afterwards, several laboratories succeeded in deriving stem cell lines directly from early embryos (Evans and Kaufman, 1981; Martin, 1981). With respect to their potential in a chimera, these primary cell lines, called embryonic stem (ES) cell lines, appeared to correspond most closely to the inner cell mass of the perimplantation embryo. In practice, this meant that they had the potential to contribute to all cells of the embryo, including the germ line (Bradley *et al.*, 1984), and also had the extraordinary capability of growing indefinitely *in vitro* as a stem cell line. The totipotent embryonic stem cell had been isolated and perpetuated in culture at last. With the help of another revolution that was taking place in molecular biology, the dream of controlled genetic manipulation of specific chromosomal genes was about to come true. Soon, chimeras were being made with ES cells that had been genetically manipulated *in vitro* by retroviral vectors (Robertson *et al.*, 1986), DNA transfection (Gossler *et al.*, 1986), metabolic selection (Hooper *et al.*, 1987; Kuehn *et al.*, 1987), and the most versatile system of all, site-directed mutagenesis by gene targeting (Thomas and Capecchi, 1987). These

chimeras transmitted the genetic alteration to their progeny and the rest is history.

Meanwhile, in parallel with this route to genetic manipulation, another means of producing transgenic animals was already proving highly successful. This was the introduction of DNA directly into the nucleus of the fertilized zygote. The success of this method was also built on improved embryo culture and manipulation methods, as well as advances in the rapidly moving field of recombinant DNA technology, which made cloned genes available in quantity. The time was ripe for rapid advances and the pace was fast and furious. Within a few short years in the late 70s and early 80s, techniques for gene transfer into cultured cells (reviewed by Solter, 1981) were translated to gene transfer into embryos, with subsequent transmission of these genes through the germ line to the next generation. It had already been demonstrated that the preimplantation embryo could incorporate foreign genes (Jaenisch and Mintz, 1974; Jaenisch, 1976). In a single year, six different laboratories, including Brinster's, reported varying degrees of success in producing transgenic mice following DNA microinjection into the nucleus or cytoplasm of the fertilized egg (Gordon *et al.*, 1980; Brinster, 1981; Costantini and Lacy, 1981; Harbers *et al.*, 1981; Wagner, E.F. *et al.*, 1981; Wagner T.E. *et al.*, 1981). Brinster's group made use of an inducible promoter and established the principle that the expression of transgenes could be controlled exogenously. Shortly thereafter, the covers of both Science and Nature showed the dramatic effects of a rat (Palmiter *et al.*, 1982) or human (Palmiter *et al.*, 1983) growth hormone transgene under the influence of an inducible metallothionein promoter (Fig. 3), a fitting testamonial to an extraordinary achievement.

Thus began an exciting era of exploration and discovery in mammalian gene expression and control. It also marked the beginning of a long-term collaboration between Ralph Brinster and Richard Palmiter that has produced an immense body of work applying transgenic technology to a variety of problems and to different species (Hammer *et al.*, 1985) (see also Brinster and Palmiter, 1986; Palmiter and Brinster, 1986; Brinster, 1993 and the articles in the current issue for reviews of some of their work).

Over the years, Brinster has continued to explore new ways of altering the genome. In 1989, he showed the feasibility of directly targeting endogenous alleles in the zygote pronucleus, via homologous recombination following DNA microinjection, by correcting a deletion in a major histocompatibility gene (Brinster *et al.*, 1989). Although in this case, no protein product was produced from the corrected allele and a number of point mutations were introduced, the transgenic animal transmitted the targeted allele directly to its offspring. Thus, this direct route to gene targeting eliminates the need to use ES cells as the intermediary for transfection, selection, and germ line transmission through chimeras. Although the extremely low success rate of this direct targeting was discouraging and seemed to preclude its common application, a "knockout" mutation has recently been produced using this approach (Susulic *et al.*, 1995). In this case, the allele in question had been shown previously to recombine very efficiently with the specific targeting vector. With advances in gene targeting methodology, this approach could yet come into its own.

The latest, spectacular contribution made by Brinster illustrates his continuing interest in stem cells, in this case adult stem

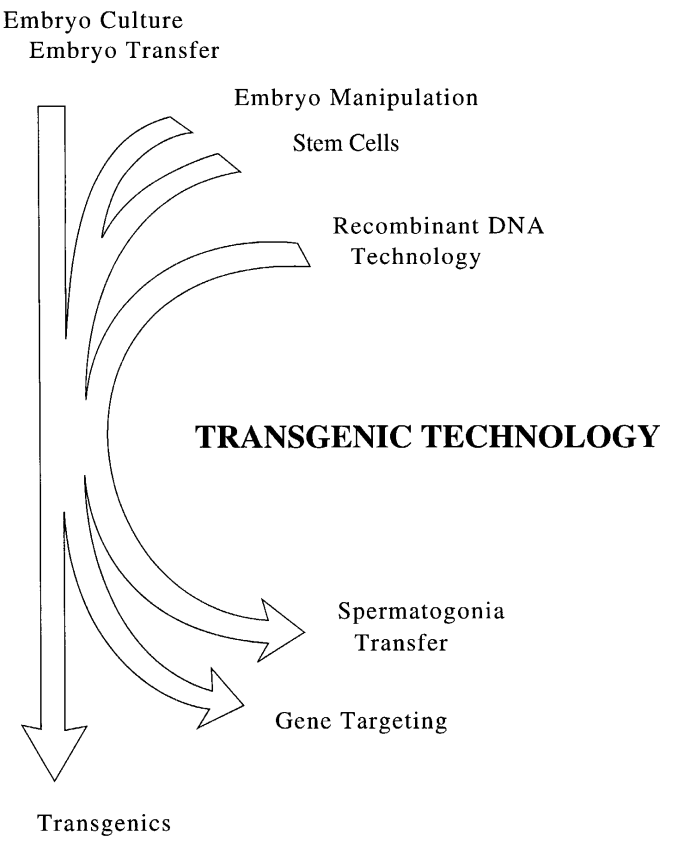

Fig. 4. The success of genetic manipulation through transgenic technology has depended on convergence of advances in diverse fields. *Combined, these allow different types of genetic alterations.*

cells of the male germ line, the spermatogonia. Brinster and his coworkers isolated spermatogonia from mouse testes, cultured them for several hours, and then reimplanted them into the seminiferous tubules of host males. Not only did these cells establish normal spermatogenesis in the host testes, but they also produced viable spermatozoa which, upon mating, could fertilize eggs and produce offspring (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). If the past history of Brinster's success is anything to go by, it will only be a matter of time before culture conditions are developed that will allow maintenance of these stem cells *in vitro* and render them amenable to genetic manipulation such as gene targeting by homologous recombination. This alternative approach to transgenesis could be particularly useful in large animals, where the difficulty and expense of embryo manipulation can be limiting factors. With uncanny perspicacity, Brinster set the stage for development of the next generation of transgenic methodology that could have an immense influence in the way we continue to probe and investigate the genome.

Acknowledgments

I thank John Biggers for providing Figure 1 and Ralph Brinster for providing the images for Figure 3. Thanks are due to Debbie Chapman for help in preparing the figures. This work was supported by the Raymond and Beverly Sackler Foundation.

References

BIGGERS, J.D. (1987). Pioneering mammalian embryo culture. In *The Mammalian Preimplantation Embryo* (Ed. B.D. Bavister). Plenum Press, New York, pp. 1-22.

BRADLEY, A., EVANS, M., KAUFMAN, M.H. and ROBERTSON, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature 309*: 255-256.

BRINSTER, R.L. (1963). A method for *in vitro* cultivation of mouse ova from two-cell to blastocyst. *Exp. Cell Res. 32*: 205-208.

BRINSTER, R.L. (1965a). Studies on the development of mouse embryos *in vitro*. I. Effect of osmolarity and hydrogen ion concentration. *J. Exp. Zool. 158*: 49-58.

BRINSTER, R.L. (1965b). Studies on the development of mouse embryos *in vitro*. II. The effect of energy source. *J. Exp. Zool. 158*: 59-68.

BRINSTER, R.L. (1965c). Studies on the development of mouse embryos *in vitro*. III. The effect of fixed-nitrogen source. *J. Exp. Zool. 158*: 69-77.

BRINSTER, R.L. (1965d). Studies on the develoment of mouse embryos *in vitro*. IV. Interaction of energy sources. *J. Reprod. Fertil. 10*: 227-240.

BRINSTER, R.L. (1970). *In vitro* cultivation of mammalian ova. *Adv. Biosci. 4*: 199.

BRINSTER, R.L. (1974). The effect of cells transferred into the mouse blastocyst on subsequent development. *J. Exp. Med. 140*: 1049-1056.

BRINSTER, R.L. (1981). Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell 27*: 223-231.

BRINSTER, R.L. (1993). Stem cells and transgenic mice in the study of development. *Int. J. Dev. Biol. 37*: 89-99.

BRINSTER, R.L. and AVARBOCK, M.R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proc. Natl. Acad. Sci. USA 91*: 11303-11307.

BRINSTER, R.L. and PALMITER, R.D. (1986). Introduction of genes into the germ line of animals. *The Harvey Lectures Series 80*: 1-38.

BRINSTER, R.L. and ZIMMERMANN, J.W. (1994). Spermatogenesis following male germ-cell transplantation. *Proc. Natl. Acad. Sci. USA 91*: 11298-11302.

BRINSTER, R.L., BRAUN, R.E., LO, D., AVARBOCK, M.R., ORAM, F. and PALMITER, R.D. (1989). Targeted correction of a major histocompatibility class II $E\alpha$ gene by DNA microinjected into mouse eggs. *Proc. Natl. Acad. Sci. USA 86*: 7087-7091.

COSTANTINI, R. and LACY, E. (1981). Introduction of rabbit β-globin gene into the mouse germ line. *Nature 294*: 92-94.

EVANS, M.J. and KAUFMAN, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature 292*: 154-156.

GARDNER, R.L. (1968). Mouse chimaeras obtained by the injection of cells into the blastocyst. *Nature 220*: 596-597.

GORDON, J.W., SCANGOS, G.A., PLOTKIN, D.J., BARBOSA, J.A. and RUDDLE, F.H. (1980). Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. USA 77*: 7380-7384.

GOSSLER, A., DOETSCHMAN, T., KORN, R., SERFLING, E. and KEMLER, R. (1986). Transgenesis by means of blastocyst-derived embryonic stem cell lines. *Dev. Biol. 83*: 9065-9069.

HAMMER, R.E., PURSEL, V.G., REXROAD JR., C.E., WALL, R.J., BOLT, D.J., EBERT, K.M., PALMITER, R.D. and BRINSTER, R.L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. *Nature 315*: 680-683.

HAMMOND, J., Jr. (1949). Recovery and culture of tubal mouse ova. *Nature 163*: 28-29.

HARBERS, K., JAHNER, D. and JAENISCH, R. (1981). Microinjection of cloned retroviral genomes into mouse zygotes: integration and expression in the animal. *Nature 293*: 540-542.

HOOPER, M., HARDY, K., HANDYSIDE, A., HUNTER, S. and MONK, M. (1987). HPRT-deficient (Lesch- Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature 326*: 292-295.

JAENISCH, R. (1976). Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. *Proc. Natl. Acad. Sci. USA 73*: 1260-1264.

JAENISCH, R. and MINTZ, B. (1974). Simian Virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocystss injected with viral DNA. *Proc. Natl. Acad. Sci. USA 71*:1250-1254.

KUEHN, M.R., BRADLEY, A., ROBERTSON, E.J. and EVANS, M.J. (1987). A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. *Nature 326*: 295-298.

LE DOUARIN, N. and MCLAREN, A., EDS. (1984). *Chimeras in Developmental Biology*. London, Academic Press.

MARTIN, G. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA 78*: 7634-7638.

MCLAREN (1976). *Mammalian Chimaeras*. Cambridge, Cambridge University Press.

MCLAREN, A. and BIGGERS, J.D. (1958). Successful development and birth of mice cultivated *in vitro* as early embryos. *Nature (Lond.) 182*: 877-878.

MINTZ, B. (1964). Formation of genetically mosaic mouse embryos, and early development of 'lethal (t^{12}/t^{12})-normal' mosaics. *J. Embryol. Exp. Morphol. 157:* 273-292.

MINTZ, B. and ILLMENSEE, K. (1975). Normal genetically mosaic mice produced from malignant teratocarcinoma cells. *Proc. Natl. Acad. Sci. USA 72*: 3585-3589.

MOUSTAFA, L. and BRINSTER, R.L. (1972a). The fate of transplanted cells in mouse blastocysts *in vitro*. *J. Exp. Zool. 181*: 181-192.

MOUSTAFA, L. and BRINSTER, R.L. (1972b). Induced chimerism by transplanting embryonic cells into mouse blastocysts. *J. Exp. Zool. 181*: 193-202.

PALMITER, R.D. and BRINSTER, R.L. (1986). Germ-line transformation of mice. *Annu. Rev. Genet. 20*: 465-499.

PALMITER, R.D., BRINSTER, R.L., HAMMER, R.E., TRUMBAUER, M.E., ROSENFELD, M.G., BIRNBERG, N.C. and EVANS, R.M. (1982). Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature 300*: 611-615.

PALMITER, R.D., NORSTEDT, G., GELINAS, R.E., HAMMER, R.E. and BRINSTER, R.L. (1983). Metallothionein-human GH fusion genes stimulate growth of mice. *Science 222*: 809-814.

PAPAIOANNOU, V.E. (1979). Interactions between mouse embryos and teratocarcinomas. In *Cell lineage, Stem Cells and Cell Determination INSERM Symposium No. 10* (Ed. N. Le Douarin). Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 141-155.

PAPAIOANNOU, V.E. (1981). Experimental chimaeras and the study of differentiation. In *Mammalian Genetics and Cancer: The Jackson Laboratory Fiftieth Anniversary Symposium Progress in Clinical and Biological Research* (Y): 77-91.

PAPAIOANNOU, V.E. and ROSSANT, J. (1983). Appendix Table 3, EC-embryo chimeras. In *Teratocarcinoma Stem Cells, Cold Spring Harbor Conferences on Cell Proliferation* (Eds. L.M. Silver, G.R. Martin and S. Strickland, 10). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 734-735.

PAPAIOANNOU, V.E., MCBURNEY, M.W., GARDNER, R.L. and EVANS, M.J. (1975). Fate of teratocarcinoma cells injected into early mouse embryos. *Nature 258*: 70-73.

PAPAIOANNOU, V.E., ROSSANT, J. and GARDNER, R.L. (1978). Stem cells in early mammalian development. In *Stem Cells and Tissue Homeostasis. British Society for Cell Biology Symposium 2* (Eds. B.I. Lord, C.S. Potten and R.J. Cole). Cambridge University Press, Cambridge, pp. 49-69.

ROBERTSON, E., BRADLEY, A., KUEHN, M. and EVANS, M. (1986). Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. *Nature 323*: 445-448.

RUSSELL, L.B., Ed. (1978). *Genetic Mosaics and Chimeras in Mammals*. New York, Plenum Press.

SLACK, C., MORGAN, R.H.M. and HOOPER, M.L. (1978). Isolation of metabolic cooperation-defective variants from mouse embryonial carcinoma cells. *Exp. Cell Res. 117*: 195-205.

SOLTER, D. (1981). Gene transfer in mammalian cells. In *New Technologies in Animal Breeding*. Academic Press, New York, pp. 201-218.

STEVENS, L.C. (1970). The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. *Dev. Biol. 21*: 364-382.

SUSULIC, V.S., FREDERICH, R.C., LAWITTS, J., TOZZO, E., KAHN, B.B., HARPER, M.-E., HIMMS-HAGEN, J., FLIER, J.S. and LOWELL, B.B. (1995). Targeted disruption of the β-adrenergic receptor gene. *J. Biol. Chem. 270*: 29483-29492.

TARKOWSKI, A.K. (1961). Mouse chimaeras developed from fused eggs. *Nature 190*: 857-860.

THOMAS, K.R. and CAPECCHI, M.R. (1987). Site-directed mutagenesis by gene tageting in mouse embryo-derived stem cells. *Cell 51*: 503-512.

WAGNER, E.F., STEWART, T.A. and MINTZ, B. (1981). The human β-globin gene and a functional viral thymidine kinase gene in developing mice. *Proc. Natl. Acad. Sci. USA 78*: 5016-5020.

WAGNER, T.E., HOPPE, P.C., JOLLICK, J.D., SCHOLL, D.R., HODINKA, R.L. and GAULT, J.B. (1981). Microinjection of a rabbit β-globin gene into zygotes and its subsequent expression in adult mice and their offspring. *Proc. Natl. Acad. Sci. USA 78*: 6376-6380.

WEISSMAN, I.L., BAIRD, S., GARDNER, R.L., PAPAIOANNOU, V.E. and RASCHKE, W. (1977). Normal and neoplastic maturation of T-lineage lymphocytes. In *Cold Spring Harbor Symposium on Quantitative Biology* , XLI. Cold Spring Harbor Laboratory, Cold Spring Harbor, pp. 9-21.

WHITTEN, W.K. (1956). Culture of tubal mouse ova. *Nature (Lond.) 177*: 96.

WHITTEN, W.K. (1957). Culture of tubal ova. *Nature 179*: 1081-1082.