Ontogeny, pathology, oncology

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ABSTRACT This article traces the history of using embryo-derived stem cells for genetic manipulation — first teratocarcinoma stem cells and then embryonic stem cells. It encompasses several decades of research investigating the similarity between cellular mechanisms of normal growth and differentiation in the embryo and abnormal growth and differentiation in neoplasia. The limited developmental potential of teratocarcinoma-derived, embryonal carcinoma (EC) stem cells is contrasted to the totipotentiality displayed by embryonic stem (ES) cells derived directly from early embryos. From early attempts to select mutants in EC cells in culture to the spectacular success of targeting genes in ES cells by homologous recombination, the different lines of developmental, genetic and cancer research have converged to open vast new areas of possibility in genetic manipulation.

KEY WORDS: *teratocarcinoma, embryonic stem cells, oncogenes, gene targeting*

Introduction

From embryo to tumor and back to embryo again; this is the story of an extraordinary connection between embryos and tumors and the unfolding of techniques to probe the workings of them both. It encompasses several important decades in the recognition of the similarity between cellular mechanisms of normal growth and differentiation in the embryo and abnormal growth and differentiation in neoplasia. It springs from many sources but two that are central to this part of the story are, first, the study of a peculiar tumor, the teratocarcinoma, and secondly, the important discovery that the genetic basis for certain aspects of neoplasia resides in the normal genome in the form of proto-oncogenes, genes with an ominous name that belies their necessary benign function in normal development.

The teratocarcinoma connection

In the study of development, one very special cell type stands out as unique in sexually reproducing organisms. This is the germ cell, the link between generations, the cell that produces the gametes of one generation and gives rise to the stem cells of the embryo in the next. The life cycle of organisms is a vehicle for the perpetuation of this cell type and thus the perpetuation of a species. From the germ cells in the gonads, the highly specialized gametes differentiate, their primary function being to provide the germ for the next generation. Fertilization triggers the release of the gametes' developmental potential, which includes the blueprint for the development of every cell type and organ in the new individual. The fertilized egg shares with some early embryonic cells this property of totipotency, and ontogeny can be seen as an orderly, gradual and largely irreversible reduction in cell potency as differentiated cell types arise, each expressing a characteristic subset of genes. Of all the cells in the body, however, the primordial germ cells retain, or acquire anew each generation, this property of totipotency, the capacity to make an entire organism including the gametes. How this occurs and how the realization or suppression of totipotency is controlled is a central mystery of biology (Papaioannou *et al.*, 1978b; McLaren, 1981).

For many years, a relationship has been recognized between developing systems and the pathology of tumor development and growth. Barry Pierce was one of the pioneers in exploring this connection and the developmental implications of differentiation in neoplasms using an illustrative model system, the teratocarcinoma (Pierce and Beals, 1964; Pierce, 1967). These tumors of the germ cells characteristically contain undifferentiated stem cells with embryonic phenotypes known as embryonal carcinoma (EC) cells, as well as differentiated elements of many cell types. The dynamic relationship between the malignant features of these tumor stem cells and the benign, differentiated elements of the tumors has provided rich material for investigating factors that tip the balance between controlled versus uncontrolled proliferation, and between proliferation versus differentiation (Pierce and Cox, 1978; Pierce *et al.*, 1983).

Teratocarcinomas, which can have serious consequences in people, are a curiosity also arising spontaneously in certain mouse

Abbreviations used in this paper: EC, embryonal carcinoma; ES, embryonic stem.

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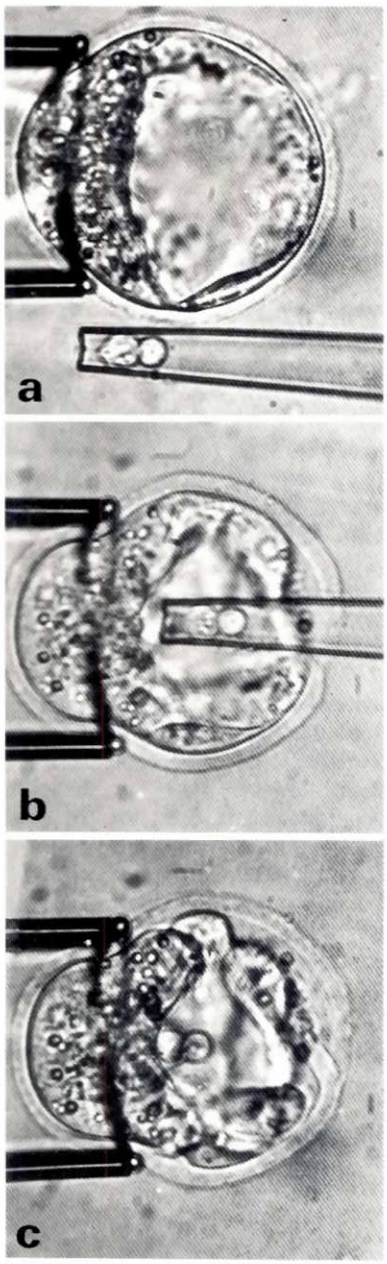

Fig. 1. Injection of cells into the blastocoelic cavity of a preimplantation mouse embryo. (a) *Blastocyst is held in place with a holding pipette and EC cells are held in an injection pipette.* **(b)** *The injection pipette is introduced into the blastocoelic cavity and then* **(c)** *withdrawn after the cells are released. Photo courtesy of C. Babinet.*

strains. They arise from primordial germ cells in the gonads of either sex, either directly, as in the fetal male testes, or from activated eggs in females (Stevens, 1983). The growth of a tumor results from the derangement of normal developmental events. Although the tissues produced are the same, the tumors are grotesque parodies of embryos, lacking spatial and temporal order and retaining a population of undifferentiated, immature cells that account for continued tumor growth. The discovery that normal embryos transplanted to ectopic sites could give rise to teratocarcinomas identical to the spontaneous germ cell tumors (Stevens, 1970; Stevens and Pierce, 1975), presented an intriguing puzzle to embryologists. Within the normal repertoire of embryonic development there must reside the potential for uncontrolled growth. What cells of the embryo have or can acquire this malignant potential? What genes are responsible for the control of this potential in normal development that can be epigenetically disrupted by ectopic transplantation? The job of the fertilized egg is to develop into an organism with differentiated somatic tissues, but also to retain totipotency, or the capacity for totipotency, in some of the cells of the developing fetus in order to produce the germ cells for the next generation. Control of this fundamental dichotomy in cell behavior appears to be disturbed in teratocarcinomas. Understanding the nature of the tumor stem cell could reveal how these features are balanced in normal development to produce both germ cells and soma in a controlled and reproducible manner.

It was in the pursuit of this information that embryologists began to explore the potential of the stem cells of the teratocarcinoma, the embryonal carcinoma (EC) cells, using classic embryological techniques. Transplantation studies, in which marked embryonic tissue is transplanted from one embryo to another, has long been a tool of the experimental embryologist. In the 1960s, this technique was applied to mammals and it was found that considerable developmental flexibility allowed quite drastic cut-and-paste experiments to be carried out. Methods for the introduction of cells into preimplantation blastocysts (Fig. 1) were developed to study embryonic cell fate and potential (Gardner, 1968). The result was a chimeric animal, a composite of host and injected cell types, with the extent of contribution of the injected cell type dependent upon its developmental potential. In the 1970s, several groups began to explore the potential of EC stem cells in this way by injecting cells into blastocysts and allowing the composite to develop further. The pattern of contribution of the injected cell could be documented by genetic markers either in the adult or fetal stages. In some of these experiments, the EC cells were isolated directly from tumors for blastocyst injection. However, the work was greatly facilitated by the capacity of EC cells for continuous growth *in vitro* under conditions that prevent their differentiation. By examining the chimeric animals that resulted from blastocyst injection, it was found that EC cells, whether derived from cultures or directly from tumors, could contribute normally differentiated cells to a developing embryo when introduced into preimplantation stages (Brinster 1974; Mintz and Illmensee, 1975; Papaioannou *et al.*, 1975, 1978a) (Fig. 2a). It appeared that the undifferentiated stem cells of the tumor had enough features in common with early embryonic cells that they could respond to the embryonic environment, differentiating in a normal manner, and coming under the controlling influence of the orderly embryonic developmental program, even after long periods *in vitro*.

The embryonic potential of EC cells was not unlimited, however. Different cell lines were used with different results. The pattern of EC cell contribution to most chimeras was not the uniform, fine-grain chimerism expected from an embryonic cell (McLaren, 1976). Most chimeras showed evidence of only sporadic and meagre EC cell contributions to the developing fetus. Cells that were extracted directly from tumors appeared to make more substantial contributions to chimeras (Mintz *et al.*, 1975) but could not be characterized

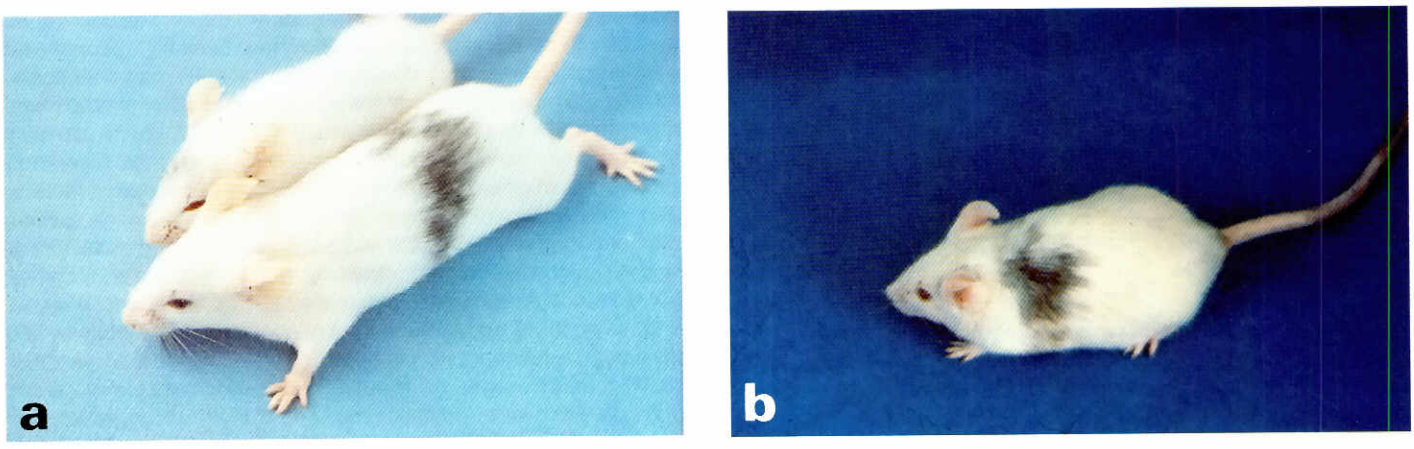

Fig. 2. Mouse chimeras made from cultured stem cells. (a) *A chimera made by the injection of EC cells of a pigmented genotype into an albino embryo.* **(b)** *A chimera made by the injection of ES cells. In this case the host embryo was pigmented and the ES cells were albino.*

or manipulated as well as EC cells in culture. Furthermore, the malignant properties of the EC cells proved not to be completely reversible. Although there was no evidence that fully differentiated cells in a chimera resumed malignant proliferation, many chimeras developed tumors in addition to showing EC cell-derived differentiated tissue (Papaioannou and Rossant, 1983). These results pointed to heterogeneity of potential among EC cells and to subtle differences between EC cells and the embryonic or germ cells from which they were derived. It was of critical interest to determine the source of these differences, whether they were genetic or epigenetic and whether they were the cause or result of the formation of tumors from embryonic cells, since by this time it had become obvious that EC cells might hold the key to a powerful new means of genetic manipulation. If EC cells had the potential to form primordial germ cells even following culture *in vitro*, then it should be possible to manipulate them *in vitro* to produce specified genetic changes which could be returned to the animal *via* the germ line of a chimera.

The next phase of work with EC cells involved a considerable effort to characterize the similarities and differences between EC and embryonic cells. Although many lines could contribute to chimeras and could be differentiated into normal cell types *in vitro*, either their derivation from tumors or their extended sojourn *in vitro* rendered them so dissimilar from early embryonic cells that they rarely, if ever, had full embryonic potential. Many had obvious karyotypic abnormalities but even normal diploid lines showed restricted potential (Papaioannou *et al.*, 1979). Although specific genetic mutations could be selected in ES cell lines *in vitro* (Slack *et al.*, 1978), none was ever propagated to the next generation through a chimera.

The embryonic stem cell

Throughout this period, the idea that the embryo harbored a totipotent stem cell with its proliferative potential under strict control encouraged efforts to release that potential without the intermediary of ectopic tumor growth. In the early 1980s, three laboratories independently succeeded in deriving stem cell lines directly from early embryos, all using different blastocyst culture conditions (Evans and Kaufman, 1981; Martin, 1981; Axelrod, 1984). These primary cell lines, called embryonic stem (ES) cell lines, corresponded closely to cells of the inner cell mass of the blastocyst in their behavior and developmental potential, and appeared to be derived from the inner cell mass at a peri-implantation stage. In these studies, blastocysts were allowed to attach and outgrow *in vitro*, a behavior that is reminiscent of implantation in the uterus. The inner cell mass was then disaggregated and grown as a cell line. The permissive conditions that led to the establishment of ES cell lines were various: the use of implantation delayed embryos, growth on inactivated feeder cells, supplementation with medium conditioned by EC cells, or culture in a small volume. Although the importance of these factors in the initial successes remains unknown, the now almost routine derivation of ES cell lines can be accomplished by culture of embryos in medium supplemented with a specific differentiation inhibitor (LIF; Smith *et al.*, 1988) and/or inactivated feeder cells that presumably secrete LIF. Nonetheless, their derivation still leaves as a mystery for the time being how the intact embryo harnesses and controls the proliferative and differentiative capacity of its cells and how this potential is released *in vitro*.

EC and ES cells are similar in many ways. They are easily cultivated, grow indefinitely and are multipotential in chimeras (Fig. 2b). However, ES cells have retained certain embryonic features that distinguish them from the carcinoma-derived EC cells and make them an ideal vehicle for transgenesis: on the whole they retain a normal karyotype; although they contribute extensively to chimeras, they rarely, if ever, give rise to tumors in these animals; most importantly, they maintain the potential to contribute to the germ line of chimeras giving rise to gametes that can transmit the ES cell genotype to the next generation (Bradley *et al.*, 1984; Robertson 1986).

The past decade has seen the rapid exploitation of the property of ES cells to contribute to the germ line. Several ingenious methods for probing and manipulating the genome have been applied to ES cells *in vitro* and genetic alterations such as retroviral insertions and «promoter trap» marker constructs have been returned to the whole animal through germ-line chimeras (Robertson, 1991). One such manipulation which has enormous potential for mutational analysis is the so-called gene targeting approach for producing specific

mutations in known genes. Constructs of altered genes are transfected into ES stem cells, which are then selected for homologous recombination and returned to the embryo for eventual transmission of the altered chromosomal gene to offspring. Thus the embryonic stem cell came out of the realm of developmental biology in the guise of a sophisticated tool for mutational analysis of the genetic basis of complex biological systems.

Homologous recombination and targeted mutagenesis in ES cells

Selective media have long been used to isolate spontaneous mutants of certain metabolic genes in cultured cells including EC cells. When ES cells became available, this method was applied as a means of obtaining mice deficient in HGPRT (Hooper *et al.*, 1987; Kuehn *et al.*, 1987). This mouse model of the Lesch-Nyhan syndrome was the first instance of a mutation selected in culture being placed into the germ line of an experimental animal. It represented the fulfillment of a long-sought goal. Hard on the heels of this success was the application of more complicated selective procedures that theoretically allowed for the targeted mutation of any endogenous gene (Smithies *et al.*, 1985; Capecchi, 1989). These procedures relied not on the selectable phenotype of a specific mutation but on the introduction of a selectable marker as the mutagen. Using the powerful methods of recombinant DNA technology, genetic constructs were devised in which bacterial or viral genes were inserted into coding regions of cloned genomic DNA in such a way as to disrupt transcription of the endogenous mouse gene. Following the transfection of these constructs into ES cells, integration of the construct into any chromosomal site could be detected and selected for by antibiotic treatment, and the relatively rare event of homologous recombination at endogenous loci could be screened by Southern analysis of restriction digests in clones of resistant cells. Provided the ES cells survived this transfection, selection and cloning with developmental potential intact, the way was clear for the introduction of specific null mutations into the germ line by way of ES cell chimeras.

Very rapidly, the technique of gene targeting by homologous recombination in ES cells was applied to many different types of genes. With the realization that gene expression was not a requirement for successful targeting (Johnson *et al.*, 1989), no gene seemed out of reach. Only the limitations of time and interest restricted the application and many labs began to attack different types of genes using this method of mutational analysis. Genes suspected of playing fundamental roles in the control of developmental processes were an early application (e.g. *int-1*, Thomas and Capecchi, 1990; *En-2*, Joyner *et al.*, 1991), as were genes affecting growth (*IGF-2*, DeChiara *et al.*, 1990) and genes involved in immune function (e.g. Zijlstra *et al.*, 1989; Koller *et al.*, 1990; and from our own laboratory $A\beta^b$, Grusby *et al.*, 1991 and *Rag-1*, Mombaerts *et al.*, 1992). Targeting genes with highly tissue-specific differentiated functions (e.g. *adipsin* and *aP2*, Johnson *et al.*, 1989 and unpublished) also shows promise for understanding the control of differentiated cell function.

Oncogenes and development

Another category of gene heavily targeted is the cellular proto-oncogene (c-*onc*), the cellular counterpart to the acutely oncogenic retrovirus genes (v-*oncs*). As could be predicted from the effects of the transforming viral oncogenes, mutants of the cellular oncogenes have growth deregulating effects. They have been highly conserved in evolution, despite their involvement in the malignant phenotype, indicating a more benign, central role in normal cellular processes of growth and differentiation. Indeed, the protein products of c-*oncs* have variously been identified as growth factors, their receptors, transducers of growth factor responses, and transcription factors. Many proto-oncogenes are expressed in a tissue- and stage-specific manner during embryonic life and are thus implicated as factors in signaling pathways crucial for the control of growth and proliferation during normal development (Adamson, 1987). Mutational analysis of these genes is a powerful means of analyzing their biological and physiological function, as has been illustrated by the discovery that the *W* locus, an old mutant affecting the development of several cell types, is the c-*kit* proto-oncogene, and the *Sl* locus, which has mutants with similar phenotypic effects, encodes the Kit ligand. Gene targeting has been used as a means of producing mutants in many oncogenes for which naturally occurring mutants have not been found.

A growing number of proto-oncogenes with embryonic expression patterns have been mutated by gene targeting, including growth factors, intracellular kinases and transcription factors (Forrester *et al.*, 1992). The results have sometimes been surprising in relation to the expression patterns and to the malignancies associated with the activated forms of the genes. For example, a c-*src* targeted mutation results in osteopetrosis due to impaired osteoclast function, while the tissues that normally express the highest levels of c-*src* appear unaffected (Soriano *et al.*, 1991). Several transcription factors have been targeted with results more in line with expression patterns; c-*myb* expression is normally associated with hematopoietic progenitor cells and c-*myb* null embryos die during embryogenesis from hematopoietic defects (Mucenski *et al.*, 1991).

We have been targeting genes for the transcription factors c-*fos* and c-*jun* (Johnson *et al.*, 1989, 1992 and unpublished results) and have found for c-*fos* null mice at least, a less severe phenotype than would be predicted by the broad range of expression of this gene in normal embryogenesis. An observed bone defect in the mutant, however, might have been predicted by the association of v-*fos* with osteosarcomas. Both c-*fos* and c-*jun* are implicated in cell growth and differentiation in many developing tissues by correlative evidence and are thought to be of fundamental importance in controlling cell proliferation. Many studies in cell culture have clarified their biochemical activities (Angel and Karin, 1991). Now, perhaps, a mutational analysis based on gene targeting will lead to a clarification of their biological roles in the developing organism.

With the application of ES cell-mediated, targeted mutagenesis to the study of the most fundamental question of what controls the proliferation and differentiation of cells in the embryo, we have come full circle. The quest to understand teratocarcinomas and the malignant potential of stem cells within the embryo is now being facilitated by the very technology these cells spawned. A mutational analysis of the genes involved in the control of rapid switches between proliferation and differentiation characteristic of embryonic development will be critical to our understanding of the process by which tissues and organs develop in the correct time and space in the embryo.

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References

ADAMSON, E.D. (1987). Oncogenes in development. *Development 99:* 449-471.

ANGEL, P. and KARIN, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta 1072:* 129-157.

AXELROD, H.R. (1984). Embryonic stem cell lines derived from blastocysts by a simplified technique. *Dev. Biol. 101:* 225-228.

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

CAPECCHI, M.R. (1989). Altering the genome by homologous recombination. *Science 244:* 1288-1292.

DECHIARA, T.M., EFSTRATIADIS, A. and ROBERTSON, E.J. (1990). A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature 345:* 78-80.

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

FORRESTER, L.M., BRUNKOW, M. and BERNSTEIN, A. (1992). Proto-oncogenes in mammalian development. *Curr. Op. Genet. Dev. 2:* 38-44.

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

GRUSBY, M.J., JOHNSON, R.S., PAPAIOANNOU, V.E. and GLIMCHER, L.H. (1991). Depletion of CD4+ T cells in MHC class II deficient mice. *Science 253:* 1417-1420.

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:* 292-298.

JOHNSON, R.S., SHENG, M., GREENBERG, M.E., KOLODNER, R.D., PAPAIOANNOU, V.E. and SPIEGELMAN, B.M. (1989) Targeting non-expressed genes in embryonic stem cells via homologous recombination. *Science 245:* 1234-1236.

JOHNSON, R.S., SPIEGELMAN, B.M. and PAPAIOANNOU, V. (1992). Pleiotrophic effects of a null mutation in the c-*fos* proto-oncogene. *Cell 71:* 577-586.

JOYNER, A.L., HERRUP, K., AUERBACH, B.A., DAVIS, C.A. and ROSSANT, J. (1991). Subtle cerebellar phenotype in mice homozygous for a targeted deletion of the En-2 homeobox. *Science 251:* 1239-1243.

KOLLER, B.H., MARRACK, P., KAPPLER, J.W. and SMITHIES, O. (1990). Normal development of mice deficient in ~2M, MHC class I proteins and CD8+ T cells. *Science 248:* 1227-1231.

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.

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

McLAREN, A. (1981). *Germ Cells and Soma: A New Look at an Old Problem*. Yale University Press.

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

MINTZ, B., ILLMENSEE, K. and GEARHART, J.D. (1975). Developmental and experimental potentialities of mouse teratocarcinoma cells from embryoid body cores. In *Teratomas and Differentiation* (Eds. M.I. Sherman and D. Solter). Academic Press, Inc., London, pp. 59-82.

MOMBAERTS, P., IACOMINI, J., JOHNSON, R.S., HERRUP, K., TONAGAWA, S. and PAPAIOANNOU, V.E. (1992). RAG-1 deficient mice have no mature B and T lymphocytes. *Cell 68:* 869-877.

MUCENSKI, M.L., MCLAIN, K., KIER, A.G., SWERDLOW, S.H., SCHREINER, C.M., MILLER, T.A., PIETRYGA, D.W., SCOTT, W.J., Jr. and POTTER, S.S. (1991). Functional c-*myb* gene is required for normal murine fetal hepatic hematopoiesis. *Cell 65:* 677-689.

PAPAIOANNOU, V.E. and ROSSANT, J. (1983). Effects of the embryonic environment on proliferation and differentiation of embryonal carcinoma cells. *Cancer Surv. 2:* 165-183.

PAPAIOANNOU, V.E., EVANS, E.P., GARDNER, R.L. and GRAHAM, C.F. (1979). Growth and differentiation of an embryonal carcinoma cell line (c145b). *J. Embryol. Exp. Morphol. 54:* 277-295.

PAPAIOANNOU, V.E., GARDNER, R.L., MCBURNEY, M.W., BABINET, C. and EVANS, M.J. (1978a) Participation of cultured teratocarcinoma cells in mouse embryogenesis. *J. Embryol. Exp. Morphol. 44:* 93-104.

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. (1978b). 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. Potter and R.J. Cole). Cambridge University Press, Cambridge, pp. 49-69.

PIERCE, G.B. (1967). Teratocarcinoma: model for a developmental concept of cancer. *Curr. Top. Dev. Biol. 2:* 223-246.

PIERCE, G.B. and BEALS, T.F. (1964). The ultrastructure of primordial germinal cells of the fetal testes and of embryonic carcinoma cells of mice. *Cancer Res. 24:* 1553-1567.

PIERCE, G.B. and COX, W.F. (1978). Neoplasms as caricatures of tissue renewal. In *Cell Differentiation and Neoplasia* (Ed. G.F. Saunders). Raven Press, New York, pp. 57-66.

PIERCE, G.B., PODESTA, A. and WELLS, R. (1983). Malignancy and differentiation: the role of the blastocyst trophectoderm in control of colony formation. In *Teratocarcinoma Stem Cells* (Eds. L.M. Silver, G.R. Martin and S. Strickland). Cold Spring Harbor Conferences on Cell Proliferation 10: 15-22.

ROBERTSON, E.J. (1986). Pluripotential stem cells lines as a route into the mouse germ line. *Trends Genet. 2:* 9-14.

ROBERTSON, E.J. (1991). Using embryonic stem cells to introduce mutations into the mouse germ line. *Biol. Reprod. 44:* 238-245.

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

SMITH, A.G., HEATH, J.K., DONALDSON, D.D., WONG, G.G., MOREAU, J., STAHL, M. and ROGERS, D. (1988). Inhibition of pluripotential embyronic stem cell differentiation by purified polypeptides. *Nature 336:* 688-690.

SMITHIES, O., GREGG, R.G., BOGGS, S.S., KORALEWSKI, M.A. and KUCHERLAPATI, R.S. (1985). Insertion of DNA sequences into the human chromosomal ß-globin locus by homologous recombination. *Nature 317:* 230-234.

SORIANO, P., MONTGOMERY, C., GESKE, R. and BRADLEY, A. (1991). Targeted disruption of the c-*src* proto-oncogene leads to osteopetrosis in mice. *Cell 64:* 693-702.

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

STEVENS, L.C. (1983). The origin and development of testicular, ovarian and embryo-derived teratomas. In *Teratocarcinoma Stem Cells* (Eds. L.M. Silver, G.R. Martin and S. Strickland). Cold Spring Harbor Laboratory 10: 23-36.

STEVENS, L.C. and PIERCE, G.B. (1975). Teratomas: definitions and terminology. In *Teratomas and Differentiation* (Eds. M.I. Sherman and D. Solter). Academic Press Inc., New York and London, pp. 13-14.

THOMAS, K. and CAPECCHI, M.R. (1990). Targeted disruption of the murine *int-1* protooncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature 346:* 847-850.

ZIJLSTRA, M., LI, E., SAJJADI, F., SUBRAMANI, S. and JAENISCH, R. (1989). Germ-line transmission of a disrupted ß2-microglobulin gene produced by homologous recombination in embryonic stem cells. *Nature 342:* 435-438.