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Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells

(embryonic stem cells/inner cell masses/differentiation *in vitro*/embryonal carcinoma cells/growth factors)

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ABSTRACT **This report describes the establishment directly from normal preimplantation mouse embryos of a cell line that forms teratocarcinomas when injected into mice. The pluripotency of these embryonic stem cells was demonstrated conclusively by the observation that subclonal cultures, derived from isolated single cells, can differentiate into a wide variety of cell types. Such embryonic stem cells were isolated from inner cell masses of late blastocysts cultured in medium conditioned by an established teratocarcinoma stem cell line. This suggests that such conditioned medium might contain a growth factor that stimulates the proliferation or inhibits the differentiation of normal pluripotent embryonic cells, or both. This method of obtaining embryonic stem cells makes feasible the isolation of pluripotent cells lines from various types of noninbred embryo, including those carrying mutant genes. The availability of such cell lines should make possible new approaches to the study of early mammalian development.**

Teratocarcinomas are tumors that arise with relatively high efficiency when normal 1- to 7.5-day-old inbred mouse embryos are transplanted to an extra-uterine site in a histocompatible host (1, 2). The stem cell lines that are isolated from them are known as embryonal carcinoma cell (ECC) lines. Such cells have morphological, biochemical, and immunological properties in common with pluripotent embryonic cells and therefore have been used extensively as an *in vitro* model system for the study of the developing embryo (3–5). In some cases these properties include the ability to differentiate *in vitro* in a manner that closely parallels the normal behavior of the isolated embryonic inner cell mass (ICM) (6, 7). The most compelling evidence for the close relationship between the tumor stem cells and normal embryonic cells is the fact that stem cells taken either from teratocarcinomas or from embryonal carcinoma cell cultures can participate in the development of completely normal adult mice when combined with embryonic cells by the technique of blastocyst injection (8, 9).

These findings, schematically represented in Fig. 1, raise questions about the origin of the teratocarcinoma stem cell. One hypothesis is that the tumor stem cells arise as a consequence of a stable, but reversible, epigenetic change in normal pluripotent embryonic cells. Such "transformed" cells presumably continue proliferation in the undifferentiated state because neoplastic conversion has reduced their efficiency of response to the normal signals for differentiation (11). An alternative idea is that embryonal carcinoma cells are not transformed but rather represent a selected population of completely normal embryonic cells that are programmed to divide until they receive the appropriate signals for differentiation; when the cells are in an extra-embryonic site the signals they receive are apparently not conducive to completely normal embryogenesis.

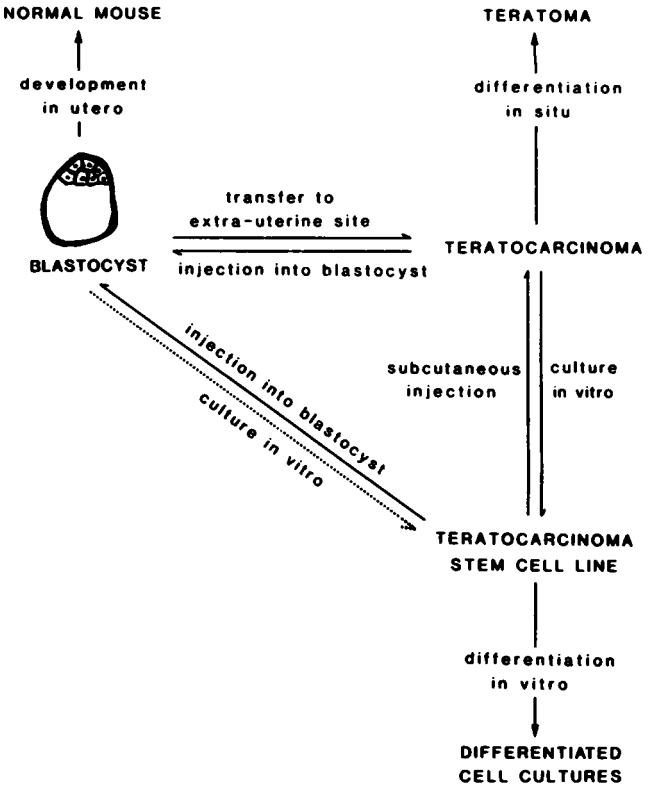

FIG. 1. Relationship between normal embryos and teratocarcinoma stem cells. Adapted from Martin (10).

It is difficult to study the process by which teratocarcinoma stem cells derive from normal embryonic cells as long as the transforming or selective events occur *in vivo*. As indicated by the dotted line in Fig. 1, it has long seemed logical to assume that pluripotent stem cells capable of forming teratocarcinomas might be isolated directly from embryos. Recently, Evans and Kaufman (12) carried out experiments in which pregnant strain 129 female mice were subjected to a treatment, involving ovariectomy and appropriate hormonal stimulation, that prevents normal implantation of blastocysts. Cells with the properties of teratocarcinoma stem cells were isolated from the embryos that had been maintained in such "delay" in the reproductive tract. Other attempts have been made to isolate cells with the properties of teratocarcinoma stem cells directly from normal early

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Abbreviations: ICM, inner cell mass; DME medium, Dulbecco's modified Eagle's medium; ESC, embryonic stem cell(s).

mouse embryos that have not been subjected to alteration *in vivo*. The approaches employed included embryo culture in a variety of media, and the use, as a starting material, of normal embryos at different stages of development and of giant embryonic cell masses created by embryo aggregation. None of these approaches has led to the establishment of pluripotent cell cultures, although several differentiated cell lines have been isolated from early embryos (13).

This report describes a method, involving culture in conditioned medium, for isolating and establishing pluripotent cell lines with the properties of teratocarcinoma stem cells directly from normal early mouse embryos *in vitro*. This method should be useful not only for further elucidating the relationship between teratocarcinoma stem cells and their normal embryonic progenitors but also for generating new, genetically marked pluripotent cell lines that can be used for studying various aspects of early mammalian development.

MATERIALS AND METHODS

Embryonal Carcinoma Cell Culture and Preparation of Conditioned Medium. PSA-1 embryonal carcinoma cells were used to generate conditioned medium. These cells were maintained in the undifferentiated state by coculture with fibroblastic STO feeder cells (6) in Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% calf serum (GIBCO). To prepare conditioned medium the undifferentiated PSA-1–feeder cultures were disaggregated by treatment with 0.05% trypsin and 0.02% EDTA in saline (trypsin/EDTA/saline). The resulting single cell suspension was first seeded at approximately 4×10^7 cells per 10-cm tissue culture dish and incubated for 30 min at 37°C. The nonadherent PSA-1 cells were then collected and seeded at approximately 10^7 cells per 10-cm tissue culture dish. This "preplating" removes feeder cells from the PSA-1 culture. After 2 days of growth at 37°C the serum-containing medium was removed from the PSA-1 cultures, and the cells were washed five times with phosphate-buffered saline and incubated in serum-free DME medium supplemented with insulin at 10 μg/ml and transferrin at 5 μg/ml. Forty-eight hours later this conditioned serum-free medium was collected and the cell debris was removed by low-speed centrifugation. Each 200 ml of conditioned medium was dialyzed against 10 liters of 20 mM NH_4HCO_3 for 2 days, during which time the dialysis buffer was changed four to six times. The dialyzed conditioned medium was then lyophilized, resuspended in 10 ml of serum-free DME medium, sterilized by passage through a 0.2-μm-pore-diameter Millipore filter, and supplemented with 10% calf serum and 0.1 mM 2-mercaptoethanol. This conditioned medium, thus concentrated approximately 20-fold, can be stored at −70°C without apparent loss of activity.

Embryo and Embryonic Stem Cell Culture. Embryos were obtained by mating superovulated random bred ICR female mice (Simenson Laboratories, Gilroy, CA) with SWR/J males (The Jackson Laboratory). Early blastocysts were flushed from the uterus approximately 76 hr after detection of a copulation plug. Fully expanded late blastocysts were obtained by culturing the embryos overnight in DME medium supplemented with 10% Hyclone fetal calf serum (Sterile Systems, Logan, UT). ICMs were isolated from these blastocysts by immunosurgery (14).

All experiments involving ICM-derived cells were carried out in DME medium supplemented with 10% calf serum and 0.1 mM 2-mercaptoethanol. When conditioned medium was used, the concentrated material described above was diluted 1:4 in this culture medium. Isolated ICMs were seeded in a 35-mm tissue culture dish containing a confluent layer of mitomycin C-treated STO fibroblasts (6). When colonies with a typical embryonal carcinoma cell morphology were observed in the dissecting microscope, the culture was washed with phosphate-buffered saline and treated with trypsin/EDTA/saline. By using a micropipette, colonies were carefully pulled away from the feeder cells and transferred, with some disaggregation, to fresh feeder layers. All colonies that proliferated were subsequently passaged in a similar manner at approximately weekly intervals. As the cultures of embryo-derived cells became denser, they were passaged to larger dishes of confluent feeder cells. All subsequent handling of the cells, with respect to passage in the undifferentiated state, cloning, and studies of differentiation *in vitro* and of tumor formation, was as previously described for PSA cells (6, 7, 15, 16).

RESULTS

Establishment of Embryonic Stem Cell Lines from Isolated Mouse ICMs. The work reported here began with the premise that teratocarcinoma stem cells are derived from a small population of pluripotent stem cells in the peri-implantation embryo that proliferate normally as a consequence of the production of an endogenous factor that promotes growth, suppresses differentiation, or both. It was assumed that increasing the concentration of such a factor in the growth medium by providing an exogenous supply might result in an expansion of this stem cell population in normal embryos *in vitro*. A corollary of this idea is that once this population of stem cells is expanded it might produce a sufficiently high concentration of the factor to eliminate the need for an exogenous supply. Established teratocarcinoma stem cell cultures seemed a logical source of such an exogenous supply of growth factor in view of the hypothesis of Todaro and De Larco (17) that the autostimulatory growth factor(s) produced by certain tumor cells might be the product of a gene normally expressed by embryonic cells.

Medium conditioned by the PSA-1 embryonal carcinoma cell line was prepared and concentrated as described in *Materials and Methods*. ICMs were isolated from normal late mouse blastocysts, and approximately 30 of these were seeded on a confluent fibroblastic feeder layer in this conditioned medium. Within 1 week, four colonies of cells appeared. These showed a remarkable resemblance to those formed when the pluripotent PSA-1 embryonal carcinoma cell line is cultured on a fibroblastic feeder layer (see Fig. 2). These embryo-derived colonies were passaged to a fresh feeder layer in conditioned medium, and within 1 week this secondary culture contained a very large number of embryonal carcinoma-like colonies. In contrast, control cultures of ICM-derived colonies grown and passaged in the absence of conditioned medium did not contain continuously proliferating embryonal carcinoma-like cell colonies. The cells growing in conditioned medium were subsequently passaged at approximately weekly intervals until a mass culture was obtained. As expected, conditioned medium was not required for continued cell proliferation after five passages. These results are reproducible, because mass cultures of similar cells have also been obtained in two other experiments, both involving embryos of inbred genotypes [(C3H × C57BL/6)F_1 hybrids]. In those experiments that have yielded positive results, individual ICMs apparently gave rise to embryonal carcinoma-like colonies with a frequency of approximately 1 in 8.

As demonstrated below, the cells derived from ICMs cultured in conditioned medium have all the essential features of teratocarcinoma stem cells. Such cells were termed *embryonic stem cells* (ESC) to denote their origin directly from embryos and to distinguish them from embryonal carcinoma cells (ECC) derived from teratocarcinomas. The specific cell line described here was designated ESC-ICR.

The mass cultures of ESC-ICR cells have a growth rate similar to that of PSA-1 cells and are morphologically identical to undifferentiated PSA-1 cells, both in the presence and absence of fibroblastic feeder cells (see Fig. 2). In addition, like many embryonal carcinoma cell lines, ESC-ICR cells are diploid. Preliminary examination of G-banded chromosome spreads indicates that at least some of the cells in the culture have a normal female karyotype, with the exception of a possible deletion of the distal portion of one of the two X chromosomes.

In view of the fact that ESC-ICR cells are morphologically indistinguishable from the PSA-1 teratocarcinoma stem cells used to condition the medium in which they were grown, it was obviously necessary to demonstrate that the former did not arise by accidental contamination of the embryo cultures with PSA-1 cells. The embryos from which the ESC-ICR cells were isolated were obtained by mating random-bred ICR female mice with males of the inbred SWR/J strain. Random-bred ICR mice segregate the glucosephosphate isomerase alleles *Gpi-1a* and *Gpi-1b*, whereas SWR/J mice are homozygous for *Gpi-1b*. (ICR × SWR/J)F_1 hybrids must therefore be either *Gpi-1b* homozygotes or *Gpi-1a/b* heterozygotes. In contrast, the PSA-1 cell line (and all other embryonal carcinoma cell lines currently being cultured in this laboratory) are homozygous for *Gpi-1a*. Therefore, if the ESC-ICR cells produce the electrophoretically faster form of the enzyme glucosephosphate isomerase encoded by the *Gpi-1b* gene, they could not have arisen by contamination of the embryo cultures with established embryonal carcinoma cells. The results of electrophoresis and staining for glucosephosphate isomerase activity (18), shown in Fig. 3, indicate that the ESC-ICR cells produce the faster form of the enzyme, thus demonstrating their origin from (ICR × SWR/J) embryos.

Tumorigenicity and Pluripotency of the ESC. To determine whether embryonic stem cells are capable of tumor formation, athymic mice were injected subcutaneously with approximately

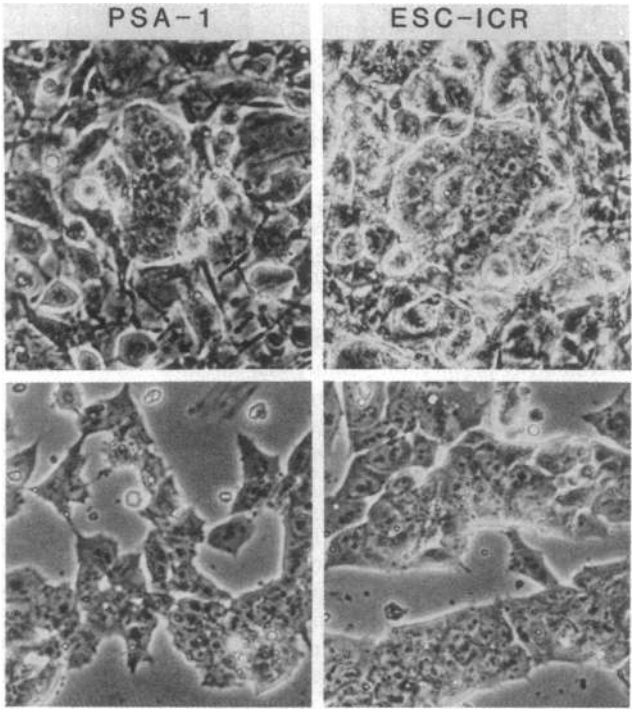

FIG. 2. Morphological similarity of embryo-derived ESC-ICR cells to PSA-1 embryonal carcinoma cells. (*Upper*) Cells growing on a fibroblastic feeder layer. (*Lower*) Mass cultures of the cells seeded in the absence of feeder cells. (Phase-contrast microscopy; approximately ×250.)

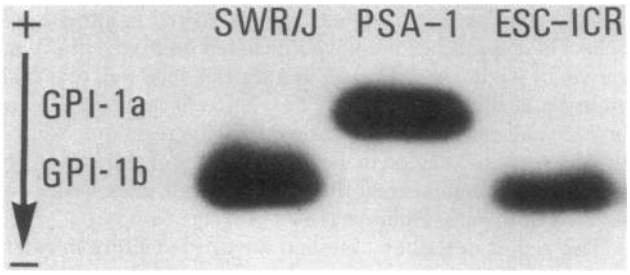

FIG. 3. Separation of electrophoretic variants of glucosephosphate isomerase (GPI). Extracts of livers from SWR/J mice (homozygous for *Gpi-1b*) and of cultures of PSA-1 cells (homozygous for *Gpi-1a*) are compared with extracts of cultures of ESC-ICR cells. Expression of the faster electrophoretic form by ESC-ICR cells demonstrates that they are not derived from PSA-1 cells.

5×10^6 ESC-ICR cells. In most cases a typical teratocarcinoma, containing derivatives of all three primary germ layers, formed within 6 weeks. These results indicated that the ESC-ICR cells are similar to embryonal carcinoma cells in their ability to form tumors and suggested that the embryo-derived cells are pluripotent. To prove this, single ESC-ICR cells were isolated in microdrops and subclonal cultures were established from them (19). Although the cloning efficiency of the ESC-ICR cells was rather low (approximately 1% as compared with at least 10% for PSA-1 cells), four single cell clones were obtained. Each clone was tested for its ability to differentiate by injection into athymic mice, and each was found to form typical teratocarcinomas (Fig. 4) containing several differentiated cell types, including endoderm and connective and epithelial tissues, as well as undifferentiated stem cells. These results conclusively demonstrate that individual cells in the ESC culture are pluripotent.

Embryonic stem cells and their clonal derivatives were also found to be capable of differentiation *in vitro* when cultured under conditions appropriate for the differentiation of PSA embryonal carcinoma cells (6, 7, 16). When the ESC-ICR cells, which are maintained in the undifferentiated state by coculture with fibroblastic feeder cells, are plated in the absence of feeder cells they form homogeneous rounded aggregates that, when placed in suspension, differentiate an outer layer of endoderm-like cells. These two-layered structures are known as embryoid bodies because of their similarity to the fetus-forming ICM of the normal mouse embryo (6). Further differentiation of the ESC-ICR cells could be obtained by allowing the embryoid

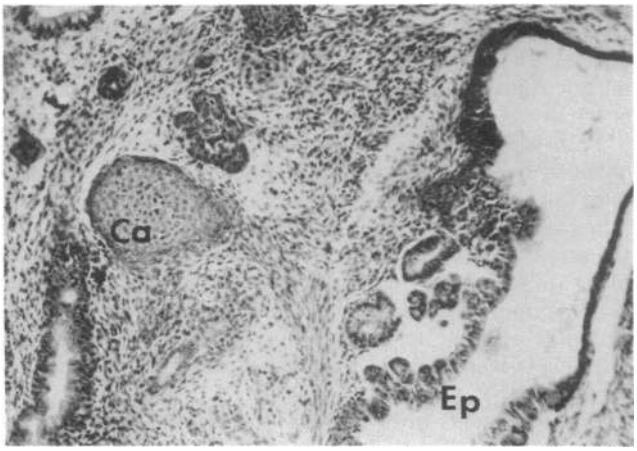

FIG. 4. Section of a tumor formed by a clonal derivative of the ESC-ICR cell line. The tumor contains a variety of differentiated cell types, including cartilage (Ca) and epithelial tissue (Ep). (Stained with hematoxylin and eosin; approximately ×100.)

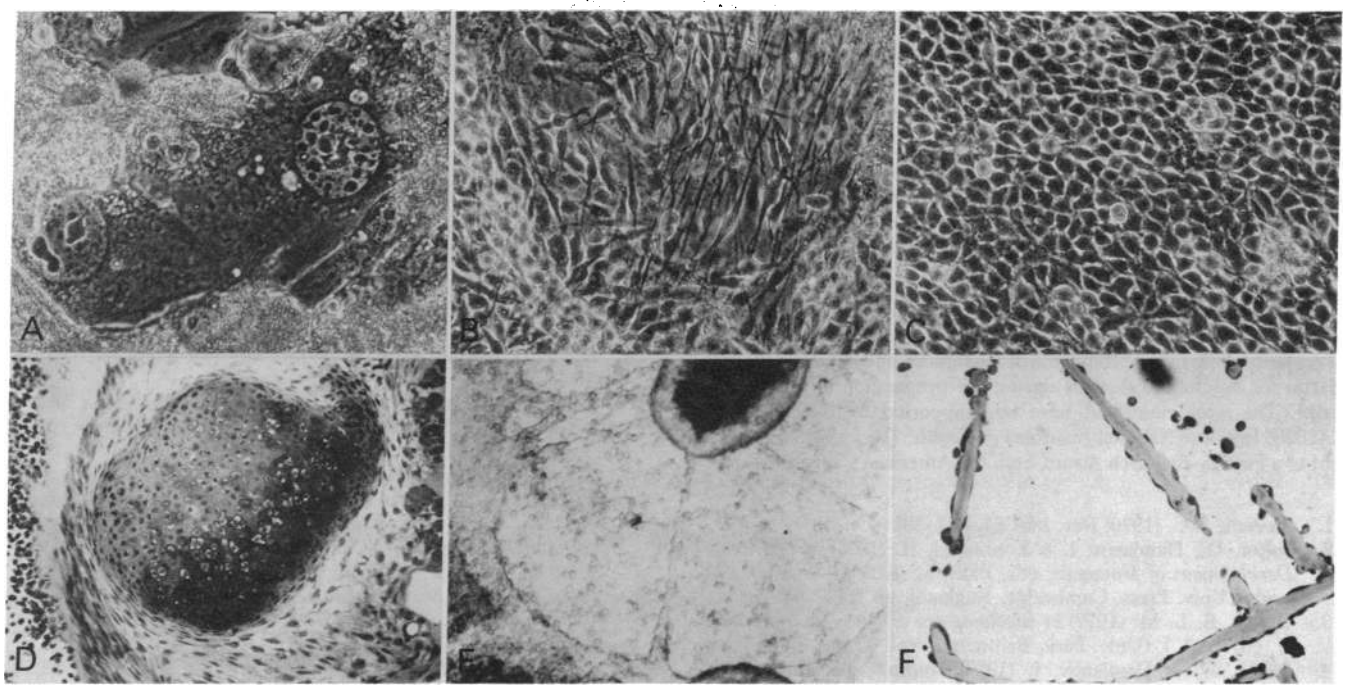

FIG. 5. Differentiation of ESC-ICR cells *in vitro*. A variety of cell types are apparent during the 6 weeks after the reattachment to tissue culture dishes of embryoid bodies formed by ESC-ICR cells. (*Upper*) Phase-contrast microscopy of live cells. (Approximately ×160). (*A*) Giant cells, (*B*) neuron-like cells, (*C*) endodermal cells. (*Lower*) (*D*) Section of plastic-embedded culture showing cartilage. (Approximately ×100.) (*E*) Live cells forming tubules. (Approximately ×35.) (*F*) Section of area shown in *E* after embedding in plastic. Tubules are filled with a granular, acellular deposit. (Approximately ×100.)

bodies to reattach to a collagen-coated tissue culture substratum. Within a few days of replating, several morphologically distinct cell types are observed migrating away from the attached embryoid body cores, and within 6 weeks such cultures contain a wide variety of differentiated cell types. Fig. 5 illustrates some of these, including giant cells, neuron-like cells, endodermal cells, cartilage, and a series of highly structured tubules.

DISCUSSION

The observations described above demonstrate that ICMs isolated from normal mouse blastocysts and cultured in medium conditioned by an established embryonal carcinoma cell line can give rise to cultures of cells with the characteristics of mouse teratocarcinoma stem cells. These properties include cell morphology, pluripotency, and the ability to form typical teratocarcinomas when injected into mice. In addition, it has been found that the embryonic stem cells described here express the SSEA-1 cell surface antigen (20) common to teratocarcinoma stem cells and early embryos but not expressed by most differentiated cell types (data not shown).

By using this culture method, it should now be possible to examine *in vitro* the way in which normal early embryonic cells give rise to teratocarcinoma stem cells. The results of such studies may ultimately serve to resolve the controversy surrounding the identity of teratocarcinoma stem cell progenitors in the early embryo and the question of whether the tumor stem cells have undergone a process of neoplastic transformation.

These experiments were undertaken on the premise that medium conditioned by teratocarcinoma stem cells might contain a factor, perhaps identical to a normal endogenous embryonic growth factor, capable of stimulating the proliferation of a small population of pluripotent cells in the normal embryo. The success of the approach reported here suggests that this working hypothesis has some validity. Although little is known at present about this factor, it seems unlikely that it is commonly produced by other cell types; fibroblastic feeder layers are generally considered potent conditioning agents, but in these experiments ICMs cultured on STO feeder layers did not give rise to ESC cells unless teratocarcinoma-conditioned medium was present in the early phases of the culture procedure. Obviously many questions remain to be answered about the teratocarcinoma-derived factor. Its purification will be difficult because the biological assay for its activity involves the growth of cells from isolated mouse embryonic ICMs. It is nevertheless interesting to speculate on its possible relationship to other known tumor-derived growth factors (17, 21). Ultimately the information that is obtained about this factor should help to elucidate the mechanism by which growth and differentiation are regulated during embryonic development.

The culture method described here, as well as the one recently reported by Evans and Kaufman (12), also has immediate practical value for the isolation of new pluripotent stem cell lines. It provides a means of circumventing the need for "converting" an embryo to a tumor *in vivo*. This makes feasible the isolation of pluripotent cells from embryos that cannot directly form teratocarcinomas when they are transplanted to an extrauterine site. These include all noninbred embryos because, for reasons that are not yet understood, stem-cell-containing teratocarcinomas can be obtained only when an embryo is transplanted to a histocompatible host; the alternative approach of obtaining stem-cell-containing teratocarcinomas by transplanting embryos to immunodeficient athymic mice has not been generally successful (2, 22). Many interesting mutations that affect early embryonic development are not maintained in inbred stocks of mice, and it has therefore not been feasible to obtain teratocarcinomas from embryos carrying these mutant genes. In this context it is noteworthy that the pluripotent ESC line described here was isolated from embryos of a noninbred genotype and thus is derived from an embryo that would not

otherwise give rise to a teratocarcinoma. Given these results, it seems likely that there will soon be available pluripotent, embryo-derived cell lines with specific genetic alterations that should make possible a variety of new approaches to the study of early mammalian development.

Note Added in Proof. The method of ESC isolation described here has now been used to establish cell cultures from embryos homozygous for the t^{w5} mutation of the *T/t*-complex.

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