

Fig. 3 Two 'intermediate' transformations. *a*, A thoracic ganglion from an animal of genotype *abx bx³ pbx/abx bx³ pbx* in horizontal plane. A metathoracic leg was backfilled and the HRP visualized using method (1) of Fig. 1 legend. *b*, A thoracic ganglion from an animal of the genotype *abx bx³ pbx/Df(3)P2* in sagittal plane, V marking the ventral surface. A metathoracic leg was backfilled and the HRP visualized using method (2) of Fig. 1 legend. Labelling as in Fig. 1.



Fig. 1 Groups of pluripotent embryo cells (arrowed) growing in monolayer culture on a background of mitomycin C-inhibited STO cells. The isolation of a definite cell line from a blastocyst takes only ~3 weeks and the pluripotent cell colonies are visible within 5 days of passage. We have had 30% yield of lines from blastocysts in one experiment. Two of the lines have been rigorously cloned by single-cell isolation but most were only colony-picked—this makes no difference.

result from a direct effect of the genes in another tissue, which in turn affects the CNS by induction or by mechanical constraints on CNS growth. Experiments using mosaic flies should determine which tissue must be mutant for the pattern of organization of leg motoneurons to be transformed. This should help to elucidate the mechanisms underlying segmental differences in the CNS.

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1. Lewis, E. B. *Nature* **267**, 565–570 (1978).
2. Mesulam, M.-M. *J. Histochem. Cytochem.* **24**, 1273–1280 (1978).
3. Hanker, J. S., Yates, P. E., Metz, C. B. & Rustioni, A. *Histochem. J.* **9**, 789–792 (1977).
4. Bacon, J. P. & Altman, J. S. *Brain Res.* **138**, 359–363 (1977).
5. Power, M. E. *J. comp. Neurol.* **88**, 347–409 (1948).
6. Lewis, E. B. *Abstr. 16th Int. Congr. Ent. Kyoto*, 161 (1980).
7. Lewis, E. B. *Drosoph. Inf. Serv.* **55**, 208 (1980).
8. Palka, J., Lawrence, P. A. & Hart, H. S. *Dev. Biol.* **69**, 549–575 (1979).
9. Ghysen, A. *Nature* **274**, 869–872 (1978).

Establishment in culture of pluripotent cells from mouse embryos

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Pluripotent cells are present in a mouse embryo until at least an early post-implantation stage, as shown by their ability to take part in the formation of chimaeric animals¹ and to form teratocarcinomas². Until now it has not been possible to establish progressively growing cultures of these cells *in vitro*, and cell lines have only been obtained after teratocarcinoma formation *in vivo*. We report here the establishment in tissue culture of pluripotent cell lines which have been isolated directly from *in vitro* cultures of mouse blastocysts. These cells are able to differentiate either *in vitro* or after inoculation into a mouse as a tumour *in vivo*. They have a normal karyotype.

Previous attempts to obtain cultures of pluripotent cells directly from a mouse embryo have been unsuccessful^{3,4}, although cells with a similar appearance have been reported to be present transiently^{5,6}. We considered that success might depend on three critical factors: (1) the exact stage at which pluripotent cells capable of growth in tissue culture exist in the embryo; (2) explantation of a sufficiently large number of these precursor cells from each embryo; and (3) tissue culture in conditions most conducive to multiplication rather than differentiation of these embryonic cells. These considerations have been discussed at greater length elsewhere⁷. An indication of the optimal stage of embryonic development might be gained by a comparison of the properties of embryonic cells at various stages with established cultures of embryonal carcinoma (EC) cells. Cell-surface antigen expression and the patterns of protein synthesis revealed by two-dimensional electrophoresis have suggested that neither the cells of the 6½-day ectoderm nor those of the 3½-day inner cell mass show homology with EC cells, but that epiblast cells of the early post-implantation embryo at 5½ days post coitum may do so⁸ (the day of finding coital plug is termed day ½). Cells from embryos of an early post-implantation stage seem to be the best candidates for direct progenitors of pluripotent cells in culture. As these embryos are difficult to isolate, and as the cell number in the isolated epiblast is small, we chose an alternative route to obtain embryo cells at this stage of development.

Mouse blastocysts may be induced to enter a state of diapause just before implantation. This delay in implantation depends on the maternal hormonal conditions, and may be induced experimentally by ovariectomy at an appropriate stage⁹. Embryos in implantational delay hatch from the zona but remain free-floating in the uterine lumen. A gradual increase in cell number occurs¹⁰, and the primary endoderm may be formed but no further development takes place until implantation occurs, under the control of hormonal stimuli.

129 SvE mice were caged in pairs and examined for mating plugs each morning. They were ovariectomized on the afternoon of day 2½ of pregnancy, injected subcutaneously with 1 mg Depo-Provera (Upjohn), and delayed blastocysts were recovered 4–6 days later. The blastocysts were cultured intact in

groups of about six embryos in small drops of tissue culture medium under paraffin oil on tissue culture plastic Petri dishes for 4 days. The blastocysts attached within 48 h and the trophoblast cells grew out and differentiated into giant trophoblast cells. The inner cell mass cells subsequently developed into large egg cylinder-like structures, with a group of small round cells surrounded by endodermal cells growing attached to the Petri dish. The egg cylinder-like structures were picked off the dish, dispersed by trypsin treatment and passaged on to gelatin-pretreated Petri dishes containing mitomycin C-inactivated STO fibroblasts. All culture was carried out in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum and 10% newborn calf serum. The cultures were examined daily and passaged by trypsinization every 2-3 days. Actively proliferating colonies of cells closely resembling EC cells were apparent from an early stage. These colonies were picked out, passaged and mass cultures grown. The cell cultures had the appearance and general growth characteristics of feeder-dependent EC cells (Fig. 1).

The embryos used to initiate these cultures are from normal 129 SvE strain mice, that is, from the same strain of mice as many EC cell lines, in particular those grown in this laboratory. Therefore it was important to exclude any possibility of contamination of these cultures with EC cells from established cell lines. Cell cultures were established from different embryos in three separate experimental series, but the best indication of their separate identity came from their karyotype. Cultures were initiated from 6-12 embryos, thus it might be expected that both male and female cells should be present. None of the 129 embryonal carcinoma cell lines in this laboratory have a normal karyotype, and, in particular—in common with most available embryonal carcinoma cell lines—they do not contain a Y chromosome. These embryo-derived cells have a completely normal karyotype. An XY karyotype is shown in Fig. 2. Three additional cell lines have been analysed; two of these are normal 40XX and one is normal 40XY. We have termed these directly embryo-derived cells EK to distinguish them from EC cells. EK cells grow rapidly in culture and have been maintained for over 30 passages *in vitro*.

Cultures of EK cells were collected by trypsinization, and ~10⁶ cells injected subcutaneously into the flank of syngeneic male mice. Tumours grew in all cases, and histological examination of these revealed that they were teratocarcinomas. When the EK cells were passaged without feeder cells they formed embryoid bodies which, when kept in suspension, became cystic.



Fig. 2 Karyotype of an embryo-derived pluripotent cell line, 40XY. Over 80% of the spreads of this clonal line possessed 40 chromosomes and had a clearly identifiable Y chromosome.

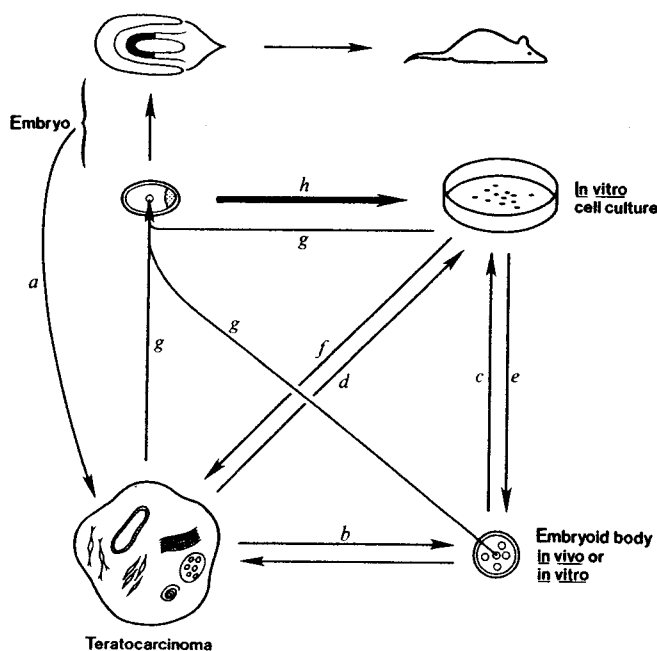


Fig. 3 Inter-relationships of cell lines, teratocarcinomas and embryoid bodies with normal mouse embryos. Arrows indicate routes of cell transfer: a, formation of teratocarcinoma by ectopic implantation of embryos; b, formation of embryoid bodies from teratocarcinoma and vice versa; c, derivation of cell culture from embryoid bodies; d, cell culture obtained directly from solid tumours; e, differentiation to embryoid bodies from culture; f, formation of solid tumours on reinjection of cells from culture; g, transfer of embryonal carcinoma cells either from cell culture or from the core of an embryoid body or from a solid tumour back to a blastocyst. All these procedures may result in chimaerism of the resulting mouse; h, the missing link supplied here.

Embryoid bodies allowed to attach to a Petri dish spread out and differentiated in the usual way into a complex of tissues. Preliminary observations indicate that, like early ectoderm cells of the mouse embryo and EC cells, EK cells carry the cell-surface antigens recognized by M1-22-25 (Forssman)^{8,11} and anti-I Ma (lacto-N-iso-octaosyl ceramide)^{12,13} and also that two dimensional gel electrophoretic separations of their proteins very closely resemble those of the EC cell line PSMB.

We have demonstrated here that it is possible to isolate pluripotent cells directly from early embryos and that they behave in a manner equivalent to EC cells isolated from teratocarcinomas. The network of inter-relationships between the mouse embryo and pluripotent cells derived from it has previously lacked only the direct link between the embryo and cells in culture for completion. We have now demonstrated this (Fig. 3).

Teratocarcinoma cells are now being widely used as a model for the study of developmental processes of early embryonic cell commitment and differentiation. Their use as a vehicle for the transfer into the mouse genome of mutant alleles, either selected in cell culture or inserted into the cells via transformation with specific DNA fragments, has been presented as an attractive proposition. In many of these studies the use of pluripotent cells directly isolated from the embryos under study should have great advantages. We have now shown that these EK cell lines are readily established from cultures of single blastocysts and so far have 15 lines of independent embryonic origin, some of which have been isolated from non-129, outbred mouse stocks. We are now studying the chimaeric mice formed from these cells.

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1. Gardner, R. L. & Papaioannou, V. E. in *The Early Development of Mammals* (eds Balls, M. & Wild, A. E.) 107-132 (Cambridge University Press, 1975).
2. Stevens, L. C. *Dev Biol.* **21**, 364-382 (1970).
3. Cole, R. J. & Paul, J. in *Preimplantation Stages of Pregnancy* (eds Wolstenholme, G. E. W. & O'Connor, M.) 82-122 (Churchill, London, 1965).
4. Sherman, M. I. *Cell* **5**, 343-349 (1975).
5. Atienza-Samols, S. B. & Sherman, M. I. *Dev Biol.* **66**, 220-231 (1978).
6. Solter, D. & Knowles, B. *Proc. natn. Acad. Sci. U.S.A.* **72**, 5099-5102 (1975).
7. Evans, M. J. *J. Reprod. Fert.* **62**, 625-631 (1981).
8. Evans, M. J., Lovell-Badge, R. H., Stern, P. L. & Stinnakre, M.-G. *INSERM Symp.* **10**, 115-129 (1979).
9. McLaren, A. *J. Endocr.* **50**, 515-526 (1971).
10. Kaufman, M. H. in *Progress in Anatomy* Vol. 1 (eds Harrison, R. J. & Holmes, R. L.) 1-34 (Cambridge University Press, 1981).
11. Stern, P. L. *et al. Cell* **14**, 775-783 (1978).
12. Feizi, T. *Blood Transfusion Immunohaemat.* **23**, 563-577 (1980).
13. Kapadia, A., Feizi, T. & Evans, M. J. *Expl Cell Res.* **131**, 185-195 (1980).