J. Embryol. exp. Morph. **87**, 27–45, (1985)
Printed in Great Britain © *The Company of Biologists Limited 1985*

The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium

THOMAS C. DOETSCHMAN, HARALD EISTETTER, MARGOT KATZ, WERNER SCHMIDT AND ROLF KEMLER

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstr. 37–39, D-7400 Tübingen, West Germany

SUMMARY

The *in vitro* developmental potential of mouse blastocyst-derived embryonic stem cell lines has been investigated. From 3 to 8 days of suspension culture the cells form complex embryoid bodies with endoderm, basal lamina, mesoderm and ectoderm. Many are morphologically similar to embryos of the 6- to 8-day egg-cylinder stage. From 8 to 10 days of culture about half of the embryoid bodies expand into large cystic structures containing alphafoetoprotein and transferrin, thus being analagous to the visceral yolk sac of the postimplantation embryo. Approximately one third of the cystic embryoid bodies develop myocardium and when cultured in the presence of human cord serum, 30% develop blood islands, thereby exhibiting a high level of organized development at a very high frequency. Furthermore, most embryonic stem cell lines observed exhibit similar characteristics. The *in vitro* developmental potential of embryonic stem cell lines and the consistency with which the cells express this potential are presented as aspects which open up new approaches to the investigation of embryogenesis.

INTRODUCTION

Blastocyst-derived embryonic stem (ES) cells are established *in vitro* from substrate-attached blastocysts without passage of the cells through tumours. They are maintained in an undifferentiated pluripotent state by culturing on an embryonic fibroblast feeder layer and spontaneously differentiate in the absence of feeder layer cells. Several methods have been applied successfully to the establishment of ES cell populations. Evans & Kaufman (1981) produced lines of pluripotent cells from the epiblast of delayed-implantation blastocysts. At the same time Martin (1981) established cell lines from cultures of immunosurgically isolated inner cell mass cells which were grown in medium conditioned by teratocarcinoma-derived embryonal carcinoma (EC) cells. It is now known that ES cell lines can be established without delayed-implantation blastocysts or EC-cell-conditioned medium (Robertson, Evans & Kaufman, 1983; Axelrod, 1984). There is no indication that there are significant differences between the cell populations derived in these ways.

Key words: ES cells, visceral yolk sac, blood islands, myocardium, mouse embryo.

Teratocarcinoma-derived EC cells are produced from a limited number of mouse strains which have the capacity to develop teratocarcinomas and have been used as an *in vitro* model system for embryonic development (Solter & Damjanov, 1979; Martin, 1980). The *in vitro* differentiation potentiality of EC cells has been studied either with the help of chemical inducers (Jones-Villeneuve, McBurney, Rogers & Kalnins, 1982; McBurney, Jones-Villeneuve, Edwards & Anderson, 1982; Paulin *et al.* 1982) or with cell lines which spontaneously differentiate under varying culture conditions (Rosenthal, Wishnow & Sato, 1970; Martin & Evans, 1975*a, b*; Sherman & Miller, 1978; Darmon, Bottenstein & Sato, 1981; Pfeiffer *et al.* 1981; Rizzino, 1983). Few EC cell lines are capable of spontaneous differentiation, and of these very few have the capacity to form cystic structures with phenotypic similarities to the postimplantation embryo (Rosenthal *et al.* 1970; Martin, Wiley & Damjanov, 1977; Cudennec & Nicolas, 1977).

The cells of blastocyst-derived ES cell lines may be quite similar to normal embryonic cells and in most cases are probably less altered by their *in vitro* environment than are the cells of most teratocarcinoma-derived cell lines by a tumour environment. This is most clearly evidenced by the remarkably high frequency with which ES cells can be used in blastocyst injection experiments to form chimaeras of a broad tissue spectrum as well as germ-line chimaeras (Bradley, Evans, Kaufman & Robertson, 1984). Other advantages of ES cells lie in the fact that they can be made from mouse strains which carry recessive lethal mutations (Magnuson, Epstein, Silver & Martin, 1982) or from parthenogenic embryos (Robertson *et al.* 1983). The degree, however, to which the use of ES cell lines will be beneficial in investigating the lesions occurring in such strains will be largely dependent upon the degree to which the lesion-bearing lines and the non-lesion-bearing lines of the same genetic background will be able to provide some semblance of organized *in vitro* development. It is therefore necessary to know the developmental potential of these cells in order that the fullest possible range of questions can be directed within the boundaries of this potential.

All blastocyst-derived ES cell lines so far described spontaneously differentiate and form cystic embryoid bodies (Evans & Kaufman, 1981; Martin, 1981; Robertson *et al.* 1983). The degree to which organized development similar to that of the embryo occurs within them, however, has not been described. The investigation reported here has done this by analysing the most advanced embryonic-like structures developed by a blastocyst-derived cell line, ES-D3. It has compared the extent of this development, as well as that of several other ES cell lines from 129 and C57 mouse strains, to the postimplantation embryo. It is shown that the blastocyst-derived cells can differentiate at a remarkably high frequency to form heart and blood cell-containing cystic structures similar to the visceral yolk sac of the embryo. A close analysis is made of the fluid content of the cystic structures, the erythrocytes of the blood islands and the morphology of the heart-like structures. The unique advantages which ES cells may provide to the study of embryonic development are outlined.

MATERIALS AND METHODS

Establishment and maintenance of cell lines

The ES cell line ES-D3 was derived from eight 129/Sv +/+ 4-day blastocysts, day of plug detection being set at 1 day of embryonic development. The ES-D3/4 and ES-D3/7, and ES-D3/10/5 cells are first- and second-order colony subclones, respectively, of the ES-D3 cells. ES-632 cells are a single-cell subclone similarly established from C57BL/6 blastocysts. After approximately 1–2 days of culture on a feeder layer of BALB/c 16- to 18-day embryonic fibroblasts (generously provided by Dr U. Koszinowski, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, W. Germany, see also Wobus, Holshausen, Jäkel & Schöneich, 1984) in Nunclon Delta SI tissue culture dishes, the inner cell mass cells were picked out, mechanically dissociated by gentle pipetting and transferred to a new feeder layer. Embryonal medium, consisting of 10 % foetal calf serum, 10 % newborn calf serum and 0·1 mM-β-mercaptoethanol in DMEM (Robertson *et al.* 1983), was changed every 2 days and the ES cells were transferred to new feeder layers about twice weekly. Embryonal medium was used during the establishment of all ES cell lines. After the lines were stable, they were maintained in the undifferentiated state on feeder layer cells. The maintenance medium was 15 % foetal calf serum in DMEM to which β-mercaptoethanol was added to 0·1 mM. The feeder layers were produced by treating the embryonic fibroblasts with 10 μg.ml^{-1} mitomycin-C for 3·5 h. The feeder layer cells were plated at approximately 5×10^6 cells per 90 mm tissue culture dish.

Cell culture under differentiation conditions

All ES and EC cells were cultured in the absence of embryonic fibroblasts in standard medium (15 % foetal calf serum in DMEM) either in tissue culture dishes, or in suspension in bacterial dishes or bottles placed on a rotary shaker. Cells were cultured in tissue culture dishes either under monolayer conditions using Falcon or hydrophilic petriperm (Heraeus) dishes at approximately 2×10^5 cells.ml^{-1} or under micromass culture conditions in which 10μl droplets of 20 000 cells each were added to 24-well tissue-culture dishes (Costar) or to hydrophilic petriperm dishes. After allowing the cells to attach for 4 h the dishes were gently flooded with medium. Suspension culture in bacterial dishes (Greiner) also contained 2×10^5 cells.ml^{-1}. Suspension culture on a rotary shaker was performed at 70 r.p.m. with 20 mM-Hepes-buffered standard medium with approximately 10^5 cells.ml^{-1}. No significant differences could be found in the developmental potentiality of ES cells between the two types of suspension culture, or between monolayer and micromass cultures. 'Days of culture' will refer to the days of culture after switching the cells to differentiation conditions.

Karyotype

Chromosome spreads were performed as described (Triman, Davisson & Roderick, 1975) using the modifications kindly provided by S. Adolph (Klinische Genetik der Universität Ulm, W. Germany). Before spreading onto microscope slides pretreated with ethanol/ether (1:1), cells were treated 1–2 h with 10 μg.ml^{-1} colcemid, 10–15 min with 0·56 % KCl and 10, 20 and 39 min successively with methanol/acetic acid (3:1) at 4 °C. G-banding was done as described (Seabright, 1971).

Histology and immunofluorescence

Indirect immunofluorescence tests with the monoclonal antibody against trophectodermal cytokeratin-like filaments TROMA-1 (Brûlet, Babinet, Kemler & Jacob, 1980) and benzidine staining of erythrocytes in blood islands were performed on methanol-fixed (−20 °C, 10 min) cryostat sections of cystic embryoid bodies. The anti-mouse macrophage monoclonal antibody (MAS 034, Sera-Lab) was used in indirect immunofluorescence tests on the easily dissociable cells from mechanically disrupted cystic embryoid bodies. After pipetting the cystic embryoid bodies in and out of a Pasteur pipette several times, the single cells were centrifuged onto a gelatine-

coated microscope slide using a cytocentrifuge (Cytospin 2, Shandon) and fixed with 4 % paraformaldehyde (4 °C, 10 min).

Immunoprecipitation and gel electrophoresis

Immunoprecipitation and electrophoresis of the fluid content of the *in vitro* cystic embryoid bodies was done by using the *Staphylococcus aureus* procedure of Kessler (1975) as applied by Vestweber & Kemler (1984). Cystic embryoid bodies were incubated in methionine-poor standard medium in the presence of [^{35}S]methionine (50 μCi.ml^{-1}) for 18 h after which the cavity fluid was collected either with a small Hamilton syringe or by gently breaking the cavities open and washing out the contents. Alphafoetoprotein (AFP) antiserum and affinity-purified anti-transferrin were kindly provided by E. Adamson (La Jolla Cancer Research Foundation, California) and were used at 20 μg.ml^{-1} (IgG fraction) and 3 μg.ml^{-1}, respectively, in the immunoprecipitations.

Blood cells for isoelectric focusing of haemoglobins were isolated and treated according to a modification of the method described by Cudennec, Delouvee & Thiery (1979). Briefly, adult 129/Sv blood cells were washed once in PBS, resuspended in 0·25 M-sucrose (containing 1 % (v/w) trasylol (Sigma) and 0·1 % KCN), centrifuged, and the cell pellet lyophilysed. Entire day-11 visceral yolk sacs (129/Sv) and blood-island-containing ES-D3 *in vitro* cystic embryoid bodies were ruptured and rinsed several times in PBS before resuspension in the above buffer. The 0·12 mm-thick isoelectric focusing gels were prepared and run on a flat-bed gel apparatus (LKB-Ultrophor) as described (LKB technical bulletin, modified by Dr Peter Symmons, Max-Planck-Institute for Developmental Biology, Tübingen, W. Germany) using pH 7–9 ampholines (Serva).

Electron microscopy

Samples were fixed for 2 h in 2·5 % glutaraldehyde in PBS, postfixed 1 h each in 1 % osmium tetroxide in PBS and 2 % uranyl acetate in 70 % alcohol, embedded in Epon, sectioned, contrasted 8 min at 25 °C in lead citrate in an LKB 2168 Ultrostainer and viewed with a Siemens Elmiskop 102 electron microscope at 80 kV.

RESULTS

Establishment and maintenance of the embryonic stem cells

After approximately 1–2 days of culturing 129/Sv +/+ blastocysts on a mitomycin-C-treated embryonic fibroblast feeder layer, clumps of inner cell mass cells from eight blastocysts (Fig. 1A) were picked out, mechanically dissociated by pipetting and transferred to a new feeder layer. In our hands 129/Sv ES cell lines could be established from approximately 5 % of the attached blastocysts and C57BL/6 cell lines from about 10 %. As long as the cells were maintained on a dense layer of feeder cells and replated every two days on a new feeder layer, differentiation was inhibited. This was determined morphologically (Fig. 1B) and by immunofluorescence with antibody markers specific for undifferentiated and differentiated embryonic cells (Kemler *et al.* 1981; Kemler, 1981; not shown). When carefully maintained in culture in the undifferentiated state as described above, ES cell lines can be kept in culture from 3 months to a year and can be freeze thawed several times without any apparent loss in developmental potential.

If the cells were grown on tissue culture plates without a feeder layer, differentiating, substrate-attached cells began growing out from the undifferentiated cell

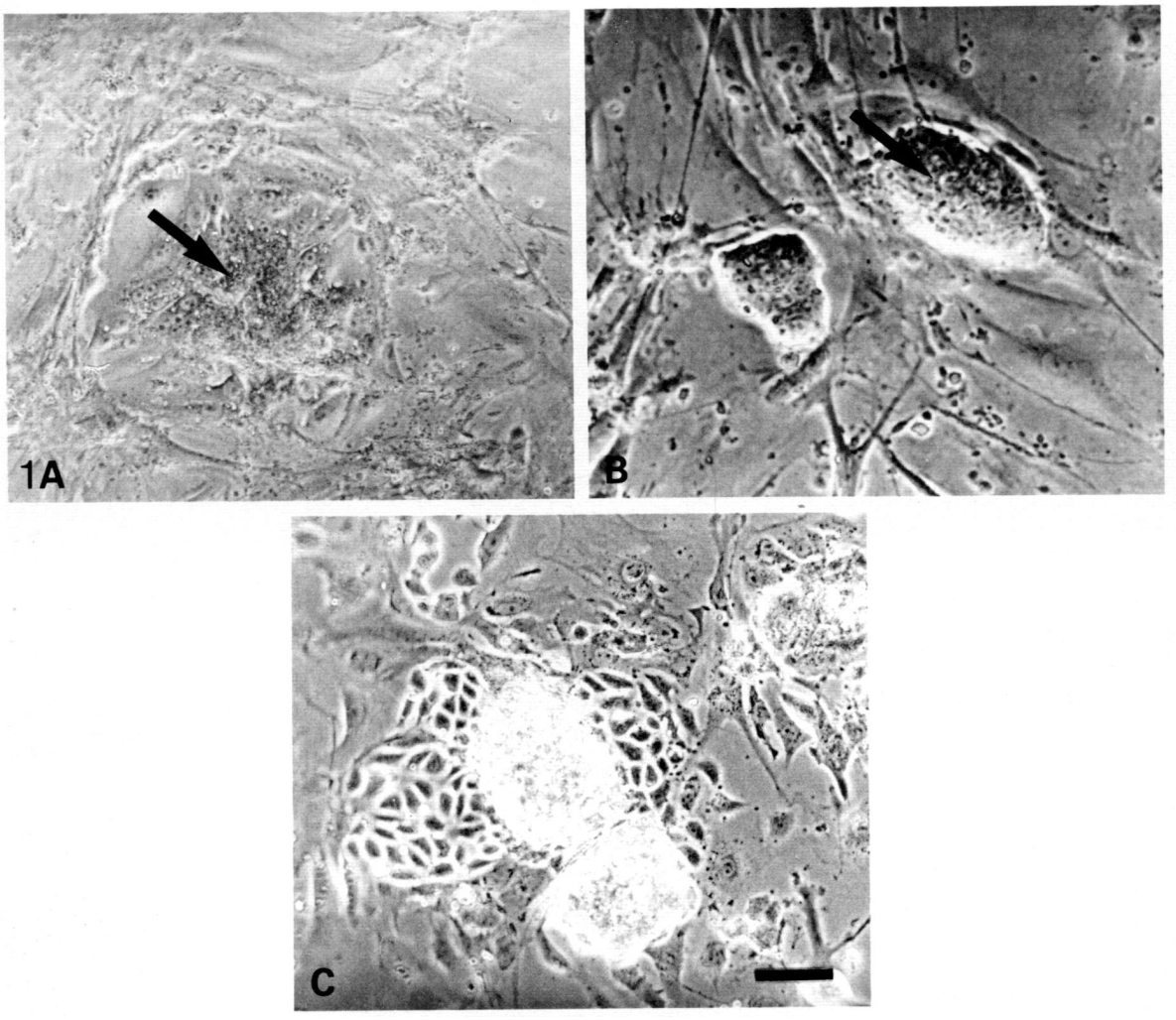


Fig. 1. ES cells during the establishment of cell lines, during maintenance in the undifferentiated state, and under differentiation conditions. 4-day 129/Sv blastocysts were allowed to attach to an embryonic fibroblast feeder layer. (A) Attached blastocysts when the inner cell mass cells (arrow) are removed and transferred to a new feeder layer after 2 days of culture. (B) Clumps of undifferentiated cells (arrow) being maintained on a feeder layer. (C) Differentiating ES-D3 cells after 2 days of culture on a gelatin-coated tissue culture dish in the absence of feeder layer. (Gelatine treatment is not necessary for ES cell differentiation.) Note the flat, differentiated cells growing out from the stem cell clumps. The few feeder layer cells remaining after transfer of ES cells to feeder-layer-free dishes are not sufficient to prevent differentiation. A–C: Bar = 100 μm.

clumps within 24 h (Fig. 1C). In all experiments the various ES cell lines seemed to behave identically. The blastocyst-derived cell lines have normal diploid karyotypes. Forty chromosomes were found in 62 % and 45 % of the cells, respectively, and both cell lines were XY. The G-banding pattern from one cell was analysed and revealed no translocations or metacentric chromosomes (Fig. 2).

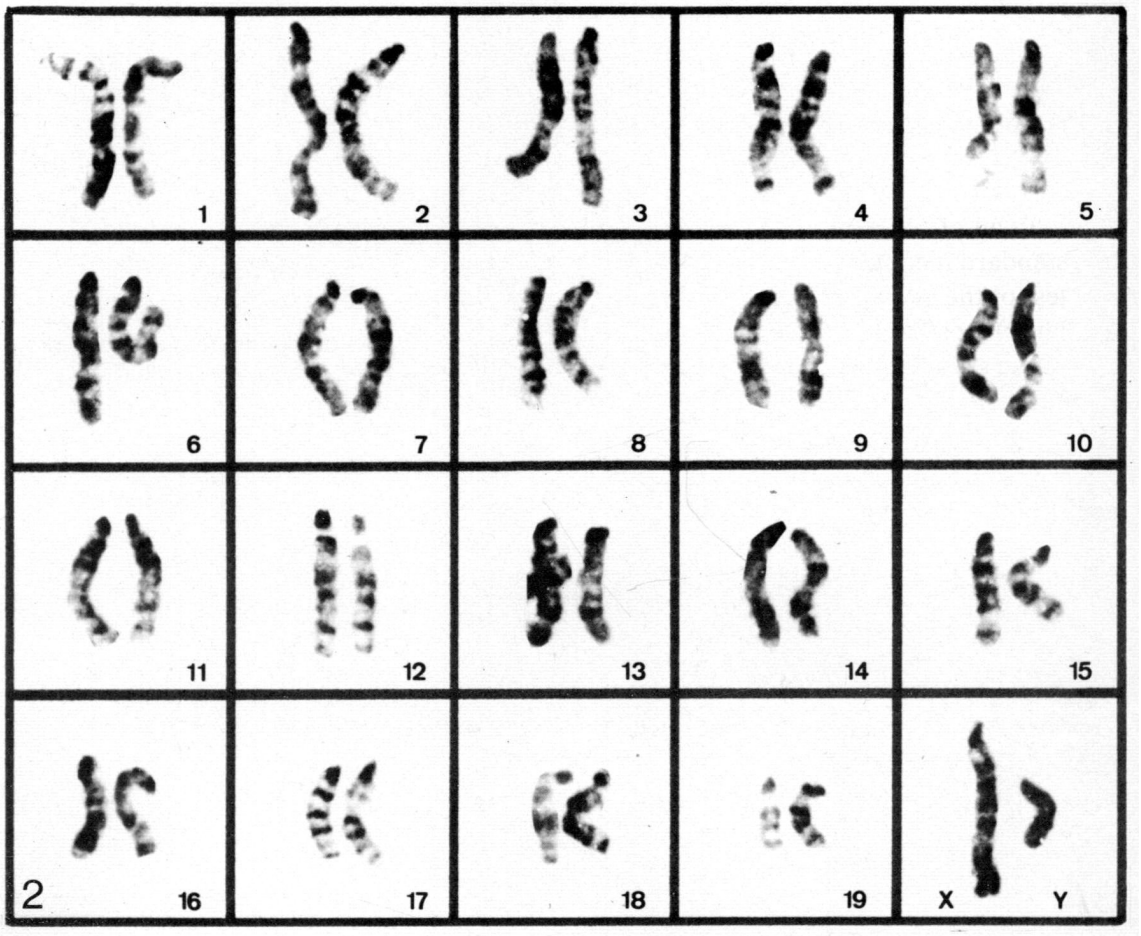


Fig. 2. Karyotype of a typical ES-D3/10/5 cell which has a normal diploid set of 40 chromosomes, has an XY constitution and contains no detectable abnormalities. ES-D3 cells: Out of 31 metaphase cells 62 % had 40, 25 % less than 40 and 13 % more than 40 chromosomes. ES-D3/10/5 cells: Out of 49 metaphase cells 45 % had 40, 32 % less than 40 and 22 % more than 40 chromosomes.

In vivo *formation of tumours*

To demonstrate that the ES cells could differentiate into products of all three germ layers, they were injected either subcutaneously or intraperitoneally into syngeneic mice, and the resulting tumours were analysed. When injected subcutaneously, solid teratocarcinomas were formed which contained large vacuoles enclosed by ciliated epithelial cells (preliminary evidence suggesting that these vacuoles have similarities to the brain ventricles), cross-striated muscle, cartilage, calcified cartilage, melanocytes and keratin sworls (not shown). When injected intraperitoneally, a mixture of mesenterically adherent solid tumours and unattached cystic embryoid bodies were formed. The latter contained outer and inner epithelial layers with areas of mesoderm in-between. The mesodermal areas

contained blood islands with embryonic haemoglobin-containing erythrocytes (see Fig. 8B for example) and contracting embryonic heart muscle cells (not shown). Immunofluorescence tests on cryostat sections of the cysts (not shown) showed that the cystic cavities contained large quantities of AFP.

In vitro *cultures*

In all of the experiments described below differentiating cells were cultured in standard medium without the addition of factors or inducers of any kind. Regardless of the type of culture (suspension (bacterial dishes or rotary shaker) or monolayer) most of the cells formed aggregates. If the aggregates were allowed to attach to the substrate (or remained attached), cells proliferated out from them along the substrate and differentiated into a wide variety of structures morphologically

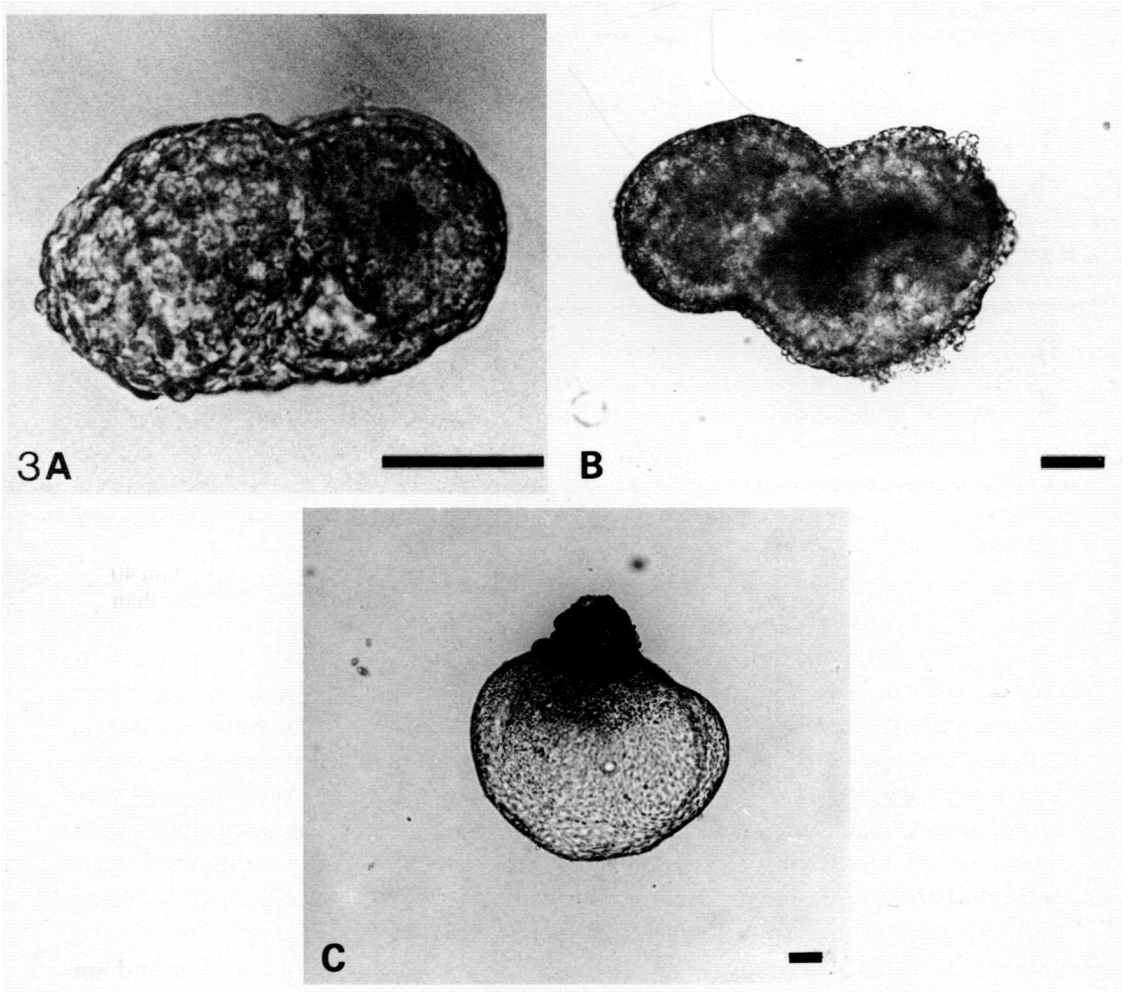


Fig. 3. Embryoid body development. ES-D3 cells were cultured under differentiation conditions in bacterial dishes for 4 days (A), 8 days (B) or 11 days (C). Bars = 100 μm.

identified at the light or electron microscopic levels as glandular, heart (see Fig. 5, for example), skeletal and smooth muscle, cartilage, nerve cells, keratin sworls, melanocytes and embryoid bodies (not shown). If, however, the aggregates were maintained in suspension, they developed only into embryoid bodies.

After 5 days of culture about 60 % of the embryoid bodies had developed into structures with an outer layer of endoderm bordered by a basal lamina within which the inner cells had condensed into a layer of columnar ectoderm-like cells. Many of these complex embryoid bodies were found to be polarized into two parts and appeared to be similar to the egg-cylinder stage of the 5-day embryo (Fig. 3A). Whether the two portions correspond to the extraembryonic and embryonic parts of the egg-cylinder-stage embryo is unclear. The markers we employed do not clearly distinguish between embryonic visceral on the one hand and extraembryonic visceral, primitive or parietal endoderm on the other. No trophoblast giant cells were ever seen during the entire culture period. During the next few days of culture there was a great deal of growth (compare Fig. 3A to 3B). By 8 days of culture an endodermal transition to the visceral type occurred along with the transition from complex to cystic embryoid bodies. After approximately 11 days of culture, many cystic structures were present (Fig. 3C) which looked similar to 8- to 10-day yolk sacs. After 3 weeks of culture, most developmental processes as well as growth had ceased, even though the cystic structures were viable for several more weeks.

Identification of cystic embryoid body as visceral yolk sac

The production of AFP and transferrin is characteristic of visceral yolk sac endoderm (Dziadek & Adamson, 1978; Adamson, 1982). Consequently, the fluid content of [^{35}S] methionine-labelled cystic embryoid bodies was electrophoretically analysed for total content (Fig. 4, lane 2) and by immunoprecipitations with anti-AFP and anti-transferrin (Fig. 4, lanes 3 and 4; the respective immunoprecipitations from 12-day embryonic visceral yolk sac: lanes 5 and 6). The total protein composition of the cavities consisted predominantly of AFP and transferrin with a few minor proteins of approximately 25 000, 45 000 and 300 000 relative molecular mass (M_r), presumably apolipoproteins A-I, A-IV and B, respectively (see Shi & Heath, 1984; Meehan *et al.* 1984) the 300 000 M_r protein which was precipitated by anti-transferrin was not precipitated from the labelling medium of embryonic visceral yolk sac cells (Fig. 4, lane 6). Electron micrographs of the outer endoderm cells of the cystic embryoid bodies (not shown) revealed many microvilli, electron-transparent cytoplasmic vesicles and junctional complexes as previously reported for EC cell cystic embryoid bodies (Martin *et al.* 1977), all of which are characteristic of visceral endoderm. Immunofluorescence tests on cryostat sections of cystic embryoid bodies using monoclonal antibodies TROMA 1 (positive for visceral and parietal endoderm, Kemler *et al.* 1981) and TROMA 3 (positive only for parietal endoderm, Boller & Kemler, 1983) (not shown) were consistent with the findings that the endodermal layer of the cystic bodies consisted predominantly of visceral yolk sac.

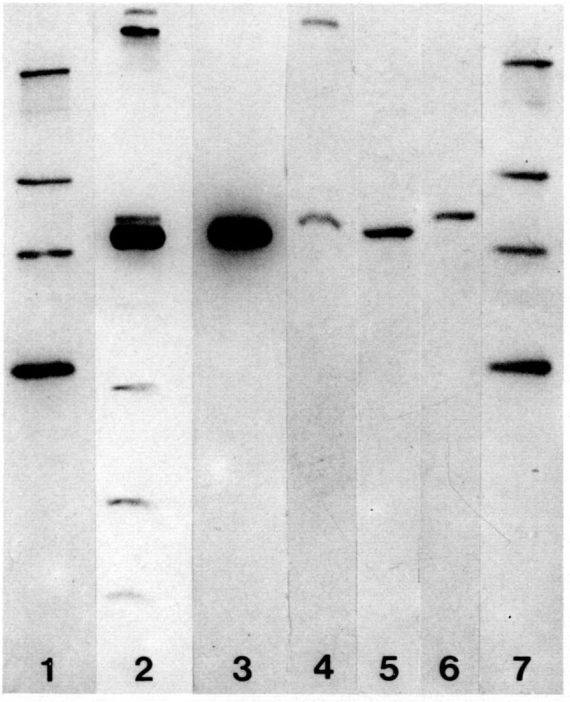


Fig. 4. SDS–PAGE and immunoprecipitation analysis of the protein content of ES cell cystic embryoid body cavities. Large, 2–4 mm in diameter ES-D3 or ES-D3/10/5 cystic embryoid bodies cultured for 2–4 weeks in bacterial or hydrophobic petriperm dishes (lanes 2–4), or freshly prepared 12-day visceral yolk sac (lanes 5, 6) were incubated with [^{35}S] methionine. Total labelled protein from cystic cavities (lane 2). Immunoprecipitation of cavity content (lanes 3,4) or labelled culture supernatant (lanes 5,6) with anti-AFP (lanes 3,5) or anti-transferrin (lanes 4,6). Note the high relative molecular mass protein coprecipitated by anti-transferrin from the cystic cavity contents but not from embryonic visceral yolk sac culture supernatant. Lanes 1, 7: protein relative molecular mass markers myosin heavy chain, 200 000; phosphorylase, 97 000; albumin, 68 000; and ovalbumin, 45 000.

Myocardium

After at least 8 days of suspension culture about one third of the ES cell cystic embryoid bodies began rhythmically contracting in areas where their surface was quite thick. Identically contracting structures could be found in micromass cultures and were analysed with respect to tissue organization and muscle type. Micrographs of video sequences of a highly organized beating structure (Fig. 5A, B) show the relaxed and contracted phase, respectively, of one contraction. The arch-shaped ridges (arrows) are phenotypically analogous to myocardium. An aggregate of cells was trapped in the endocardial-like cavity and moved back and forth during the contraction (arrowheads). Also associated with this structure were endothelial capillaries some of which also contained trapped cells which moved with the contractions (not shown). Electron micrographs showed that the muscle cells inside

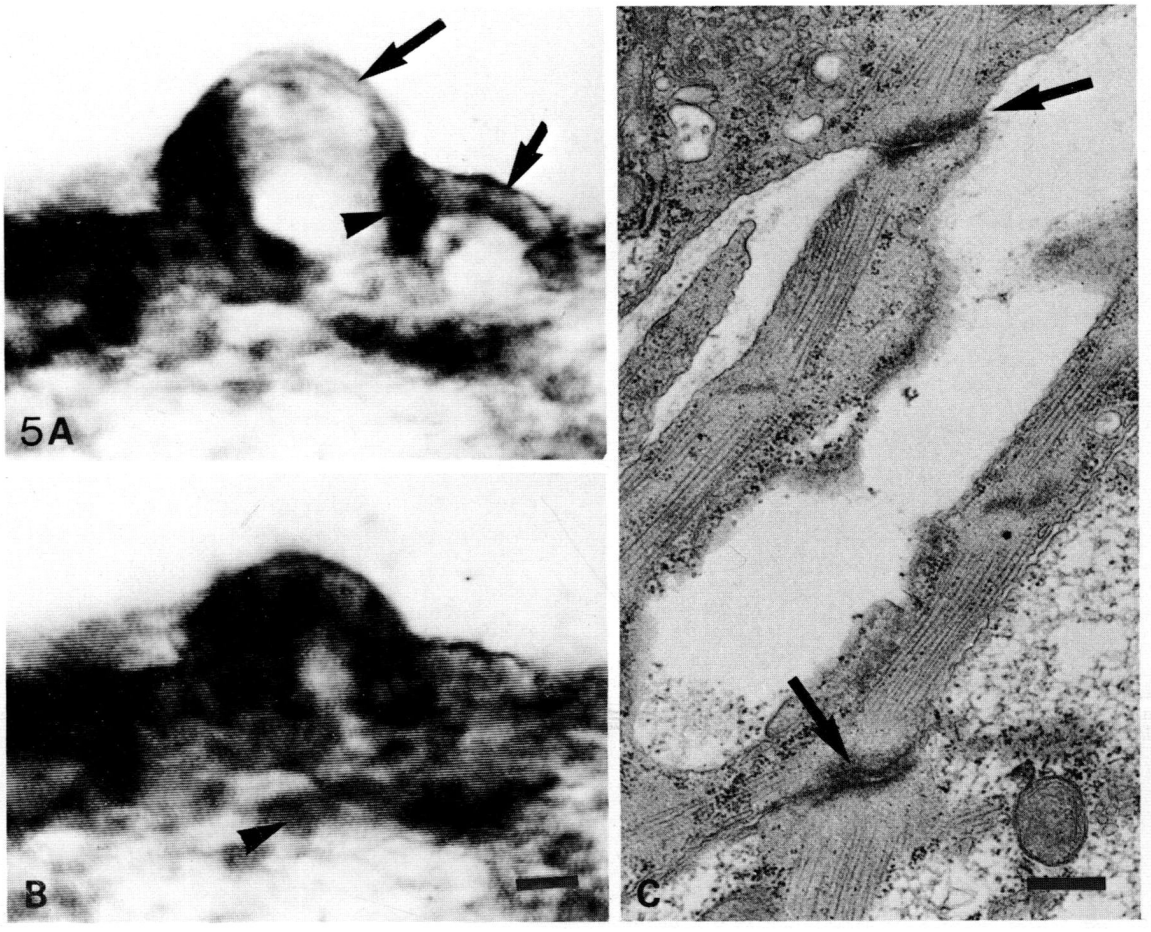
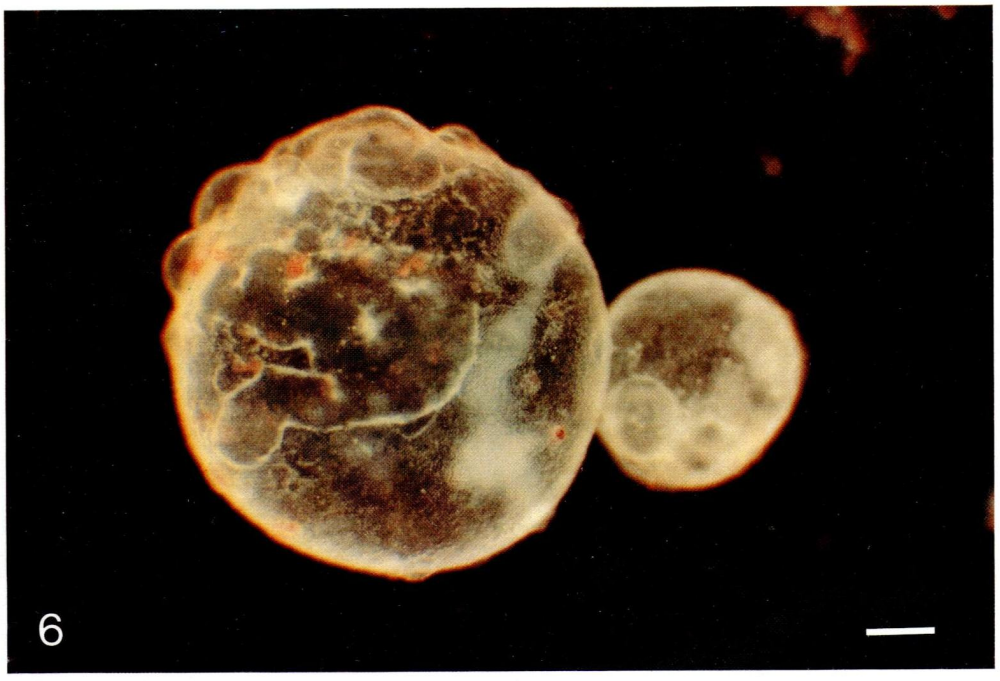
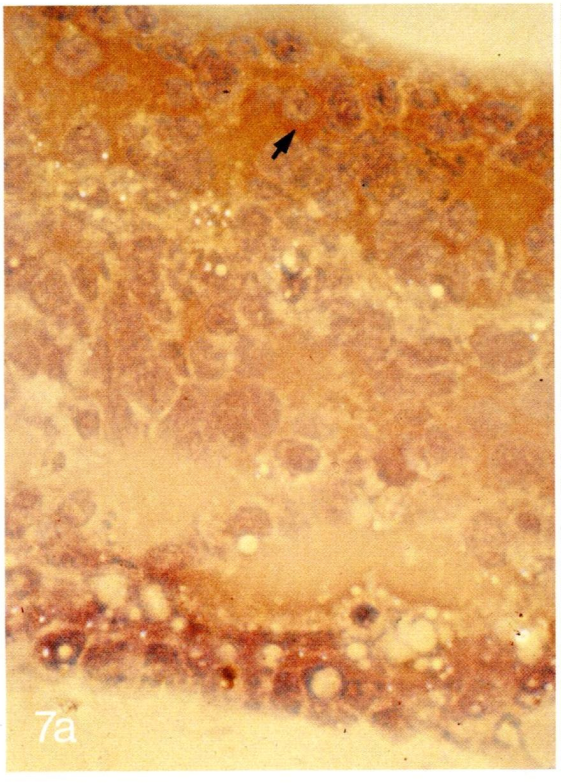


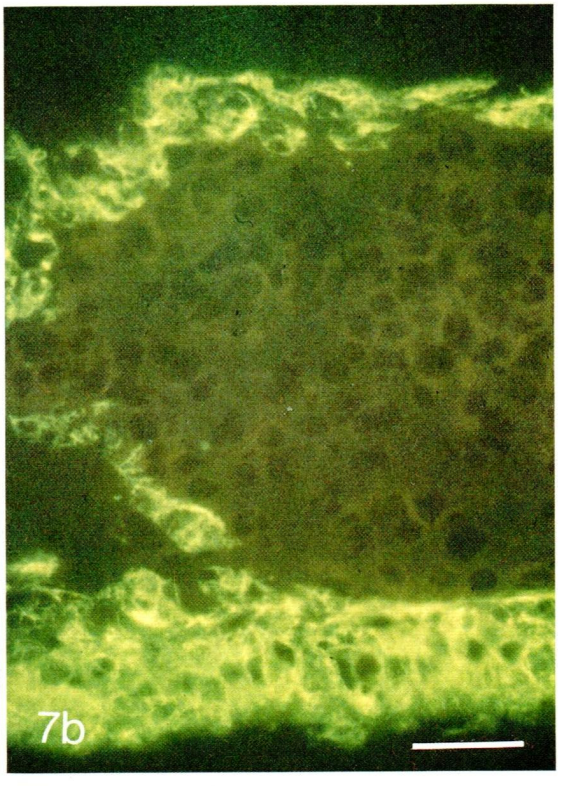
Fig. 5. Video micrographic and electron microscopic analysis of ES myocardial cells. (A,B). Polaroid photographs of video sequences of the relaxed and contracted phases, respectively, of one contraction. ES-D3 cells were grown in micromass culture. Myocard-like ridges (arrows) can be easily recognized in the relaxed phase of the contraction. Inside the endocard-like cavity a cell aggregate can be seen (arrowheads) which moves about 30 μm during the contraction. (C) Electron micrograph of ES-D3 cells which had been rhythmically contracting. The cells had been cultured for 4 days in suspension followed by 17 days on hydrophilic petriperm dishes. Note the intercalated disks which lie at the myofibrillar Z-bands of adjacent cells (arrows), structures characteristic of cardiac muscle cells. A,B: bar = 100 μm. C: bar = 0·5 μm.

Fig. 6. Dark-field photograph of ES cell cystic embryoid body. ES-D3/10/5 cells were cultured for 12 days on bacterial dishes. Besides the red blood islands, mesodermal thickenings (white areas) as well as endodermal subcompartmentalization can be seen. Bar = 200 μm.

Fig. 7. Histological and immunofluorescence analysis of ES cell cystic embryoid body blood islands. Cryostat sections of one blood-island-containing cystic embryoid ascites tumour removed 4 weeks after intraperitoneal injection of 5×10^6 ES-D3/10/5 cells. (A) Benzidine stain. In the inner (top) and outer (bottom) endothelial cell layers, only the nuclei are stained. Between these layers the cytoplasm of the erythrocytes (arrow) are stained as well. (B) Indirect immunofluorescence with TROMA 1. Endothelial cells are positively stained and mesodermal cells, including erythrocytes, are unstained. A,B: Bar = 50 μm.


6


7a


7b

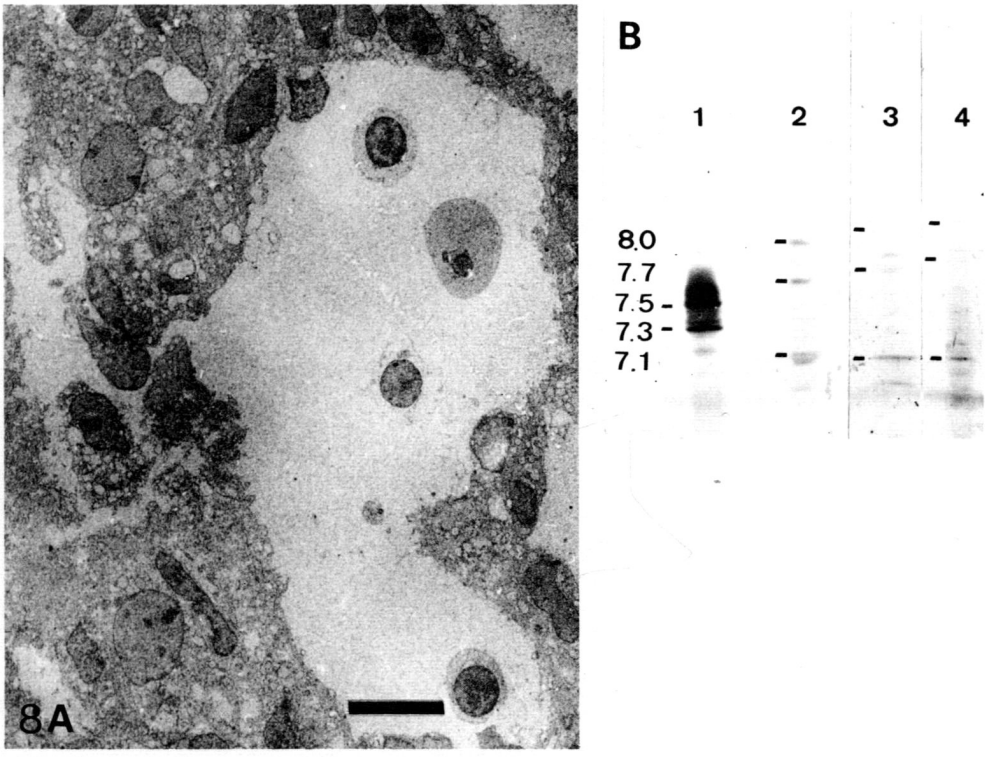


Fig. 8. Characterization of erythrocytes from ES cell cystic embryoid bodies and cystic tumours. (A) Electron micrograph of a blood island in an ES-D3/10/5 cystic embryoid body after 13 days of culture in a hydrophobic petriperm dish. The blood island consists of an endoderm-bordered cavity which contains unattached, nucleated erythrocytes typical of all yolk sac-derived red blood cells. (B) Isoelectric focusing of haemoglobin from three different gels (lanes 1 and 2, lane 3, and lane 4). The pH values indicated at the side are designated by a small line at the left side of each gel. Lane 1: adult globin chains from 129/Sv blood focus at pH 7·5 and 7·3. Lanes 2 and 3: embryonic globins from disrupted and washed visceral yolk sacs of 11-day 129/Sv embryos focus at pH 8·0, 7·7 and 7·1. Lane 4: embryonic globins found in disrupted and washed *in vitro* cystic embryoid bodies after 14 days in culture in bacterial dishes. The cells were cultured the first 10 days in standard medium and the last 4 days in 20 % foetal calf serum in Iscove's modified Dulbecco's medium. Benzidine reaction (lanes 1 and 2) and Coomassie blue staining (lanes 3 and 4). The cystic embryoid bodies were electrophoresed in their entirety in order to minimize the loss of erythrocytes. The visceral endodermal cells which greatly outnumber the erythrocytes in our samples are probably the source of the non-haemoglobin bands in lanes 2–4. Bar = 10 μm.

the myocard-like structures contained intercalated disks (Fig. 5C) which are heart and somitic myotome-specific intercellular junctions found where Z-bands of the apposing myofibrils of adjacent cells come together. The muscle cells could continue beating for more than a week. The morphological development of the *in vitro* beating structures was usually complete by the time the contractions were first observed.

Visceral yolk-sac-derived blood islands

At the light microscopic level red areas could be detected just under the surface of approximately 1 % of the cystic embryoid bodies after 12 days of suspension culture (Fig. 6). Closer examination of similar blood islands found in cystic tumours using benzidine (Fig. 7A; stained erythrocyte cytoplasm being indicated by arrow) and fluorescence staining with TROMA 1 (Fig. 7B) revealed a pocket of blood cells surrounded by two endothelial layers. An electron micrograph (Fig. 8A) shows that the blood island erythrocytes of an *in vitro*-formed cystic embryoid body were nucleated — characteristic of the blood cells of embryonic visceral yolk sac. The haemoglobins of blood island cells in *in vitro* cystic embryoid bodies were determined by isoelectric focusing to be embryonic (Fig. 8B, lane 4; control adult, lane 1; and control embryonic, lanes 2 (benzidine) and 3 (Coomassie blue)). The double control shows that Coomassie-blue staining can also be used to detect haemoglobins in these structures. In cystic tumours the blood islands also contained exclusively embryonic haemoglobin (not shown), thus demonstrating that host erythrocytes had been excluded from the cystic tumours. As may be the case in the mouse embryo, the blood islands in the *in vitro* cystic embryoid bodies usually disappeared after 2–6 days.

In order to increase the frequency of appearance of blood islands, culture conditions used for blood stem cell cultures (Iscove's modified Eagle's medium) and for mouse embryo *in vitro* cultures (20 % human cord serum instead of foetal calf serum; Hsu, 1979) were combined. These culture conditions increased the percentage of cystic embryoid bodies which contained blood islands from 1 % to 30 % (Table 1). Four different cell lines from two different mouse strains gave similar results. These data suggest that except for possible small quantitative

Table 1. *Blood island production in various differentiated ES cell lines.*

Mouse strain	Cell line	No. of exps.	Blood-island-containing cystic structures	
			FCS	HCS
129	ES-D3/7	5	3/165 (2%)*	78/249 (30%)
129	ES-D3/10/5	1	1/281 (1%)	22/218 (19%)
129	ES-D3/4	1	0/102 (0%)	19/76 (25%)
C57	ES-632	2	3/34 (10%)	16/48 (33%)

ES cells were cultured in bacterial dishes in the absence of embryonic fibroblasts and in standard medium for 10 days. On day 10 the medium was changed to Iscove's modified Eagle's medium containing either 20% foetal calf serum (FCS) or human cord serum (HCS). Every 2 days thereafter each cystic, visceral yolk sac structure was examined under dark-field stereo optics. Each cystic structure containing one or more blood islands was scored as one. The scores from days 14–18 of culture were combined. Data were taken only from experiments in which both culture conditions were used.

* Number of blood-island-containing cystic embryoid bodies/total number examined.

differences between ES cell lines, they all display qualitatively similar developmental potentialities.

Immunofluorescence tests with a monoclonal antibody specific for macrophage cells (Fig. 9A and B) suggest that the cystic embryoid bodies may also contain

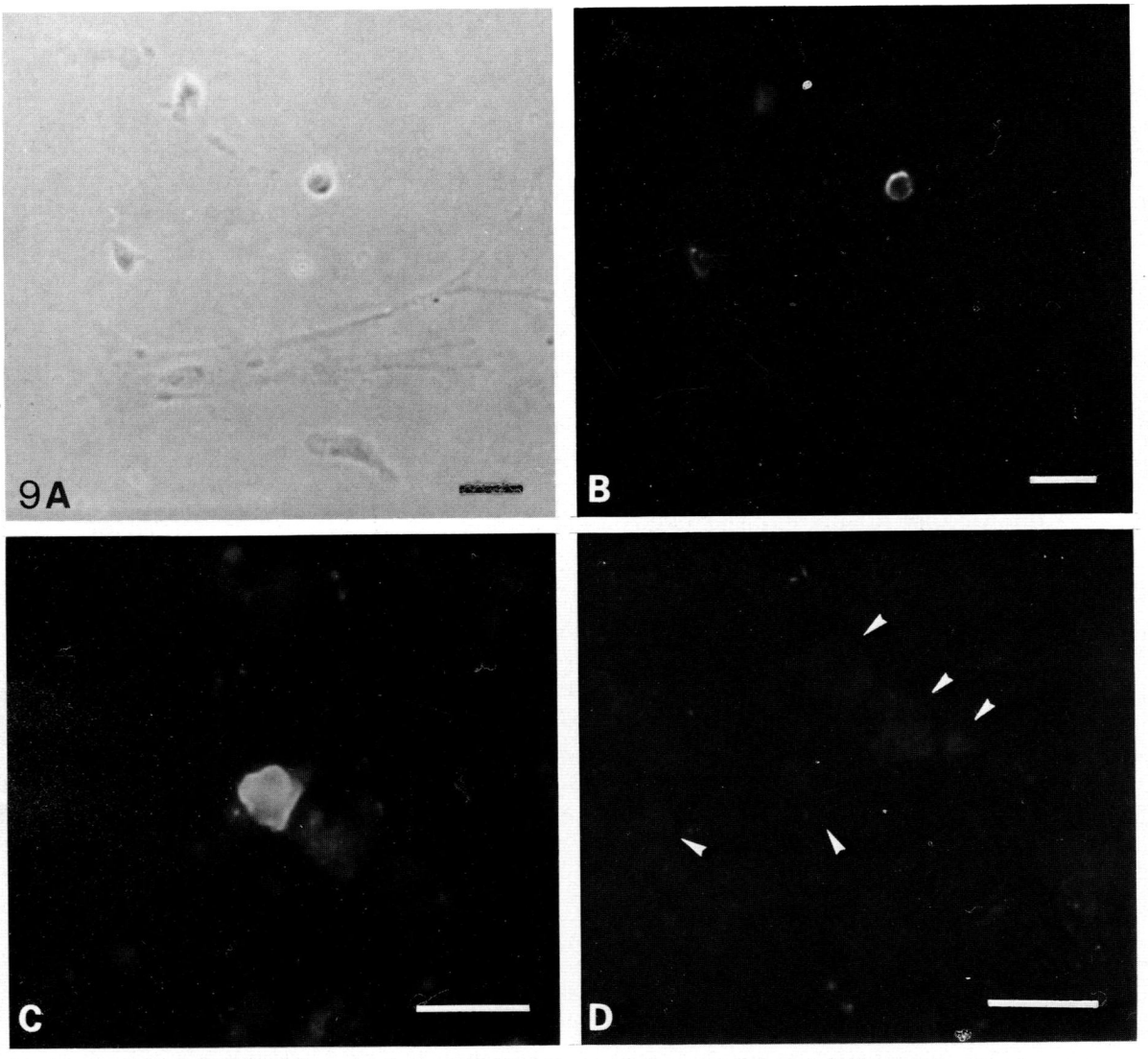


Fig. 9. Indirect immunofluorescence test with anti-macrophage monoclonal antibodies. (A,B) Positive control: Mouse peritoneal fluid was placed in tissue culture. After 2 days of culture, the attached cells were fixed and indirectly stained with antibody. Note the fibroblastic and non-fibroblastic cells which do not stain. (C,D) Cells were mechanically dissociated (see Materials and Methods section) from 18 day *in vitro* ES-D3 cystic embryoid bodies and cytocentrifuged onto microscope slides. (C) Indirect immunofluorescence test with antibody. (D) Control: Fluorescent second antibody alone. Arrowheads indicate location of cells. (A) Phase contrast. B–D, Fluorescence. Bars = 50 μm.

macrophages (Fig. 9C). Preliminary results using methyl cellulose culture conditions also indicate the presence of macrophage colony-forming cells in the cystic embryoid bodies (Gordon Keller, Basel Institute of Immunology, Switzerland).

Comparison to EC cells

Two teratocarcinoma-derived cell lines have been reported to produce cystic structures which occasionally contained erythrocytes *in vitro* (Martin *et al.* 1977; Cudennec & Nicolas, 1977). We have compared a subclone (EC-PSA1/NG2) of one of them to our blastocyst-derived cell lines. The greatest difference between the ES and EC lines was of a quantitative nature (not shown). There appeared to be more mesoderm-derived structures in the ES cells, and although the teratocarcinoma-derived cells could form similar highly organized structures, the frequency with which they formed them was strikingly less.

DISCUSSION

There are four aspects of blastocyst-derived embryonic stem cell lines which together make them potentially useful as a model system for embryonic development. The first is that they can be established from individual blastocysts of nearly any genotype. This makes it possible to investigate interstrain differences which may become apparent during embryogenesis. ES cell lines have been used for the investigation of cells homozygous for a lethal recessive mutation carried by inbred mouse strains (Magnuson *et al.* 1982), of gross chromosome abnormalities such as metacentricity (Kaufman, Robertson, Handyside & Evans, 1983), and of parthenogenesis (Robertson *et al.* 1983). Other uses could be to investigate the effects of mono- and trisomy (Gropp, 1982) and to investigate whether the differences between mouse strains which do and do not form spontaneous teratomas at high frequencies are caused by differences in the embryonic cells or in their host environment. Investigations of this nature could be of potential importance to the problem of transformation.

The second aspect, the consistency from one cell line to the next with respect to the *in vitro* developmental process, is necessary if the homozygous mutant lines referred to above are to be fruitfully compared to their respective background lines. We have now closely observed six ES cell lines from three different genetic backgrounds as well as many of their subclones (single cell as well as colony subclones) and have found them all to be remarkably similar with respect to their developmental characteristics.

In light of their developmental consistency, the third aspect of ES cell lines, namely their ease of establishment, is of special significance. One now has the advantage of always being able to work with cells which are temporally close to the embryo. It is generally accepted that the more time an embryonic cell spends outside of the embryonic environment, the more likely it will be selected for growth in the new environment. Consequently, both EC and ES cells can be expected to

lose their pluripotent characteristics over time. The special advantage of the blastocyst-derived ES cells is that under such circumstances a new line can easily be made with the same original potentialities of the old.

The fourth aspect of ES cells which make them suitable as a model system for embryonic development is their *in vitro* expression of a large degree of developmental potential. Since ES cells are capable of forming germ-line chimaeras at a frequency much higher than teratocarcinoma cells (Bradley *et al.* 1984), one would also expect higher *in vitro* frequencies of well-developed embryonic structures. This can be realized through two types of culture conditions: under two-dimensional (substrate-attached) culture conditions ES cells can differentiate into a large variety of cell types (Martin, 1981; this report). Such conditions may be well suited for investigations into some of the determining events which lead to terminal differentiation, especially if humoral factors are involved. In three-dimensional suspension culture ES cells form highly organized cystic embryoid body structures which are in many respects analogous to postimplantation embryos. With these structures one should be able to answer more easily questions concerning 'development' of the embryo rather than simply 'differentiation' of cell types. They may also be suitable for studying the developmental regulation of the expression of genes, normal or altered, inserted into ES cells, thereby offering all of the analytical advantages of *in vitro* systems. It is on these highly organized cystic embryoid bodies that we have focused our attention in this report.

Comparison of ES cell development to that of the embryo and EC cells

The major proteins synthesized by the ES cell cystic embryoid bodies and secreted into their cavities are AFP and transferrin — two of the major products of the visceral yolk sac. It is noteworthy that other than these two proteins very few others are detectable. The minor proteins of approximately 25 000 and 45 000 M_r, but not the one of 300 000 M_r, have been observed previously in visceral yolk sac fluid (Adamson, 1982; Janzen, Andrews & Tamaoki, 1982). Recently, apolipoproteins of all three sizes have been found to be produced by visceral yolk sac endoderm (Shi & Heath, 1984; Meehan *et al.* 1984). We do not know why an approximately 300 000 M_r protein is coprecipitated by anti-transferrin serum though not recognized by this same serum in immunoblots. This protein could not be coprecipitated from embryonic visceral yolk sacs metabolically labelled *in vitro* and is therefore unlikely to be apolipoprotein B.

In the embryo blood islands appear within the mesodermal layer of the visceral yolk sac on day 8. These primitive erythrocytes are large, contain nuclei and synthesize primitive haemoglobins (Craig & Russell, 1964). *In vitro* erythropoiesis has been shown to occur in two teratocarcinoma-derived EC cell lines. The large, nucleated red blood cells produced by the cystic structures of EC-PSA1 (Martin *et al.* 1977) and EC-PCC3/A/1 (organ culture conditions; Cudennec and Nicolas, 1977) develop blood islands from mesodermal thickenings on the inner side of

endodermal vesicles. The EC-PCC3/A/1 blood cells contain embryonic haemoglobin (Cudennec, Thiery & Le Douarin, 1979).

Under standard culture conditions approximately 1 % of the blastocyst-derived ES cell cystic bodies contain islands of large, nucleated, embryonic haemoglobin-containing erythrocytes after two weeks in culture. A 30-fold increase in the percentage of blood island-containing cystic bodies can be induced by human cord serum. That these cells could make adult haemoglobin under appropriate conditions, however, cannot be ruled out. This is important in light of evidence that some sera used in culture (Stamatoyannopoulos, Nakamoto, Kurachi & Papayannopoulou, 1983) as well as other embryonic tissue (Cudennec *et al.* 1981; Ripoche & Cudennec, 1983; Labastie, Thiery & Le Douarin, 1984) can induce yolk sac erythrocytes to produce adult haemoglobin. The cystic embryoid bodies may also contain stem cells for macrophages. The existence of other stem cells of the haemopoietic lineage is presently under investigation. The presence of haemopoietic stem cells in the cystic structures may provide investigators with a purely *in vitro* model system for unravelling some of the complexities of the haemopoietic cell lineages.

In the embryo the first muscle cells to appear are in the myocardium and the somitic myotome. Whereas the myotome-derived muscle anlagen produce multinucleated cells, the myocardial cells remain by and large mononucleated and develop intercalated disks which serve to join the myofibrillar apparatus of adjoining cells (rev. by Manasek, 1973). Contractile protein isoforms also seem to follow this pattern in that cardiac isoforms are found both in myotome and myocard, but not in skeletal muscle (Toyota & Shimada, 1981; Sweeney *et al.* 1984). The only distinguishing characteristic then between the earliest myocardial and myotomal muscle is the rhythmic contraction of the primitive heart cells.

The production of beating muscle cells by two teratocarcinoma-derived cell lines has been reported. EC-PSA1 cells produce such cells both in monolayer culture (Martin *et al.* 1977) and in cystic embryoid bodies (our observations). EC-P19 cells, which require chemical inducers to differentiate, form beating structures in monolayer culture (McBurney *et al.* 1982). The development of associated endocardial tissue by these cell lines has not been described previously.

About one third of the cystic embryoid bodies produced by the blastocyst-derived cells develop rhythmically contracting, intercalated disk-containing myocardial cells. The associated endocardial tissue found in cultures of substrate-attached cells can also form in the cystic structures (not shown). These data show that ES cells have the potential to develop into several cardiac cell types in a well-organized manner, suggesting that they may be suitable for investigations of heart organogenesis.

It is pertinent to this discussion that only the embryonic and not the extra-embryonic portion of egg-cylinder-stage embryos can 1) form teratocarcinomas (Diwan & Stevens, 1976) or 2) develop *in vitro* into structures similar to those described here (Hogan & Tilly, 1981). Likewise, it is noteworthy that the structures

described in this report are similar to those produced by some of the earlier attempts at embryo culture (Hsu, 1972). These data are consistent with the chimaera experiments mentioned above (Bradley *et al.*, 1984) and lead to the conclusion that ES cells are in fact quite similar to the pluripotent cells of the blastocyst. We are confident that the developmental similarity of most of the ES cell lines produced, their ease of establishment, their ability to form highly organized structures analogous to those of the embryo, and their amenability to the production of interstrain variants, should provide investigators with new approaches to the study of embryonic development.

We are grateful to Drs F. Bonhoeffer and H. Frank of the Max-Planck-Institute for Developmental Biology, Tübingen, W. Germany for assistance with the video micrography and electron microscopy/photography, respectively; to Dr S. Adolph for helpful discussions concerning the chromosome banding patterns; to Dr J. -P. Thiery, Institut d'Embryologie du Centre National de la Recherche Scientifique et du Collège de France, Nogent-sur-Marne, France, and Dr P. Symmons, Max-Planck-Institute for Developmental Biology, Tübingen, W. Germany, for advice concerning the haemoglobin isoelectric focusing gels; to Drs K. Schuster-Gossler and K. Bühler of the Kreiskrankenhaus, Albstadt, W. Germany, and the Frauenklinik, Tübingen, W. Germany, respectively, for their generous gifts of human cord serum, to Drs W. Birchmeier and S. Goodman for critically reading and thereby improving the manuscript; to C. Baradoy, J. Bidermann and B. Kafka for technical assistance; and R. Brodbeck for assistance in typing the manuscript. H. E. is supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

ADAMSON, E. D. (1982). The location and synthesis of transferrin in mouse embryos and teratocarcinoma cells. *Devl Biol.* **91,** 227–234.

AXELROD, H. R. (1984). Embryonic stem cell lines derived from blastocysts by a simple technique. *Devl Biol.* **101,** 225–228.

BOLLER, K., & KEMLER, R. (1983). *In vitro* differentiation of embryonal carcinoma cells characterized by monoclonal antibodies against embryonic cell markers. In *Teratocarcinoma Stem Cells* (eds. L. M. Silver, G. R. Martin, and S. Strickland), Vol. **10,** pp. 39–49, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

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

BRÛLET, P., BABINET, C., KEMLER, R. & JACOB, F. (1980). Monoclonal antibodies against trophectoderm-specific markers during mouse blastocyst formation. *Proc. natn. Acad. Sci., U.S.A.* **77,** 4113–4117.

CRAIG, M. L. & RUSSELL, E. S. (1964). A developmental change in hemoglobins correlated with an embryonic red cell population in the mouse. *Devl Biol.* **10,** 191–201.

CUDENNEC, C. A., DELOUVEE, A. & THIERY, J. -P. (1979). Embryonic hemoglobin production by teratocarcinoma-derived cells in *in vitro* cultures. In *Cell Lineage, Stem Cells and Cell Determination* (ed. N. Le Douarin), pp. 163–172, Amsterdam: Elsevier/North-Holland Biomedical Press.

CUDENNEC, C. A. & NICOLAS, J. -F. (1977). Blood formation in a clonal cell line of mouse teratocarcinoma. *J. Embryol. exp. Morph.* **38,** 203–210.

CUDENNEC, C. A., THIERY, J. -P. & LE DOUARIN, N. M. (1981). *In vitro* induction of adult erythropoiesis in early mouse yolk sac. *Proc. natn. Acad. Sci., U.S.A.* **78,** 2412–2416.

DARMON, M., BOTTENSTEIN, J. & SATO G. (1981). Neural differentiation following culture of embryonal carcinoma cells in a serum-free defined medium. *Devl Biol.* **85,** 463–473.

DIWAN, S. B. & STEVENS, L. C. (1976). Development of teratomas from the ectoderm of mouse egg cylinders. *J. natn. Canc. Inst.* **57,** 937–939.

DZIADEK, M. & ADAMSON, E. (1978). Localization and synthesis of alphafoetoprotein in post-implantation mouse embryos. *J. Embryol. exp. Morph.* **43,** 289–313.

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

GROPP, A. (1982). Value of an animal model for trisomy. *Virchows Arch. (Pathol. Anat.)* **395,** 117–131.

HOGAN, B. L.M., BARLOW, D. P. & TILLY, R. (1983). F9 teratocarcinoma cells as a model for the differentiation of parietal and visceral endoderm in the mouse embryo. *Cancer Surveys* **2,** 115–140.

HOGAN, B. L. M. & TILLY R. (1981). Cell interactions and endoderm differentiation in cultured mouse embryos. *J. Embryol. exp. Morph.* **62,** 379–394.

HSU, Y. -C. (1972). Differentiation *in vitro* of mouse embryos beyond the implantation stage. *Nature* **239,** 200–202.

HSU, Y. -C. (1979). *In vitro* development of individually cultured whole mouse embryos from blastocyst to early somite stage. *Devl Biol.* **68,** 453–461.

JANZEN, R. G., ANDREWS, G. K. & TAMAOKI, T. (1982). Synthesis of secretory proteins in developing mouse yolk sac. *Devl Biol.* **90,** 18–23.

JONES-VILLENEUVE, E. M. V., MCBURNEY, M. W., ROGERS, K. A. & KALNINS, V. I. (1982). Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell Biol.* **94,** 253–262.

KAUFMAN, M. H., ROBERTSON, E. J., HANDYSIDE, A. H. & EVANS M. J. (1983). Establishment of pluripotential cell lines from haploid mouse embryos. *J. Embryol. exp. Morph.* **73,** 249–261.

KEMLER, R. (1981). Analysis of mouse embryonic cell differentiation. *Fortschr. Zool.* **26,** 175–181.

KEMLER, R., BRÛLET, P., SCHNEBELEN, M. -T., GAILLARD, J. & JACOB, F. (1981). Reactivity of monoclonal antibodies against intermediate filament proteins during embryonic development. *J. Embryol. exp. Morph.* **64,** 45–60.

KESSLER, S. W. (1975). Rapid isolation of antigens from cells with a staphylococcal protein A-antibody absorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115,** 1617–1624.

LABASTIE, M. -C., THIERY, J. -P. & LE DOUARIN, N. M. (1984). Mouse yolk sac and intra-embryonic tissues produce factors able to elicit differentiation of erythroid burst-forming units and colony-forming units, respectively. *Proc. natn. Acad. Sci., U.S.A.* **81,** 1453–1456.

MAGNUSON, T., EPSTEIN, C. J. SILVER, L. M. & MARTIN G. R. (1982). Pluripotent embryonic stem cell lines can be derived from t^{w5}/t^{w5} blastocysts. *Nature* **298,** 750–753.

MANASEK, F. J. (1973). Some comparative aspects of cardiac and skeletal myogenesis. In *Developmental Regulation, Aspects of Cell Differentiation* (ed. S. J. Coward), pp. 193–218, New York: Academic Press.

MARTIN, G. R. (1980). Teratocarcinomas and mammalian embryogenesis. *Science* **209,** 768–776.

MARTIN, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. natn. Acad. Sci., U.S.A.* **78,** 7634–7638.

MARTIN, G. R. & EVANS, M. J. (1975*a*). Multiple differentiation of clonal teratocarcinoma stem cells following embryoid body formation *in vitro*. *Cell* **6,** 467–474.

MARTIN, G. R. & EVANS, M. J. (1975*b*). Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies *in vitro*. *Proc. natn. Acad. Sci., U.S.A.* **72,** 1441–1445.

MARTIN, G. R., WILEY, L. M. & DAMJANOV, I. (1977). The development of cystic embryoid bodies *in vitro* from clonal teratocarcinoma stem cells. *Devl Biol.* **61,** 230–244.

MCBURNEY, M. W., JONES-VILLENEUVE, E. M. V., EDWARDS, M. K. S. & ANDERSON, P. J. (1982). Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature* **299,** 165–167.

MEEHAN, R. R., BARLOW, D. P., HILL, R. E., HOGAN, B. L. M. & HASTIE, N. D. (1984). Pattern of serum protein gene expression in mouse visceral yolk sac and fetal liver. *EMBO J.* **3,** 1881–1885.

PAULIN, D., JAKOB, H., JACOB, F., WEBER, K. & OSBORN, M. (1982). *In vitro* differentiation of mouse teratocarcinoma cells monitored by intermediate filament expression. *Differentiation* **22.** 90–99.

PFEIFFER, S. E., JAKOB, H., MIKOSHIBA, K., DUBOIS, P., GUENET, J. L., NICOLAS, J. -F., GAILLARD, J., CHEVANCE, G. & JACOB, F. (1981). Differentiation of a teratocarcinoma line: Preferential development of cholinergic neurons. *J. Cell. Biol.* **88,** 57–66.

RIPOCHE, M. -A. & CUDENNEC, C. A. (1983). Adult hemoglobins are synthesized in yolk sac microenvironment obtained from murine cultured blastocysts. *Cell Differentiation* **13,** 125–131.

RIZZINO, A. (1983). Two multipotent embryonal carcinoma cell lines irreversibly differentiate in defined media. *Devl Biol.* **95,** 126–136.

ROBERTSON, E. J., EVANS, M. J. & KAUFMAN, M. H. (1983). X-chromosome instability in pluripotential stem cell lines derived from parthenogenetic embryos. *J. Embryol. exp Morph.* **74,** 297–309.

ROSENTHAL, M. D., WISHNOW, R. M. & SATO, G. H. (1970). *In vitro* growth and differentiation of clonal populations of multipotential mouse cells derived from a transplantable testicular teratocarcinoma. *J. natn. Canc. Inst.* **44,** 1001–1014.

SEABRIGHT, M. (1971). A rapid banding technique for human chromosomes. *Lancet* **ii,** 971–972.

SHERMAN, M. I. & MILLER, R. A. (1978). F9 embryonal carcinoma cells can differentiate into endoderm-like cells. *Devl Biol.* **63,** 27–34.

SHI, W. -K. & HEATH, J. K. (1984). Apolipoprotein expression by murine visceral yolk sac endoderm. *J. Embryol. exp. Morph.* **81,** 143–152.

SOLTER, D & DAMJANOV, I (1979). Teratocarcinoma and the expression of oncodevelopmental genes. *Methods Cancer Res.* **18,** 277–332.

STAMATOYANNOPOULOS, G., NAKAMOTO, B., KURACHI, S. & PAPAYANNOPOULOU, T. (1983). Direct evidence for interaction between human erythroid progenitor cells and a hemoglobin switching activity present in fetal sheep serum. *Proc. natn. Acad. Sci., U.S.A.* **80,** 5650–5654.

SWEENEY, L. J., CLARK JR., W. A., UMEDA, P. K., ZAK, R. & MANASEK, F. J. (1984). Immunofluorescence analysis of the primordial myosin detectable in embryonic striated muscle. *Proc. natn. Acad. Sci., U.S.A.* **81,** 797–800.

TOYOTA, N. & SHIMADA, Y. (1981). Differentiation of troponin in cardiac and skeletal muscles in chicken embryos as studied by immunofluorescence microscopy. *J. Cell Biol.* **91,** 497–504.

TRIMAN, K. L., DAVISSON, M. T. & RODERICK, T. H. (1975). A method for preparing chromosomes from peripheral blood in the mouse. *Cyt. Cell Genet.* **15,** 166–176.

VESTWEBER, D. & KEMLER, R. (1984). Rabbit antiserum against a purified surface glycoprotein decompacts mouse preimplantation embryos and reacts with specific adult tissues. *Expl Cell Res.* **152,** 169–178.

WOBUS, A. M. HOLZHAUSEN, H., JÄKEL, P. & SCHÖNEICH, J. (1984). Characterization of a pluripotent stem cell line derived from a mouse embryo. *Expl Cell Res.* **152,** 212–219.

(Accepted 11 January 1985)