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Use of BRL-conditioned medium in combination with feeder layers to isolate a diploid embryonal stem cell line

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Summary. The derivation of a karyotypically normal embryonal stem (ES) cell line, E14, from inner cell masses (ICMs) isolated by immunosurgery from 129/Ola late mouse blastocysts is described. Disaggregated ICMs were cultured on mitotically-arrested fibroblast feeder layers in droplets of medium conditioned with Buffalo rat liver (BRL) cells under oil. BRL-conditioned medium inhibits the differentiation of established embryonal carcinoma (EC) and ES cell lines which can be maintained indefinitely in the complete absence of feeder cells (Smith and Hooper 1987). At clonal densities, however, a combination of BRL-conditioned medium and a feeder layer was most effective in preventing the differentiation of E14 cells. This effect was less pronounced at higher passage suggesting it may be particularly important to use a combination in the early stages of isolation. Once established, E14 has been maintained in BRL-conditioned medium alone. In non-conditioned medium on agarose, E14 cells formed embryoid bodies which when allowed to reattach differentiated into a wide variety of tissues. An HPRT-deficient subline of E14, E14TG2a, has been demonstrated to form germline chimaeras with high efficiency after injection into blastocysts (Hooper et al. 1987). The modifications to the ES cell isolation procedure described here may improve the efficiency with which karyotypically normal lines can be derived.

Key words: Embryonal stem cells – Conditioned medium – Feeder layer

Introduction

The derivation of embryonal stem (ES) cell lines from preimplantation mouse embryos in vitro is now well established (Bradley 1987). ES cells, like embryonal carcinoma (EC) cells derived from teratocarcinoma tumours, are pluripotent and differentiate into a wide variety of tissues either in vitro under certain conditions or in vivo after subcutaneous injection into syngeneic adult mice. However, unlike EC cells, ES cells generally retain a normal karyotype and following injection of cells into host blastocysts, several lines have been shown to make substantial contributions to the developing embryo and to the germline of chimaeric mice (Bradley et al. 1985).

ES cells are thought to be derived from a subpopulation of cells, the embryonic ectoderm or epiblast, in the inner cell mass (ICM) of the late blastocyst which gives rise to all of the tissues of the fetus and some components of the extraembryonic membranes in later development (Gardner and Rossant 1979). The remainder of the ICM, the primitive endoderm, only gives rise to layers of the yolk sac and Reicherts membrane. Two different approaches were used originally to isolate ES cell lines from blastocysts in vitro. Firstly, delayed blastocysts, i.e. blastocysts prevented from implanting in the uterus by ovariectomy, were allowed to attach and outgrow in culture to expose the ICM which was then disaggregated and cells cultured on mitotically-arrested fibroblast feeder layers (Evans and Kaufman 1981). Secondly, Martin (1981) isolated ICMs from blastocysts by immunosurgery and established cultures again on similar feeder layers but in the presence of medium conditioned by an EC cell line. However, subsequent attempts to isolate ES cell lines, by these and other groups, have demonstrated that neither delaying implantation nor the use of EC conditioned medium is essential. Although further minor modifications involving the use of small medium volumes in multiwell dishes (Axelrod and Lader 1983; Axelrod 1984) and primary fibroblasts for feeder layers (Doetschman et al. 1985) have been described, the efficiency with which ES cell lines can be derived is still variable.

Two factors influencing the initial isolation and maintenance of ES cells appear to be the disaggregation of the ICM to prevent further development and culture on fibroblast feeder layers which is known to facilitate the derivation of pluripotent EC cell lines from disaggregated teratocarcinomas (Solter and Damjanov 1979). Medium conditioned with STO mouse fibroblasts (Martin and Evans 1975) or primary fibroblasts has been shown to inhibit the differentiation of EC cells but not efficiently enough to allow these cell lines to be maintained in the absence of a feeder layer (Smith and Hooper 1983; Koopman and Cotton 1984). However, recently, medium conditioned with Buffalo rat liver (BRL; Coon 1968) cells has been shown to be more effective and EC and ES cell lines can be maintained indefinitely in the absence of any feeder cells (Smith and Hooper 1987).

We report here that a combination of fibroblast feeder layers and BRL-conditioned medium was most effective in preventing the differentiation of ES cells at early passages

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and describe the successful derivation of a karyotypically normal ES cell line, E14, from six ICMs isolated from late blastocysts by immunosurgery. An HPRT-deficient subline of E14, E14TG2a, selected using the toxic purine analogue 6-thioguanine, has been demonstrated to form germline chimaeras with high efficiency after blastocyst injection. In this way a mouse strain carrying this deficiency, which in Man causes a severe and eventually fatal neurological disease, Lesch-Nyhan syndrome, has been established (Hooper et al. 1987).

Materials and methods

Cells and cell culture

BRL, a clonal cell line isolated from the liver of a 5-week-old Buffalo rat (Coon 1968), and STO, a thioguanine- and ouabain-resistant derivative of a fibroblast line isolated from a mouse of strain SIM (Martin and Evans 1975) were grown in medium CMβ (Smith and Hooper 1987). BRL-conditioned CMβ was prepared as previously described (Smith and Hooper 1987), and feeder layers prepared from STO cells as described by Martin and Evans (1975). To establish feeder layers in droplets of medium under oil, the latter method was adapted as follows: Sub-confluent cultures of STO fibroblasts were incubated with 10 μg/ml mitomycin C (Sigma) for 2–3 h, washed, trypsinised and resuspended at a concentration of 3×10^5 cells/ml in non-conditioned or 60% (v/v) BRL-conditioned CMβ + 20% (v/v) foetal calf serum (FCS) ("BRL-conditioned medium"). Rows of 10 μl droplets of 1% (w/v) gelatin (swine skin; Sigma) in PBS were pipetted on to 60 mm Falcon petri dishes, covered with 5 ml of light liquid paraffin (Boots) and stored for 10 min–1 h at 4° C. The gelatin was then removed using a micropipette and replaced with 20 μl of the mitomycin-treated STO cells. The cells were incubated overnight to allow them to attach, checked for confluency and used within a week of preparation. ES cell cultures were established on STO feeder layers in BRL-conditioned medium. For feeder-free culture, the surfaces of culture vessels were precoated with gelatin (Bernstine et al. 1973). Cells were monitored periodically for mycoplasma contamination by the method of Chen (1977). None was detectable during this work.

Induction of differentiation in vitro

Cells previously grown in conditioned medium in the absence of feeders were induced to form suspension aggregates in non-conditioned medium as previously described (Smith and Hooper 1987), except that 10^{-4} M β-mercaptoethanol was present throughout, and suspension culture was carried out in tissue culture dishes coated with agarose rather than in bacteriological dishes. Two layers of agarose of different concentrations were used, as this gave good mechanical stability without appreciable toxicity to the cells. 2% (w/v) agarose (Miles Labs Ltd, Stoke Poges, England) in PBS without calcium and magnesium (Flow Labs, Irvine, England) was melted by autoclaving. 10 ml was added per 100 mm dish, and once the surface was completely covered 7 ml were removed and the residual thin layer allowed to set at room temperature. A second layer was then applied by repeating this process using 1% (w/v) agarose in PBS without calcium and magnesium. Once the upper layer had set, 10 ml of EfC_{10} medium (Smith and Hooper 1987) was added to each dish, and dishes were then incubated overnight to allow equilibration. Before use the supernatant medium was aspirated and replaced with fresh medium. Agarose layers thus produced were usually stable for at least 1 week.

Embryoid body outgrowths were formed by allowing embryoid bodies, after 6 days' suspension culture, to attach to Thermanox coverslips as previously described (Smith and Hooper 1987).

Embryo collection

Female mice, superovulated where indicated in the Results section by intraperitoneal injection of 7.5 IU of pregnant mares serum gonadotrophin followed 48 h later by 5 IU of human chorionic gonadotrophin (Intervet), were paired with males of the same strain, and examined the following day for copulation plugs (Day 1 of pregnancy). Embryos were flushed from the uteri on Day 4 in HEPES-buffered medium M2 (Quinn et al. 1982) supplemented with 4 mg/ml bovine serum albumin (BSA) and cultured overnight in medium M16 (Whittingham 1971) supplemented with 4 mg/ml BSA at 37° C in an atmosphere of 5% CO_2 in air.

Immunosurgery

Following overnight incubation, the zonae pellucidae were removed from expanded blastocysts by incubating in pronase (Handyside and Barton 1977) and inner cell masses (ICMs) isolated by immunosurgery. The zona-free blastocysts were incubated, firstly, in rabbit anti-mouse spleen cell antiserum (diluted 1:10 in M2 + BSA; Handyside and Hunter 1986) for 10 min at room temperature, washed in M2 + BSA, and secondly, in guinea pig complement (diluted 1:10 in M2 + BSA, Gibco) for 30 min at 37° C. The treated blastocysts were washed in M2 + BSA and ICMs freed of lysed trophectoderm using a finely pulled pasteur pipette.

Disaggregation of inner cell masses

ICMs, either freshly isolated or following overnight culture as specified in the Results section, were washed in Dulbecco's phosphate buffered saline A (PBSA) incubated in 0.25% (w/v) trypsin, 1 mM Na_2EDTA, 1% (v/v) chick serum in PBSA for 10–40 min at 37° C until decompaction was evident, and disaggregated in CMβ + 20% FCS using a micropipette (flame-polished, hand-pulled capillary tubing). Single cells or small groups of cells were then transferred to droplets with feeder cells containing BRL-conditioned or non-conditioned medium as specified. Droplets were examined daily for growing colonies of ES-like cells on a Leitz Diavert inverted phase contrast microscope and passaged initially into fresh feeder droplets by washing and trypsinising *in situ* and then removing the colonies by micropipette for dissociation in serum supplemented medium as above.

Karyotype analysis

Metaphase spreads were prepared as described by Robertson (1987) except that the cultures were not exposed to colcemid. After 10–14 days the chromosome preparations were incubated in 2 × SSC (0.3 M NaCl, 0.03 M trisodium

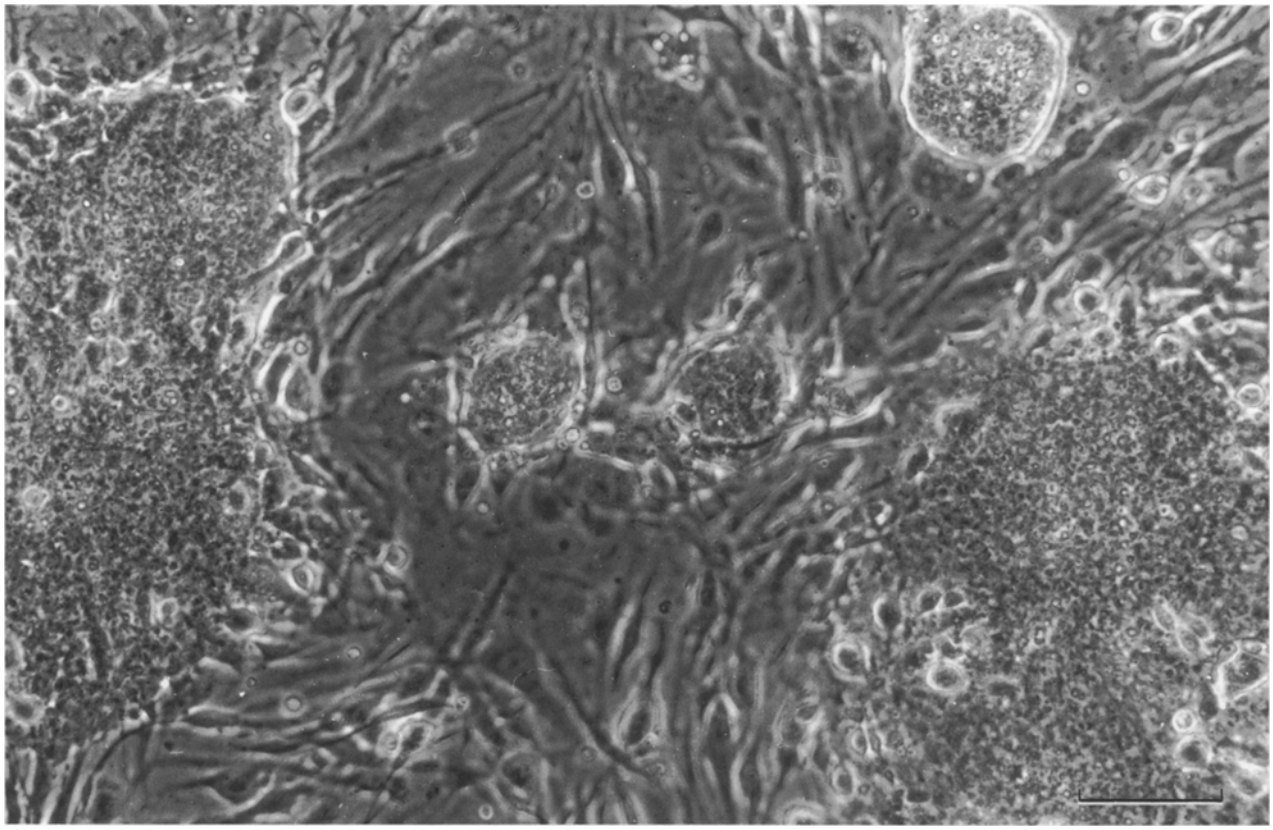


Fig. 1. ES-like colonies derived from single ICM cells (ICMs isolated by immunosurgery from MF1 × MF1 blastocysts) 4 days after seeding onto a feeder layer in CMβ + 20% FCS. Three of the colonies have retained the typical rounded morphology, while others are more flattened and spread. Scale bar = 100 μm

citrate) at 60° C for 2 h. At the end of this period, they were washed twice in distilled water and then exposed to 0.25 mg/ml trypsin (Difco) in 0.15 M NaCl for either 20 or 30 s at 25° C. The slides were washed in 0.15 M NaCl and stained in 3% (v/v) Giemsa stain in 5 mM phosphate buffer, pH 6.8 for 1.5 h. G-banded metaphase spreads were karyotyped according to the Standardized Genetic Nomenclature for mice as proposed by Nesbitt and Franke (1973).

Results

Development of ICMs and ICM cells in vitro

Initial observations were carried out on embryos of the outbred MF1 strain recovered following superovulation. Intact ICMs isolated by immunosurgery from late blastocysts and cultured in droplets under oil increased in volume and, over the first 18–24 h, generated an outer layer of primitive endoderm surrounding a core of epiblast cells. After 2–4 days these embryoid bodies cavitated and formed egg cylinder-like structures. This was unaffected by the presence of fibroblast feeder layers, BRL-conditioned medium or a combination of the two, which inhibit the development of embryoid bodies by EC and ES cells. Only a small proportion of single cells, from ICMs dissociated immediately following immunosurgery, were viable. However, over a period of 2–4 days, some of these cells developed into colonies of either ES-like or, in a few cases, endoderm-like morphology (Fig. 1). Partially disaggregated ICMs, however, tended to regenerate small double-layered structures which rapidly differentiated into embryoid bodies with cavities. Since ICMs are difficult to dissociate into single cells, often the majority of ICM cells differentiated in this way. During the first 1–2 weeks in culture, the majority of ES-like colonies underwent a process of differentiation involving initially attachment to the substratum underneath the feeder layer, spreading and a slowing down of cell division. These colonies rapidy stopped growing and viability after trypsinisation was very low.

Derivation of the embryonal stem cell line, E14

The preliminary observations described above were used to optimise the procedure employed for the isolation of the E14 cell line from embryos of the inbred strain 129/Ola (Olac 1976 Ltd) recovered following natural matings without superovulation. Because ICMs differentiated if cultured intact for more than 2 days but survival was poor following trypsinisation immediately after immunosurgery, a period of 24 h was allowed for recovery from immunosurgery before trypsinisation. Three intact ICMs were plated in a droplet of non-conditioned medium with feeder cells and three in a droplet of BRL-conditioned medium with feeder cells. The following day these ICMs were removed by micropipette and disaggregated, and cells transferred to fresh feeder droplets in the same medium.

Three days following dissociation, six colonies (three with only a small number of cells) of ES-like morphology were present. These colonies were trypsinised and the cells pooled and split between fresh feeder droplets with non-

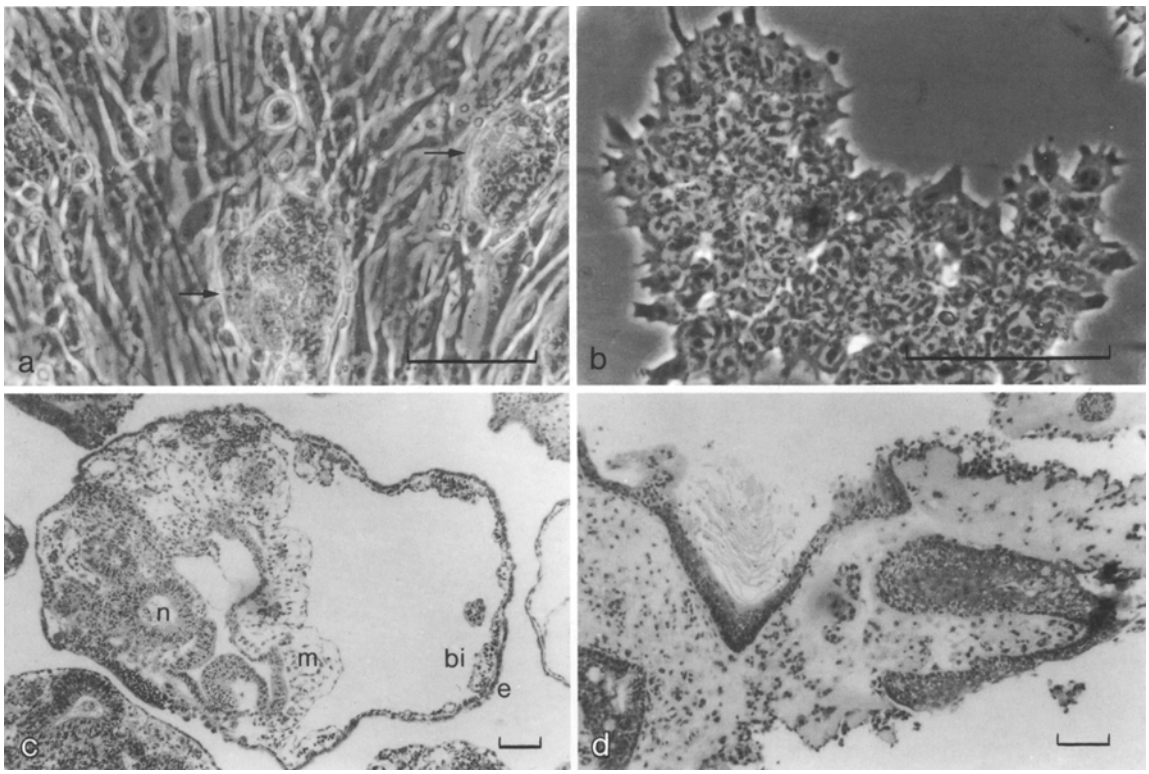


Fig. 2a–d. Morphology of undifferentiated and differentiated cultures of E14. **a** Colonies with ES morphology (*arrows*) on STO feeder layers in BRL-conditioned medium after three serial passages of inner cell mass-derived culture. **b** Undifferentiated culture following adaptation to feeder-free culture in BRL-conditioned medium. **c** Section of embryoid body after 6 days' suspension culture in non-conditioned medium. Haematoxylin and eosin stain. *bi*, blood island; *e*, endoderm; *m*, mesoderm; *n*, neuroepithelium. **d** Section of embryoid body outgrowth 4 weeks after plating on Thermanox coverslips in non-conditioned medium. Haematoxylin and eosin stain. *k*, keratinising squamous epithelium. In a to d, scale bar = 100 μm

Table 1. Colony formation and differentiation in the established E14 cell line

Feeder cells	Conc. of BRL-conditioned medium (% v/v)	Conc. of FCS (% v/v)	Colony-forming efficiency (%)	Undifferentiated colonies (%)
−	60	10	28	50
−	80	10	30	89
+	0	10	40	77
+	0	20	39	85
+	60	10	37	92
+	60	20	38	97

10^3 cells were seeded into each of duplicate 60-mm dishes in the specified medium. Medium was renewed after 4 days, and colonies stained at 1 week with Leishman's stain. Only colonies consisting solely of cells with ES morphology are scored as undifferentiated

conditioned or BRL-conditioned medium. After 24 h, ES-like colonies were present in both drops and the medium was replaced. After 48 h, the ES-like colonies in the droplet with BRL-conditioned medium were growing rapidly and were, therefore, trypsinised and moved to fresh feeder droplets with BRL-conditioned medium. The colonies in the other droplet with non-conditioned medium were passaged in the same way the following day but maintained in non-conditioned medium; the resulting colonies appeared to differentiate over the next two days. The ES-like colonies in the droplets with BRL-conditioned medium (Fig. 2a) grew progressively and were further subcultured into feeder droplets with BRL-conditioned medium. At the fifth passage, a droplet containing a single ES-like colony was trypsinised and the resulting cells used to inoculate a further droplet. Many colonies of ES cell morphology ensued and the culture was expanded onto progressively larger areas of feeder cells with each successive trypsinisation until a confluent 25 cm^2 flask was obtained at the tenth passage. The cells from this culture, designated E14, were used to inoculate a 175 cm^2 flask containing a feeder layer for the preparation of a frozen stock, and a 25 cm^2 gelatin-treated flask without feeder cells. The E14 cells adapted readily to feeder-free culture in BRL-conditioned medium (Fig. 2b).

E14 cells were tested at the thirteenth passage for their ability to form colonies under various conditions (Table 1). Under the standard conditions used for growth of EC cells in conditioned medium alone (60% (v/v) BRL-conditioned CMβ, 10% (v/v) FCS; Smith and Hooper 1987), the efficiency of colony formation was acceptably high but only half the colonies consisted solely of cells with the morphology of ES cells, the remainder being a mixture of undifferentiated and differentiated cells. Less differentiation was observed when the cells were plated on feeder layers in non-conditioned medium, but the lowest degree of differentiation was observed when both feeder layers and conditioned medium were used. Increasing the serum concentration or the concentration of conditioned medium reduced the level of differentiation. The experiment was repeated at the twen-

tieth passage: qualitatively similar results were obtained but levels of differentiation were lower than in the corresponding culture at the thirteenth passage.

When cultures of E14 cells were induced to differentiate by plating in non-conditioned medium as previously described (Smith and Hooper 1987) considerable cell death occurred and poor yields of embryoid bodies were obtained. This problem could be overcome without any apparent effect on differentiation by adding 10^{-4} M β-mercaptoethanol to the medium throughout the differentiation process. This necessitated modification of the second stage of the procedure which entails culture of cell aggregates (embryoid bodies) in suspension in bacteriological petri dishes, since β-mercaptoethanol promoted attachment of the embryoid bodies to the surface of the bacteriological dish. This could be avoided by the use of tissue-culture dishes coated with agarose in place of bacteriological dishes. Bacteriological dishes were difficult to coat with a thin layer of agarose because of their more hydrophobic surface. The detailed protocol used is described in Materials and methods. After 6 days' suspension culture, E14 aggregates showed extensive differentiation with the development of large cavities within the aggregates, the organisation of the cores of the aggregates into epithelia often resembling neuroepithelium, the formation of endoderm and mesoderm and the development of blood islands (Fig. 2c). When these aggregates were allowed to attach to a substratum extensive further differentiation occurred with the formation of a variety of cell types including muscle, cartilage and keratinising epithelium (Fig. 2d).

Metaphase spreads were prepared from an E14 culture at the twelfth passage. Chromosome counts showed a modal number of 40, with 16 out of 20 spreads having the modal number of chromosomes. In spreads with the modal chromosome number, no abnormalities in G-banding pattern were detectable (Fig. 3a, b). At the twenty-fifth passage the modal chromosome number remained at 40 but the proportion of spreads with the modal number had declined to 13 out of 20.

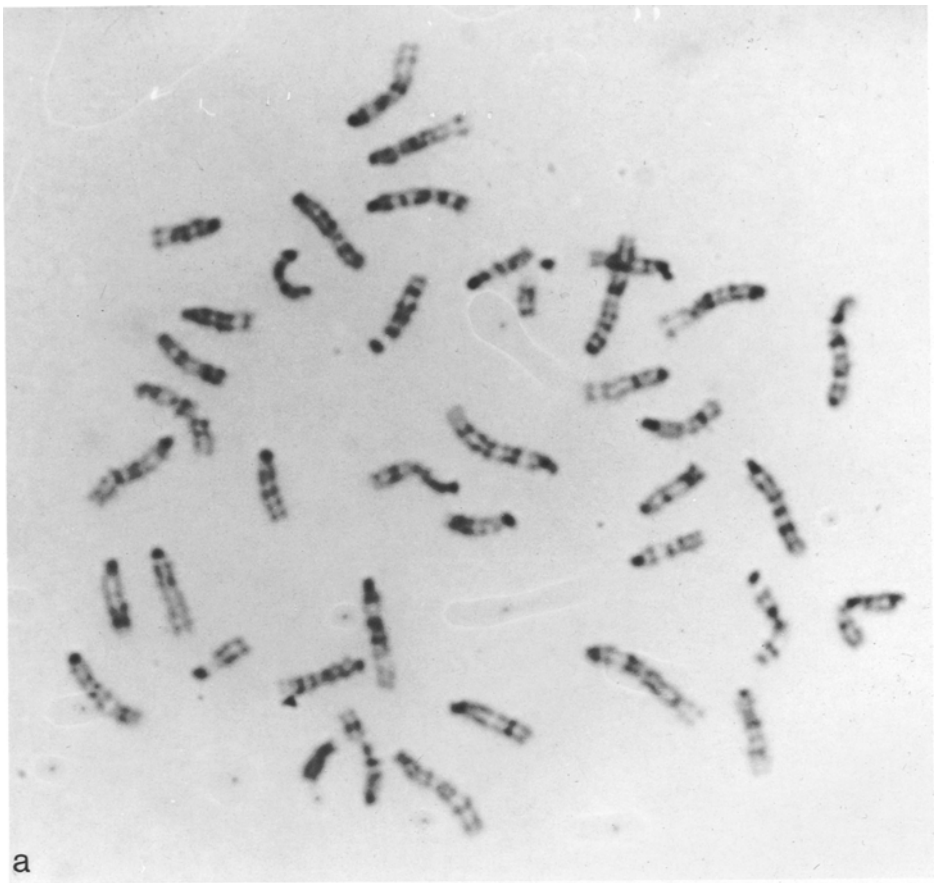
The isolation from E14 of the HPRT$^-$ derivative E14TG2a has previously been described (Hooper et al. 1987). Figure 3 (c, d) shows that no cytologically detectable chromosome abnormality is present in E14TG2a. However, Southern blot analysis of DNA from E14TG2a reveals a deletion extending from at least 10 kb upstream of the promoter of the hprt gene into its second intron (S. Thompson et al. 1989; see also Melton 1987). When induced to differentiate by formation of suspension aggregates and subsequent outgrowth as described above for E14, these HPRT$^-$ cells behaved indistinguishably from their wild-type parent cells.

Discussion

The approach to ES cell line derivation described here using droplets under oil was designed to minimise the loss of disaggregated ICM cells and facilitate observation of their behaviour at early stages in vitro. In preliminary experiments, the ability to examine closely the fate of disaggregated ICM cells demonstrated clearly two important factors in the initial isolation of ES cells. Firstly, failure to disaggregate ICMs completely to single cells, either immediately following immunosurgery or after overnight culture, led rapidly with the majority of cell aggregates to the regeneration of small embryoid bodies with an outer layer of primitive endoderm even in the presence of a feeder layer and/or conditioned medium. Martin (1981) derived ES cell lines directly from ICMs in medium conditioned by teratocarcinoma stem cells. However, the behaviour of these ICMs before trypsinisation was not described. Secondly, having established isolated ES-like colonies at early stages after ICM disaggregation, failure to prevent differentiation led to cell flattening and attachment to the substratum underlying the feeder layer rather than to the formation of embryoid bodies as occurs with EC or ES cells in suspension. Once this process occurred, viability after trypsinisation was low and the cultures rapidly died out.

Single ICM cells gave rise only to colonies of ES- or endoderm-like morphology (Fig. 1) over a period of two to four days although the colony forming efficiency was low. This suggests that there are committed subpopulations of epiblast and primitive endoderm cells already in the ICM of late blastocysts, and is consistent with the analysis of the fate of individual ICM cells injected into blastocysts (Gardner and Rossant 1979). Thus it seems likely that in partially disaggregated ICMs combinations of these two cell types reassociate and cell interactions, possibly involving growth factors (Heath and Rees 1985), result in embryoid body formation. Although not attempted during the derivation of E14, overnight culture of ICMs before disaggregation allows the separation of the external layer of primitive endoderm (which is more sensitive to trypsinisation) from the cores of epiblast cells. These epiblast cores can then be disaggregated separately. On Day 5 between 102–114 h post coitum, there were an average of 21–26 ICM cells (Handyside and Hunter 1986). Since only a proportion are epiblast cells which are the totipotent cells that give rise to the tissues of the fetus (Gardner and Rossant 1979), this extra effort may be justified for the successful derivation of ES cell lines from small numbers of embryos.

We also incorporated two modifications not previously applied to the isolation of ES cell lines, viz. the use of a trypsinising agent incorporating chicken serum and the use of BRL-conditioned medium in combination with feeder cells. It seems likely that both modifications contributed to the successful isolation of the diploid lines E14 and E14TG2a. Chicken serum contains no trypsin-inhibiting activity and its inclusion in solutions used for cell disaggregation improves cell viability (Coon 1966; Bernstine et al. 1973). The disaggregation of ICMs in order to free embryonal stem cells from endodermal derivatives requires prolonged incubation in the trypsinising agent and therefore protection against non-specific toxicity is of paramount importance. The rationale behind our use of conditioned medium was to provide better control of the level of differentiation-inhibiting activity in the cultures. With feeder layers alone this is expected to be low immediately following a medium change, while in conditioned medium alone degradation of the active molecule may result in lower activity prior to medium renewal. If both feeders and conditioned medium are used these two effects will tend to cancel, resulting in less variability with time. Table 1 shows that even once the cell line has been established, better inhibition of differentiation is indeed achieved in the presence of both feeders and conditioned medium than with either alone. This effect was less pronounced at higher passages, suggesting that it may be particularly important in the initial pas-



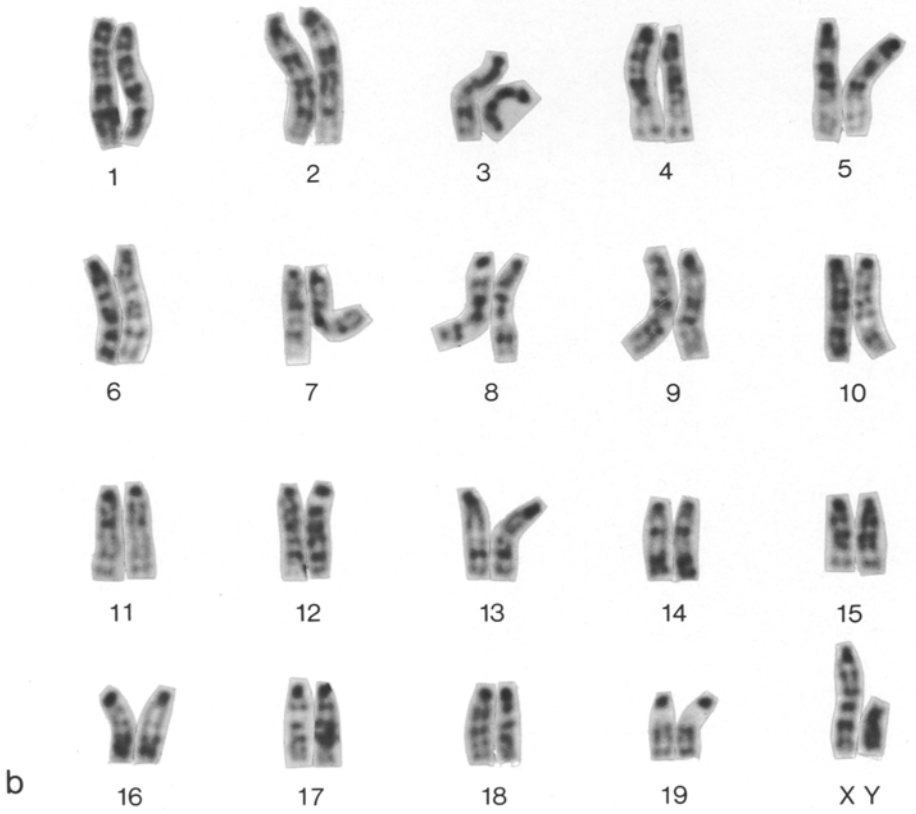


Fig. 3a–d. G-banded metaphase spreads and karyograms of E14 and E14TG2a. **a** Metaphase spread and **b** karyogram of E14 cells at the 16th passage; **c** metaphase spread and **d** karyogram of E14TG2a at the 11th passage after isolation from E14, i.e. at the 23rd passage in total after inner cell mass plating

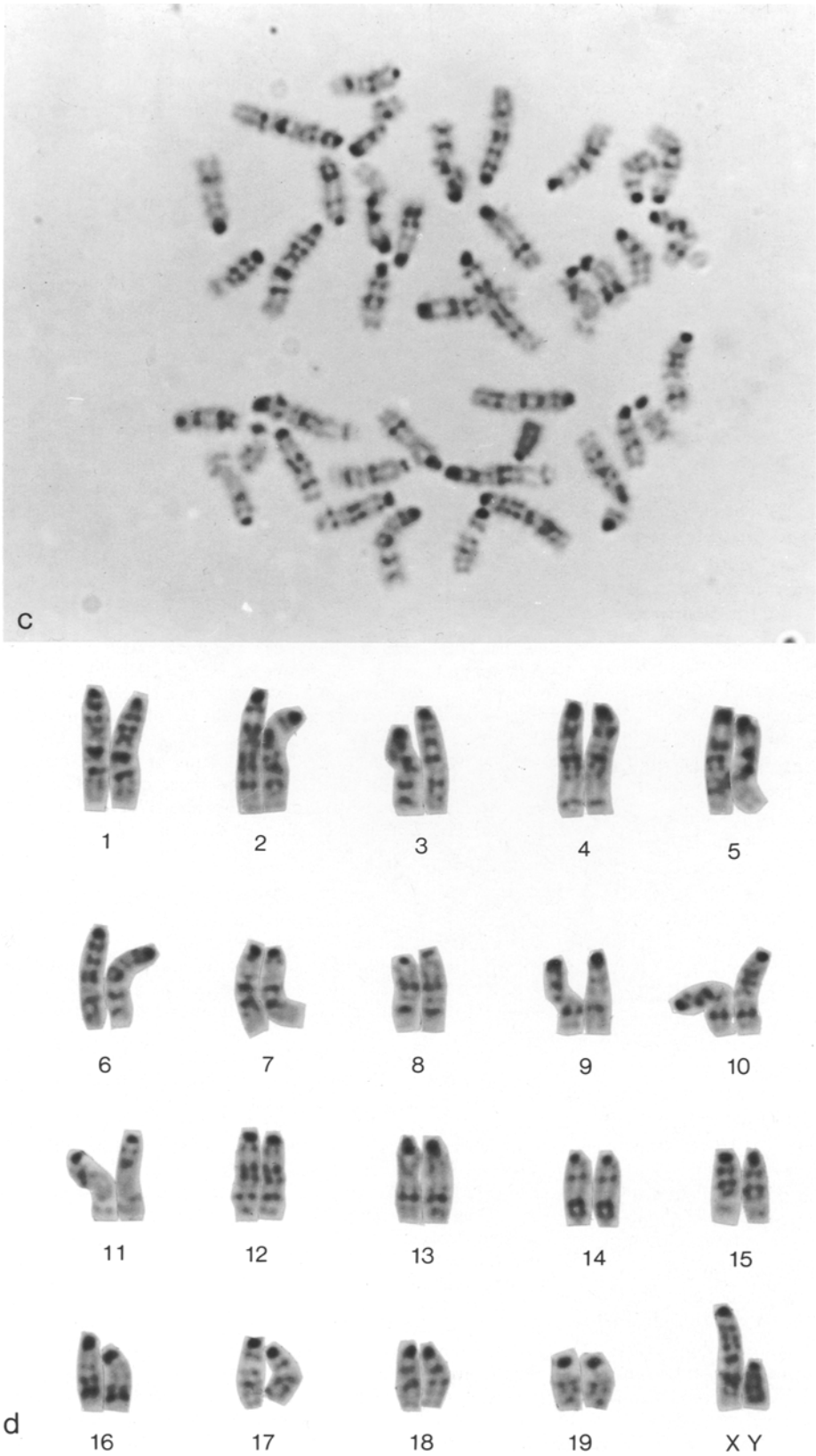


Fig. 3c, d: legend is on p. 53

sages leading to the establishment of a cell line. However, to prove that these conditions increase the efficiency with which diploid ES cells can be isolated would require the characterisation of a significant number of lines by karyotyping, testing of differentiation capacity, etc.; for the presence of cell colonies with ES-like morphology (as occurred in several other cultures) is not, in itself, a reliable criterion.

The achievement of optimal conditions for inhibition of differentiation during the establishment and maintenance of ES cell cultures is important not only to obtain a high yield of cell lines but also to avoid subjecting the cultures to unwanted selective pressure in favour of variants with reduced developmental capacity. Like previously isolated ES cell lines which give rise to germline chimaeras following blastocyst injection (Bradley et al. 1984), E14 and E14TG2a exhibit no detectable deviation from diploidy in their G-banded karyotype, providing additional support for the contention that a normal G-banded karyotype is a necessary condition for an ES cell line to produce germline chimaeras. However, previous experience suggests that while being a necessary condition it is not a sufficient one (Bradley 1987) so that the high frequency of germline chimaerism obtained with E14TG2a (Hooper et al. 1987) probably provides a more stringent test of the success of our isolation procedure in avoiding the effects of unwanted selective pressure. It also demonstrates that once established, ES cell lines can be maintained in the absence of feeder cells without adverse effects on developmental capacity. Whether it is possible to isolate ES cell lines of similar potency without the use of feeder cells at any stage remains to be established: if it is, this will enable us to exclude formally the remote possibility that the transfer of genetic material from the feeder cells has a role in the establishment of ES cell lines.

The extent of differentiation observed when E14 cells were cultured as suspension aggregates in non-conditioned medium and then allowed to form outgrowths on a substratum compares favourably with our previous experience with EC and ES cells. It is likely that two factors contribute to this: firstly, the high developmental potency of the cell line, discussed above; secondly, the fact that the cells had previously been grown in feeder-free culture so that no residual feeder cells remained to cause partial inhibition of differentiation when conditioned medium was removed (cf. Smith and Hooper 1987). Assessment of in vitro developmental capacity would appear to be a useful preliminary screening procedure to identify cell lines most likely to give germline chimaerism following blastocyst injection.

We have also used BRL-conditioned medium in combination with feeder cells in attempts to isolate ES cell lines from sheep. We have been successful in maintaining cells of ES morphology for limited periods in culture, but not in preventing their ultimate differentiation (Handyside et al. 1987). Nevertheless, while isolation of ES cell lines from larger animals may require the solution of additional problems, we conclude that the modifications to the ES cell isolation procedure described here will be of considerable benefit in the isolation of further lines from the mouse.

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