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CULTURE AND DIFFERENTIATION OF EMBRYONIC STEM CELLS

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SUMMARY: Techniques are described for the culture of murine embryonic stem cells in the absence of heterologous feeder cells and for the induction of differentiation programs. The regulatory factor differentiation inhibiting activity/leukaemia inhibitory factor (DIA/LIF) is produced at high concentration by transient expression in Cos cells and is used to suppress stem cell differentiation by addition to the culture medium. Differentiation is then induced in a controlled manner either by withdrawal of DIA/LIF or by exposure to the chemical inducers retinoic acid or 3-methoxybenzamide.

Key words: embryonic stem cells; differentiation; differentiation inhibiting activity; leukaemia inhibitory factor; retinoic acid.

I. INTRODUCTION

Embryonic stem (ES) cells are derived directly from the pluripotential inner cell mass of the preimplantation mouse embryo (4,11). They are permanent cell lines which can be propagated and experimentally manipulated in vitro. The unique feature of ES cells is that they retain the properties of normal early embryo cells and so can be reintroduced into the blastocyst where they participate fully in embryonic development. The ES cells contribute differentiated progeny to all tissues, including the production of functional gametes (1). The latter permits germ-line transmission of the ES cell genotype and thereby provides a system for introducing predetermined modifications, notably homologous recombination events, into experimental animals (9,19,24). The close identity between ES cells and normal pluripotential embryo cells also implies that they can be used directly to characterize developmental events. Such a model embryo system is required because at the stage when crucial determinative events occur the small size and innaccessibility of the mammalian embryo render it intractable to conventional biochemical analysis. In particular, ES cells may be exploited to identify and define regulatory factors that direct developmental decisions underlying the maintenance of stem cells and the establishment of differentiated lineages (6,7,17,18).

Historically, ES cells have been maintained on feeder layers of mitotically inactivated embryonic fibroblasts (12,20). Feeders sustain the propagation of undifferentiated stem cells. However, the use of feeders imposes constraints on the genetic manipulation of the ES cells (9,22) and hinders the biochemical dissection of growth and differentiation processes (6,8,22). The essential function of feeders is production of the regulatory factor differentiation inhibiting activity/leukaemia inhibitory factor (DIA/LIF) (14,17,18). This is a glycoprotein which at subnanomolar concentrations reversibly inhibits differentiation of ES cells in vitro and thereby enables their propagation as relatively homogeneous population of stem cells in the absence of feeders (21,22,25). ES cells maintained with DIA/LIF are equivalent to cells cultured on feeder layers as demonstrated by their continued ability to contribute extensively to chimeras and to colonize the germ-line. Indeed germ-line competent ES cells can be derived directly from preimplantation embryos using DIA/LIF (14). DIA/LIF thus permits the propagation and experimental manipulation of normal pluripotential ES cells under simpler, more defined conditions. In this paper the essential methodology is described for the production of DIA/LIF, the routine culture of ES cells, and the induction of controlled differentiation programs.

II. MATERIALS

A. Equipment

CO_2 incubator, model B 5060 EC/CO2, Heraeus[1]
Laminar flow hood, Envair[2]
Bench top centrifuge, no. 41135-305, MSE[3]
Inverted microscope, Olympus[4]
Stereomicroscope[4]
Filter housing, no. YF55 040 00, Millipore[5]
Dispensing pressure vessel, no. XX67 00P 05[5]
Vortex, no. 330/0055/00, BDH[6]

B. Chemicals

Phosphate buffered saline (Dulbecco A) tablets, no. BR014G, Oxoid[7]

Trypsin 1:250, no. 0152-13-1, Difco[8]
Dulbecco's modified Eagle's medium (DMEM) (powder), no. 10-331, Flow[9]
Ham's F12 medium (powder), no. 10-431[9]
Sodium bicarbonate, no. S5761, Sigma[10]
Insulin (bovine), no. I6634[10]
Transferrin (human), no. 1073 974, Boehringer Mannheim[11]
Sodium selenite, no. S9133[10]
HEPES (1 *M* solution, pH 7.4), no. 16-884-49[9]
Gelatin, no. G9391[10]
EDTA (disodium salt), no. ED2SS[10]
Calcium chloride, no. 10070 3HH[6]
BES, no. B6266[10]
Analar water, no. 10292 3C[6] or Millipore-filtered water (Milli-Q-plus)
2-Mercaptoethanol, no. M7522[10]
All-*trans*-retinoic acid, no. 22,301-8, Aldrich[12]
3-Methoxybenzamide, no. M1,005-0[12]
Dimethyl sulphoxide, no. D/4121/08, FSA[13]
Fetal bovine serum (selected batches) [Serum can be obtained from a variety of suppliers but must be tested for the ability to sustain ES cells as described by Robertson (20)].
Chicken serum, no. 29-501-49[9]
Leishman's stain, no. 34042 4 *M*[6]
Methanol, no. 10158 6B[6]

C. Cell lines and plasmids

Embryonic stem cell lines were provided by Dr. Martin Evans (Genetics, Cambridge, UK), Dr. E. Robertson (Columbia, New York, NY), and Dr. Martin Hooper (Pathology, Edinburgh, UK), or were derived in the author's laboratory (14). The derivation of these lines is described in ref 4 and ES cell line E14Tg2a has been deposited with the American Type Culture Collection, Rockville, MD.

Cos-7 cells were obtained from the Cell Bank, Sir William Dunn School of Pathology, Oxford, UK.

The human, pC10-6R, and murine, pDR10, DIA/LIF expression plasmids have been described elsewhere (13,17) and are available from the authors.

D. Materials

5- and 10-ml Plastic pipette, nos. 7543 and 7551, Falcon[14]
Tissue culture plasticware (various), Nunc[15]
Millidisk cartridge filters (0.2 μm), no. MCGL 10S03[5]
Micropipettes (various), Gilson[16]
Micropipette tips, nos. LL 1030-800 and LL 1040-800, Laser[17]
Syringe filters (Flowpore, 0.2 μm), no. 64-001-04[9]
Hemacytometer, no. 403/0061/01[6]
Bottle-top filters, no. 7111[14]

III. PROCEDURE

A. Preparation of solutions

1. Phosphate buffered saline (PBS), without divalent cations
   a. Dissolve 10 PBS(A) tablets in 1 liter analar water.
   b. Autoclave to sterilize.
   c. Store in aliquots of 100 to 250 ml at 4° C.
2. Trypsin solution
   a. In 1 liter PBS dissolve
      250 mg trypsin 1:250
      372 mg EDTA, disodium
      Add 10 ml chicken serum.
   b. Filter sterilize through 0.2-μm bottle-top filter.
   c. Store in 20-ml aliquots at −20° C.
3. Transfection solutions
   a. Filter sterilize analar H_2O. Store in aliquots of 5 to 20 ml at 4° C.
   b. Adjust concentration of plasmid DNA prepared by standard cesium chloride centrifugation procedures (10) to 1.0 mg/ml in sterile analar H_2O. Store at 4° C.
   c. Dissolve $CaCl_2$ in analar H_2O to 2.5 *M*. Filter sterilize and store in 10-to 50-ml aliquots at 4° C.
   d. To 100 ml analar H_2O add 1.63 g NaCl, 1.06 g BES, and 0.02 g $Na_2HPO_4 \cdot 2H_2O$. Dissolve and adjust to pH 6.95. Filter sterilize and store in 10- to 50-ml aliquots at 4° C.
4. Gelatin
   a. Add 0.1 g gelatin per 100 ml analar H_2O.
   b. Autoclave to sterilize.
   c. Store in aliquots of 100 to 250 ml at 4° C.
5. Culture additives
   a. 10^{-1} *M* 2-mercaptoethanol, add 0.1 ml 2-mercaptoethanol to 14.1 ml sterile PBS. Store up to 4 wk at 4° C.
   b. Sodium selenite, add 19.3 ml sterile analar H_2O to 1 mg sodium selenite in vial. Store indefinitely at 4° C. For working stock (3×10^{-5} *M*), dilute 1:10 with sterile analar H_2O. Store up to 1 yr at 4° C.
   c. 10 mg/ml insulin, add 0.5 ml 0.1 *M* sterile-filtered acetic acid to 50-mg vial insulin using aseptic technique. Mix well to dissolve, then add 4.5 ml sterile PBS. Store at 20° C in 50-1000-μl aliquots.
   d. 10 mg/ml transferrin, reconstitute 100 mg lyophilized powder in vial with 10 ml basal medium. Store up to 1 yr at 4° C.
6. Culture medium
   a. Prepare basal medium according to manufacturer's instructions using analar H_2O. Make up 50:50 mix of DMEM:Hams F12. Filter sterilize and store for 2 to 4 wk at 4° C in 100- and 500-ml aliquots.
   b. For complete medium add 2-mercaptoethanol solution 1 in 1000 (vol/vol) to give final concentration of 10^{-4} *M*, and fetal bovine serum to 10% by volume.
   c. For defined medium omit serum and add HEPES solution 1 in 100 (vol/vol), sodium selenite solution 1 in 1000 (vol/vol) to give final concentration of 3×10^{-8} *M*, and insulin and transferrin stocks 1 in 2000 (vol/vol) to final concentration of 5 μg/ml each.
7. Leishman's fix and stain

   Dissolve 1.5 g Leishman's in 1 liter methanol using heated magnetic stirrer. Filter through Whatman paper and store at room temperature.

8. Retinoic acid (10^{-2} *M*)

   Protect from direct light. Dissolve 30 mg retinoic acid in 10 ml dimethyl sulphoxide and store in 0.1-0.5-ml aliquots in the dark at −20° C.

9. 3-Methoxybenzamide (2 *M*)

   Dissolve 302 mg 3-methoxybenzamide in 1.0 ml dimethyl sulphoxide in 37° C water bath. Store at −20° C.

B. Preparation of DIA/LIF

1. Grow Cos-7 cells in complete medium to near confluence in 245-mm plates. Replace spent medium with 67.5 ml fresh complete medium per plate.
2. For each plate make up following solution in a sterile, 25-ml plastic centrifuge tube using aseptic technique:
   a. 0.15 ml DNA
   b. 3.22 ml H_2O
   c. 0.38 ml 2.5M $CaCl_2$

   Mix and add equal volume (3.75 ml) of 2× BES-buffered saline. Vortex for 30 s. Leave at room temperature for 10 to 20 min. Add dropwise to culture medium ensuring even distribution. Swirl gently to mix. Place plate(s) in an incubator pre-equilibrated with a reduced CO_2 atmosphere—2.5% CO_2 in air. Incubate for 20 h.

   Note: The transfection solution should be translucent and on addition to the culture medium the precipitate should barely be visible initially, but will develop gradually. If the transfection solution is opaque, check the pH of the BES or re-extract the DNA, or both.
3. Decant transfection medium and replace with 50 ml fresh complete medium. Incubate for 1 h in 7.5% CO_2 atmosphere.
4. Decant medium and replace with 75 ml complete medium. Incubate for 48 h, harvest, and replace with further 75 ml medium. Harvest again after further 48 h and discard cultures. Clarify harvested medium by centrifugation and filter sterilize. Store at −20° C in 0.5- and 1.0-ml aliquots.

   Note: DIA/LIF can readily be prepared in the absence of serum by rinsing cultures twice in basal medium, incubating for 1 h in 50 ml basal medium, then harvesting in defined medium.

C. Titration of DIA/LIF activity

1. Trypsinize ES cell culture (*see* D below) using physical agitation to dissociate cells. Pellet by centrifugation and resuspend vigorously to produce single cell suspension.
2. Seed ES cells into 24-well tissue culture plates at 10^4 cells · 16-mm^{-1} well · ml^{-1} complete medium. Allow to attach for 3 to 4 h in incubator.
3. Add DIA/LIF aliquots with micropipette to give duplicate dilution series from 1:100 to 1:500 000. (Dilute stock DIA/LIF supernatant with medium). Leave two wells with no addition as controls. Also set up two wells with 1:100 dilution

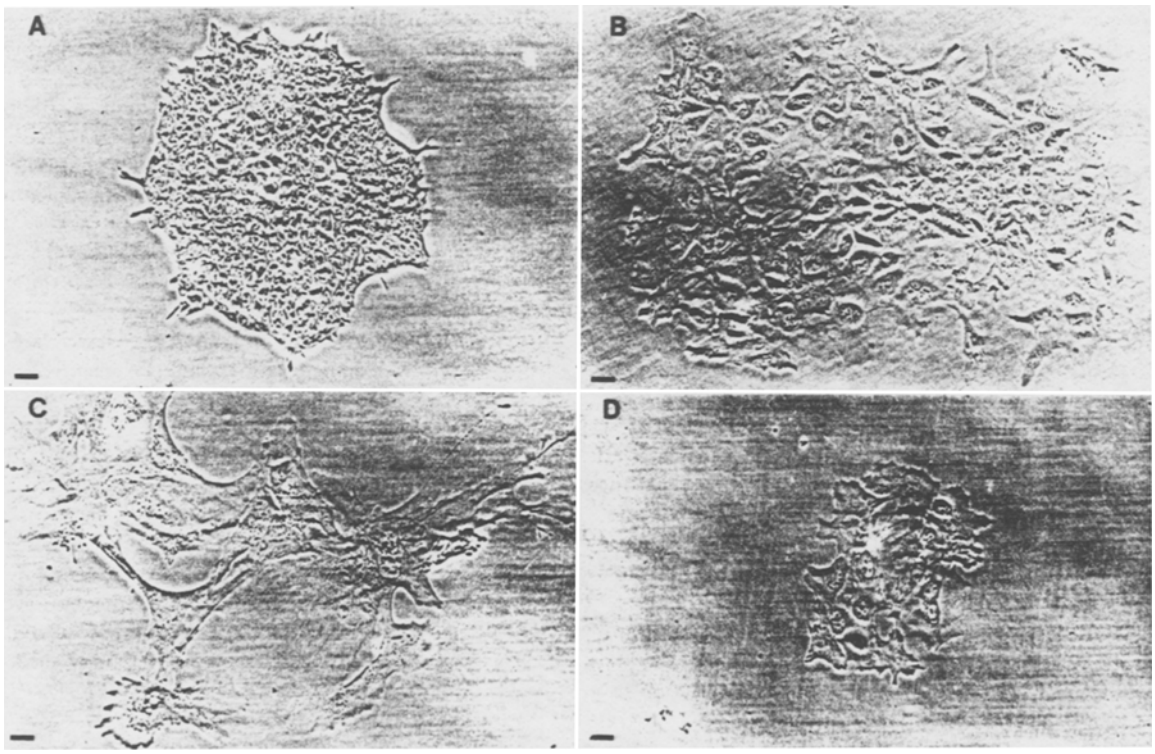

FIG. 1. Morphology of ES Cells and differentiated progeny. *A*, undifferentiated stem cell colony cultured for 5 d in the presence of 100 U/ml DIA/LIF; *B*, differentiated colony after 5 d culture in the absence of DIA/LIF; *C*, differentiated colony induced by incubation with 10^{-6} *M* retinoic acid for 5 d in the presence of 10 U/ml DIA/LIF; *D*, differentiated colony induced by incubation with 5 m*M* 3-methoxybenzamide for 4 d in the presence of 100 U/ml DIA/LIF. ×154. *Bar* = 25 μm.

of mock-transfected Cos cell supernatant and, if available, two wells with DIA/LIF of known activity. Incubate for 4 d.

4. Examine cells under phase contrast. Morphologic distinctions should be readily apparent (Fig. 1). Fix and stain cells with Leishman's as follows:
   a. Aspirate medium.
   b. Add 0.5 ml Leishman's to each well. Leave for 5 min.
   c. Add 1.0 ml H_2O to each well. Leave 1 min.
   d. Decant Leishman's and allow to air dry.
   e. Examine on light box or under stereomicroscope, or both.

   Stem cells are apparent as small rounded colonies of tightly packed, intensely stained cells, whereas differentiated cells form larger, spread-out colonies that stain relatively faintly (22).
5. Define the limiting dilution of DIA/LIF which gives a detectable inhibition of differentiation as 1 U. The DIA/LIF activity in the Cos cell supernatant should be 5 to 20 $\times$ 10^4 U/ml. Activity may be up to 10-fold lower in serum-free preparations.

D. Propagation of ES Cells in DIA/LIF
1. Add 5 ml gelatin solution per 25-cm^2 tissue culture flask and incubate for at least 1 h at 4° C. Aspirate thoroughly.
2. Seed 10^6 ES cells into a 25-cm^2 gelatin-coated tissue culture flask in 10 ml prewarmed, complete medium. Add DIA/LIF to a final concentration of 100 U/ml. Incubate at 37° C in 7.5% CO_2 atmosphere.
3. Inspect cells under phase contrast every day. Change medium every 24 h, adding fresh DIA/LIF at each change.
4. When cells are near confluent (after 3 d) pre-equilibrate complete medium, trypsin, and PBS at 37° C in water bath.
5. Aspirate medium and rinse culture twice with 4 to 5 ml PBS. Add 1 ml trypsin and disperse evenly over culture. Place in incubator for 30 s. Examine under phase contrast. As soon as cells begin to detach, knock the flask several times to ensure complete dissociation, and immediately add 9 ml complete medium. Transfer to sterile centrifuge tubes and pellet cells at 1200 rpm for 3 min.
6. Aspirate supernatant and resuspend cells in 5 ml complete medium. Disperse cell pellet by pipetting vigorously against side of centrifuge tube 3 times. Count cells on hemacytometer. Cells should predominantly be single and viability greater than 90%. Total cell number should be 1 to 2 $\times$ 10^7.
7. Repeat 2. For routine culture, stocks should show a 10- to 20-fold increase in cell numbers over 72 h and require subculturing every 3rd day. To expand cell numbers, use larger tissue culture vessels and increase seeding density and volume of medium in proportion.

   Note: ES cells can be cultured on non gelatin-coated surfaces. Under such conditions the colonies are more rounded and tend to detach from the substratum. Great care must therefore be taken during media changes and subculturing.

E. Induction of ES cell differentiation
1. Withdrawal from DIA/LIF.
   a. Trypsinize cells as normal, ensuring single cell suspension. Seed at 10^3 cells/cm^2 into gelatin-coated flasks in complete medium but without DIA/LIF.
   b. Culture for up to 7 d, with daily changes of medium after initial 72 h. Inspect daily under phase contrast. Discard any cultures in which significant numbers of stem cells persist. Culture should consist predominantly of sheets of large, flattened cells (Fig. 1 *B*), although other cell morphologies will also be apparent.

   Note: Differentiation can also be induced at higher cell densities by withdrawal of DIA/LIF. Under such conditions differentiation is more heterogeneous and many stem cells persist which can tend to overgrow the cultures (18).
2. Exposure to retinoic acid
   a. Trypsinize cells as normal ensuring single cell suspension. Seed at 10^4 cells/cm^2 into gelatin-coated flasks in complete medium plus 10 U/ml DIA/LIF. Allow cells to attach for 2 to 6 h.
   b. Dilute stock retinoic acid to 10^{-6} *M* (1 in 10 000) in complete medium. Replace culture medium with retinoic acid-supplemented medium plus 10 U/ml DIA/LIF.
   c. Change medium twice at 24-h intervals for freshly prepared retinoic acid-containing medium plus 10 U/ml DIA/LIF.
   d. After 72 h change to complete medium without retinoic acid or DIA/LIF.
   e. Culture for further 2 to 3 d, monitoring by microscopic inspection. Discard any cultures in which significant numbers of stem cells persist. Cultures should consist predominantly of large fibroblastic cells (Fig. 1 *C*) with variable amounts of refractile parietal yolk sac-like cells (8,22).

   Note: Differentiation can also be induced in the absence of DIA/LIF. Under such conditions retinoic acid should be used at lower concentration (10^{-7} *M*) as both differentiation-inducing and toxic effects are more pronounced.
3. Exposure to 3-methoxybenzamide
   a. Trypsinize cells as normal ensuring single cell suspension. Seed at 10^4 cells/cm^2 into gelatin-coated flasks in complete medium plus 100 U/ml DIA/LIF. Incubate overnight.
   b. Thaw 3-methoxybenzamide stock in 37° C water bath. Add 25 μl 3-methoxybenzamide per 10 ml prewarmed complete medium and mix well (final concentration = 5 m*M*). Return to water bath for 10 to 20 min to ensure complete dissolution of 3-methoxybenzamide. Replace culture medium with medium containing 3-methoxybenzamide plus 100 U/ml DIA/LIF.
   c. Culture 48 h, then change to complete medium without 3-methoxybenzamide or DIA/LIF.

d. Culture for further 2 to 4 d, monitoring by microscopic inspection. Cultures should form a relatively uniform monolayer of flattened epithelial cells (Fig. 1 *D*). Discard any cultures with low cell numbers due to excessive cell death.

Note: Both 3-methoxybenzamide and retinoic acid are toxic to ES cells at concentrations slightly higher than those that induce optimal differentiation. The concentrations quoted above are intended as guidelines and may require to be adjusted for other variables, such as serum batch, which may effect cell viability. In addition, the effects of chemical inducers are greater at low cell density and the concentration should be reduced accordingly to avoid undue toxicity.

IV. DISCUSSION

The exploitation of embryo-derived embryonic stem cells is receiving increasing attention both as a means of introducing predetermined genetic modifications into germ line of experimental animals (24) and as an in vitro differentiation system for the identification and characterization of gene products that regulate developmental decisions (6,7,18). The culture and experimental manipulation of ES cells is complicated by the requirement for coculture with heterologous feeder cells. In this paper procedures have been detailed for substitution of feeders with the regulatory factor DIA/LIF. Under the conditions described it is possible to maintain undifferentiated cultures of pluripotential ES cells for prolonged periods (15). As feeders are not required, the isolation of genetically modified cells is simplified, the effects of regulatory factors on growth and differentiation can be assessed effectively, and the expression of regulatory factors by the ES cells themselves can be characterized (18).

Recombinant DIA/LIF is produced by transient transfection (2) of Cos cells that harbor SV40 large T antigen with an expression plasmid containing the SV40 origin of replication. Massive overexpression of heterologous proteins is achieved in the Cos cell system by amplification of the plasmid to 100 000 copies/cell (25). DIA/LIF is secreted into the culture medium at high levels for 96 h. The protein is quite stable in frozen storage so it can be prepared in bulk quantities by transfection of mass cultures, then stored at −20° C. The DIA/LIF expressed by Cos cells is heterogeneously glycosylated (14), but this does not seem to affect biological activity.

Routine culture of ES cells is performed in medium supplemented with 100 U/ml DIA/LIF. The viability and growth rate of cells cultured in DIA/LIF are comparable to those observed on feeders (22). Requirements for high quality sera and media and for ultraclean glassware are more stringent in the absence of feeders, however, and this may lead to sickly looking cultures or high levels of cell death, or both. In such cases the source of the toxin should be identified and eliminated immediately. Under suitable culture conditions, 10 to 35% of single cells should form macroscopic colonies when plated at clonal density (10^3 cells/35-mm well).

The level of differentiation during culture in DIA/LIF should be low (<5%) but may vary with cell line, serum batch, and passaging regimes. If excessive differentiation is consistently observed, addition of newborn bovine serum (selected batches) to 5% by volume may prove beneficial. For ES cells originally obtained on feeders, significant differentiation may accompany the initial transfer to DIA/LIF. After 2 to 4 passages, however, relatively homogeneous stem cell populations should be obtained from which frozen stocks can be prepared.

Embryonic stem cells can undergo differentiation into a variety of cell types in vitro after aggregation and the formation of embryoid bodies (3). This is a complex, asynchronous system however. Characterization of the intracellular events that determine cellular commitment and differentiation may be more effectively accomplished in monolayer culture (8). Methods have been described here for the induction of relatively homogeneous ES cell differentiation. The three treatments yield distinct populations of differentiated derivatives (Fig. 1) (6,8,22) although their phenotypes are currently poorly defined.

The underlying mechanisms of differentiation are a major area of future investigation. Specific cell surface receptors for DIA/LIF are expressed by ES cells (21,26) and it is presumed that binding to these receptors activates as yet undefined signaling pathways that are required for maintenance of the stem cell phenotype and suppression of differentiation. Withdrawal of DIA/LIF results in loss of these signals, and therefore the ES cells differentiate. Retinoic acid has a variety of effects in diverse developmental systems and has been known for some time as an inducer of embryonal carcinoma and ES cell differentiation (22,23). The recent cloning of a family of nuclear retinoic acid receptors (5,16) may herald an enhanced understanding of its mode of action. DIA/LIF partially inhibits the inductive effects of retinoic acid (22) and is therefore used at a reduced concentration. By contrast, 3-methoxybenzamide can apparently completely override DIA/LIF signaling. The related compound 3-aminobenzamide has previously been reported to induce differentiation of EC-A1 embryonal carcinoma cells (15) and is effective on ES cells (unpublished data). Both inducers are inhibitors of ADP-ribosylation, but whether this is the basis of their inductive action on ES cells remains to be determined.

The methods described in this manuscript have already proven to be of considerable benefit both for ES cell-based transgenic strategies and for the analysis of embryonic growth and differentiation pathways. The ability to culture and manipulate ES cells in the absence of feeders should make a significant impact on the anticipated widespread use of ES cell technology in coming years.

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[1] Heraeus, Brentwood, Essex, UK
[2] Envair (UK), Rossendale, Lancashire, UK
[3] MSE, Loughborough, Leicester, UK
[4] Olympus (UK), London, UK
[5] Millipore, Watford, Hertfordshire, UK
[6] BDH, Atherstone, Warwickshire, UK
[7] Oxoid, Basingstoke, Hants, UK
[8] Difco, East Molsesey, Surrey, UK
[9] Flow, Rickmansworth, Hertfordshire, UK
[10] Sigma, Poole, Dorset, UK
[11] Boehringer Mannheim, Lewes, East Sussex, UK
[12] Aldrich, Gillingham, Dorset, UK
[13] FSA, Loughborough, Leicester, UK
[14] Falcon, through Becton Dickinson, Cowley, Oxford, UK
[15] Nunc, through GIBCO BRL, Paisley, Scotland
[16] Gilson, through Anachem, Luton, Bedfordshire, UK
[17] Laser Laboratory Systems, Southampton, UK