Establishment of germ-line-competent embryonic stem (ES) cells using
differentiation inhibiting activity

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Summary

The regulatory factor Differentiation Inhibiting Activity/Leukaemia Inhibitory Factor (DIA/LIF) suppresses the differentiation of cultured embryonic stem (ES) cells. In the present study, it is shown that ES cell lines can be derived and maintained in the absence of feeder layers using medium supplemented with purified DIA/LIF. These cells can differentiate normally in vitro and in vivo and they retain the capacity for germ-line transmission. DIA/LIF therefore fulfils the essential function of feeders in the isolation of pluripotential stem cells.

Key words: embryonic stem cells, differentiation inhibiting activity, leukaemia inhibitory factor.

Introduction

Embryonic stem (ES) cells are permanent cell lines established directly from the inner cell mass of the preimplantation mouse embryo (Evans and Kaufman, 1981; Martin, 1981). They retain the ability to participate in normal embryonic development and, following reprogramming to the blastocyst, they generate chimaeric animals that are mosaic in all their tissues. Mosaicism extends to the germ cell lineage and ES cells can contribute fully functional gametes (Bradley et al. 1984). Consequently ES cells are increasingly being exploited as cellular vectors for experimental manipulation of the mouse germ-line (Robertson, 1986; Thomas and Capecchi, 1987; Gossler et al. 1990). The pluripotentiality of ES cells and their capacity to respond to normal developmental cues implies that they constitute a particularly appropriate system for the experimental investigation of mammalian development. ES cells provide a resource that circumvents the inaccessibility of the early mammalian embryo to conventional biochemical approaches. In particular, they can be exploited to identify and define factors that regulate developmental decisions (Heath and Smith, 1988; Rathjen et al. 1990a,b). A knowledge of the signalling networks that govern ES cell proliferation and differentiation will significantly enhance our understanding of regulatory mechanisms in the embryo proper.

The parameters governing the successful derivation and propagation of pluripotential ES cells are poorly characterised. Historically, ES cells have been isolated and maintained on feeder layers of mitotically inactivated embryonic fibroblasts (reviewed by Robertson, 1987). The feeder cells are thought to have generalised effects, such as media detoxification, in addition to more specific effects in promoting cell attachment and viability and preventing ES cell differentiation. However, it is difficult to analyse and define in molecular terms the essential components of such a co-culture system. The presence of feeders also complicates both the genetic manipulation of ES cells (Smith and Hooper, 1987; Hooper et al. 1987) and the characterisation of specific growth and differentiation modulators.

The differentiation of ES cells in vitro can be inhibited by the regulatory factor Differentiation Inhibiting Activity (DIA) secreted by Buffalo rat liver cells (Smith and Hooper, 1987). This allows ES cells to be propagated in the absence of feeders. Pure DIA is a glycoprotein of Mr 43000 and is identical to the myeloid regulator Leukaemia Inhibitory Factor (Smith et al. 1988; Williams et al. 1988). DIA/LIF has a variety of other in vitro biological activities on haemopoietic (Hilton et al. 1988; Moreau et al. 1988; Leary et al. 1990), osteogenic (Abe et al. 1986), hepatic (Baumann and Wong, 1989), adipogenic (Mori et al. 1989) and neuronal (Yamamori et al. 1989) cell types and has multiple pathological effects in the whole animal (Metcalf and Gearing, 1989). The normal physiological role(s) of DIA/LIF remains unclear, however.
In order to define further both the ES cell requirement for feeders and the potential function of DIA/LIF in the early embryo, we have investigated the maintenance and derivation of ES cell lines in the absence of feeders in media supplemented with DIA/LIF.

Materials and methods

Cell culture

Cos-7 cells were obtained from the Sir William Dunn School of Pathology, Oxford. E14 ES cells (Handyside et al. 1989) were generously provided by Dr Martin Hooper, Department of Pathology, University of Edinburgh. E14 is homozygous for the chinchilla coat colour marker (cch cch). ES cells were cultured on gelatin-coated tissue-culture plastic, as described (Smith, 1990). Differentiation was induced by withdrawal of DIA/LIF, or by exposure to all-trans-retinoic acid (10^{-6} M) or to 3-methoxybenzamide (5 × 10^{-3} M) in the continued presence of DIA/LIF (Smith and Hooper, 1987; Smith, 1990). Embryoid bodies were formed by aggregation in the absence of DIA/LIF as previously described (Smith and Hooper, 1987).

Preparation of DIA/LIF

DIA/LIF was prepared by transfection of Cos-7 cells with the appropriate human and murine expression plasmids, pc10-6R (Moreau et al. 1988) and pDR10 (Rathjen et al. 1990a), respectively, as described (Smith, 1990). Purified DIA/LIF was obtained from serum-free supernatants of transfected Cos-7 cells by sequential hydrophobic interaction chromatography and reverse-phase HPLC using methods described (Smith and Hooper, 1987), but only fully glycosylated protein, which has an apparent M_{r}=45000 similar to native DIA (Smith, 1988). In common with other glycoproteins, heterogeneous glycosylation of DIA/LIF is observed in the Cos cell expression system (Moreau et al. 1988; see Fig. 1). All the glycoforms appear to be biologically active (unpublished data), but only fully glycosylated protein, which has an apparent M_{r}=45000 similar to native DIA (Smith et al. 1988), was employed in this study. Purity of both human and murine DIA/LIF was greater than 95% as judged by radioiodination (Smith et al. 1988), protein sequence determination (Rathjen et al. 1990a; AGS, R. Hewick and J. Heath, unpublished) and silver-staining of ultrathin SDS gels (see Fig. 1). Purified DIA/LIF was quantified by amino acid analysis.

Embryo culture and derivation of cell lines

Embryos were flushed out in PFI medium (Whittingham and Wales, 1969) supplemented with 10% foetal calf serum (Gardner, 1982). Culture of blastocysts and stem cells was performed in Dulbecco-modified Eagles medium (DMEM) supplemented with 10% foetal calf serum, 5% newborn calf serum, 10^{-4} M 2-mercaptoethanol and 1 mM non-essential amino acids (Robertson, 1987). Serum batches were selected that supported optimal growth of established ES cell lines. Cultures were maintained in a humidified atmosphere of 6% CO_{2} in air at 37°C.

Donor embryos were derived from strain 129J mice, obtained from Olac. This strain is homozygous for chinchilla (cch cch). Implantation delay was achieved by ovariectomy on the third day of pregnancy and subcutaneous injection of Depo Provarra diluted 1:4 with phosphate-buffered saline. Four days later blastocysts were flushed from the uterus. The blastocysts were transferred to gelatin-coated 16 mm tissue culture wells (Nunc) in supplemented DMEM. Up to 5 blastocysts were cultured per well, but these attached separately and the ICMs remained discrete. After 5 days of culture, the ICMs were individually disaggregated by physical dislodgement from the trophoblast with a fine glass rod and transferred to separate drops of trypsin. Following incubation at 37°C for 5 min, the trypsin was inactivated by dilution with complete medium. Cells were dissociated into small groups by repeated aspiration through a finely drawn out siliconised Pasteur pipette, then transferred to a gelatinised 16 mm culture well containing 0.5 ml pre-equilibrated complete medium. Where appropriate, fresh DIA/LIF was added every 3-4 days thereafter. Dissociation of primary stem cell colonies was performed by the same procedure when the colonies contained 50-250 cells. This was generally during the second week of culture. Primary colonies were replated in 16 mm wells. Subsequent subculturing and expansion of the cells was carried out as for established ES cell lines (Smith, 1990), with a split ratio of 1/10.

Production of chimaeras

Host blastocysts were provided by a closed colony of a random bred albino strain, PO (Pathology, Oxford). Blastocysts were flushed on the afternoon of the fourth day p.c. Donor ES cells were obtained from single cell suspensions generated by trypsinisation of healthy cultures. Micromanipulators were employed to inject 10–15 cells into each blastocyst (Bradley, 1987). Blastocysts were allowed to re-expand in culture before transfer to the uteri of pseudopregnant PO recipients. Chimaeras were identified amongst resulting offspring by the presence of patches of sandy coat colour on the albino background. Male chimaeras were test-bred to albino PO females. Germ-line transmission of the ES cell genotype was revealed by the production of heterozygous (cch/c) progeny which are grey chinchilla-coloured.

Cytogenetic analysis

Karyotype analysis of the ES cells was performed on ASG/trypsin banded chromosome spreads prepared as described (Hogan et al. 1986). Cytological screening of mice was carried out by standard methods for testis and bone marrow.

Results

Maintenance of ES cell pluripotentiality in media supplemented with DIA/LIF

ES cells irreversibly differentiate if plated in normal growth medium in the absence of feeders. The differentiation of ES cells in monolayer culture can be inhibited by subnanomolar concentrations of DIA/LIF purified from either native or recombinant sources (Smith et al. 1988). This effect is reversible, such that the ES cells rapidly differentiate on withdrawal of DIA/LIF from the culture medium. To determine whether the developmental potential of ES cells is fully maintained by this factor, however, requires an in vivo analysis via the production of chimaeric animals. The diploid ES cell line E14 (Handyside et al. 1989) was therefore serially passaged in the absence of feeders in medium supplemented with 10 ng ml^{-1} (300 pm) purified human DIA/LIF (Smith et al. 1988), before injection into host blastocysts. The cells were maintained with daily changes of medium and passaged every second or third day as required with a split ratio...
Table 1. Production of chimaeras from E14 ES cells cultured in DIA/LIF

<table>
<thead>
<tr>
<th>Series</th>
<th>Generations in DIA/LIF</th>
<th>Blastocysts injected</th>
<th>Progeny born</th>
<th>Total Chimaeras</th>
<th>Male Chimaeras</th>
<th>Germ-line Chimaeras</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>18</td>
<td>12</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>22</td>
<td>15</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III</td>
<td>24</td>
<td>18</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

E14 ES cells were serially passaged in purified human DIA/LIF prior to blastocyst injection. Chimaeras were detected by coat colour pigmentation and phenotypic males were test bred to PO albino females.

of 1 in 10, i.e. approximately 3.5 generations per passage. The results of three series of injections are summarised in Table 1.

Chimaeras exhibited patches of sandy fur on the albino host background due to homozygous expression of the chinchilla allele carried by E14. The chimaeras all developed into healthy adults. Two of the male chimaeras mated with albino females produced offspring with chinchilla coat pigmentation. Such animals are heterozygous for chinchilla due to germ-line transmission of ES-cell-derived gametes. These animals also developed normally into healthy fertile adults. Purified DIA/LIF is thus sufficient to sustain the full potential of cultured ES cells for somatic and germ-line chimaerism.

Isolation of ES cells in media supplemented with DIA/LIF

The finding that DIA/LIF is functionally equivalent to a feeder layer in the maintenance of established ES cells prompted an examination of the ability of DIA/LIF to substitute for the essential function of feeders in the derivation of new ES cell lines directly from preimplantation mouse embryos. Three series of experiments were performed on implantation-delayed blastocysts from strain 129J mice. In the first series, purified recombinant human DIA/LIF was employed at 50 ng ml\(^{-1}\) (2.5 nM) and, in the second series, human DIA/LIF was provided at 1000 i.u. ml\(^{-1}\) in the form of a 1/100 dilution of supernatant from transfected Cos-7 cells. In the third series, purified murine DIA/LIF was used at 50 ng ml\(^{-1}\) (2.5 nM). Both human and murine DIA/LIF were purified to apparent homogeneity and the fully glycosylated isoform was employed (Fig. 1, lane a).

The procedure followed to derive cell lines from whole blastocysts is essentially the same protocol as that developed for isolation of ES cell lines on feeders (Robertson, 1987). The results of culturing blastocysts in the absence of feeders and in the presence or absence of DIA/LIF are presented in Table 2.

Blastocysts readily attached to gelatin-coated tissue-culture plastic. Trophoblast cells grew out in the presence or absence of DIA/LIF whilst the ICMs tended to remain central. In general ICMs showed somewhat greater viability and growth in media supplemented with DIA/LIF. After 5 days in culture the ICMs were individually detached and dissociated and plated separately into 16 mm wells. ICMs from blastocysts cultured in the absence of DIA/LIF gave

![Fig. 1. Silver-stained ultrathin gel of purified murine DIA/LIF glycoforms. Aliquots of sequential fractions from final reverse phase HPLC purification of murine DIA/LIF were analysed by non-reducing SDS-PAGE on ultrathin 10–15 gradient gels and visualised by silver-staining with periodic acid enhancement (Dubray and Bezard, 1982). Fractions a–d were biologically active, had similar amino acid composition and yielded identical N-terminal sequence. The fully glycosylated glycoform (a) with relative molecular mass=50,000 (reduced Mr=43,000 [Smith et al. 1988; AGS, unpublished data]) was used in further studies. Molecular weight markers (Mr) were from the Sigma MW-SDS-70L kit.](image-url)
Table 3. Karyotypes of ES cell lines derived in DIA/LIF

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>% '4n'</th>
<th>% '2n'</th>
<th>Sex</th>
<th>Karyotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF1 (A)†</td>
<td>43</td>
<td>57</td>
<td>XX</td>
<td>10, 40XX</td>
</tr>
<tr>
<td>LF2 (A)</td>
<td>84</td>
<td>16</td>
<td>XX</td>
<td>10, 40XX</td>
</tr>
<tr>
<td>LF3 (A)</td>
<td>100</td>
<td>0</td>
<td>XX</td>
<td></td>
</tr>
<tr>
<td>LF4 (A)</td>
<td>48</td>
<td>52</td>
<td>XX</td>
<td>8, 40XX/2, 39XO</td>
</tr>
<tr>
<td>JN1 (B)</td>
<td>32</td>
<td>68</td>
<td>XX</td>
<td>10, 40XX-Ts6, Ms10</td>
</tr>
<tr>
<td>JN2 (B)</td>
<td>26</td>
<td>74</td>
<td>XX</td>
<td>10, 41XX-Ts8, Rb8.8</td>
</tr>
<tr>
<td>JN3 (B)</td>
<td>55</td>
<td>45</td>
<td>XX</td>
<td>10, 40XX</td>
</tr>
<tr>
<td>JN4 (B)</td>
<td>11</td>
<td>89</td>
<td>XX</td>
<td>10, 40XX-Dp6</td>
</tr>
<tr>
<td>JN5 (B)</td>
<td>5</td>
<td>95</td>
<td>XY</td>
<td>10, 41XY-Ts8</td>
</tr>
<tr>
<td>JN6 (B)</td>
<td>100</td>
<td>0</td>
<td>XX</td>
<td></td>
</tr>
<tr>
<td>JN7 (B)</td>
<td>4</td>
<td>96</td>
<td>XY</td>
<td>2, 40XY/7, 40XY-Ts6/9/1, 41XY-Ts11</td>
</tr>
<tr>
<td>P12.8 (C)</td>
<td>8</td>
<td>92</td>
<td>XY</td>
<td>10, 41XY-Ts8</td>
</tr>
<tr>
<td>EFC-1 (C)</td>
<td>0</td>
<td>100</td>
<td>XY</td>
<td>9, 40XY/1 39 XO</td>
</tr>
<tr>
<td>EFC-2 (C)</td>
<td>4</td>
<td>96</td>
<td>XY</td>
<td>4, 40XY/3, 41XY-Ts11/2, 41XY-Ts8/1, 41XY-Ts6</td>
</tr>
<tr>
<td>EFC-3 (C)</td>
<td>6</td>
<td>94</td>
<td>XY</td>
<td>6, 40XY/4, 41XY-Ts11</td>
</tr>
<tr>
<td>EFC-1+ (C)</td>
<td>12</td>
<td>88</td>
<td>XY</td>
<td>10, 40XY</td>
</tr>
</tbody>
</table>

* Data from 10 spreads.
† Letters in parentheses indicate derivation of cell line (see Table 2).
‡ Karyotyped after 10 passages.

Karyotypes were determined on ES cells between passages 4 and 6, except where otherwise indicated. For each cell line 50 metaphase spreads were counted ('4n' and '2n' indicate approximate tetraploidy and diploidy respectively) and 10 randomly selected, well-banded, '2n' spreads were analysed in detail. Standard Mouse Nomenclature is employed: /, presence of more than one population; Ts, trisomy; Ms, monosomy; Rb, Robertsonian translocation or centric fusion; Dp, duplication; T, reciprocal translocation.

ES cells derived from parthenogenetic embryos (Robertson et al. 1983a). Only one of the lines isolated in the present study, EFC-1, had a normal karyotype rise only to a few differentiated cells. ICMs grown in media supplemented with DIA/LIF when replated, again in the presence of DIA/LIF, produced occasional small clusters of differentiated cells but in addition generated primary stem cell colonies. The latter were distinguished by their characteristic ES-like morphology and their capacity for continuous proliferation. Overall, stem cell colonies were obtained from around 30% of blastocysts, though the frequency ranged from 1 in 15 to 6 out of 9 between individual experiments. Primary stem cell colonies could be readily subcultured and expanded into permanent cell lines in the presence of DIA/LIF. Established ES cell lines were obtained in this manner using both human and murine DIA/LIF (Table 2).

The karyotypes of the new ES cell lines were examined for chromosomal abnormalities after four or five passages. These data are summarised in Table 3. Most of the lines contained significant populations of tetraploid and/or aneuploid cells. Nine were karyotypically female, but these were all partially or wholly tetraploid. The significance of this is unclear. Possibly tetraploidisation confers some selective advantage in the presence of two X chromosomes. It has previously been reported that the combination of two X chromosomes is unstable in ES cells and this was reflected in the partial or complete deletion of one of the copies in
Fig. 3. Differentiation of EFC-1 ES cells in vitro. (A) Undifferentiated stem cell colony cultured for 5 days in the presence of 100 i.u. ml⁻¹ DIA/LIF. (B) Differentiated colony after 5 days culture in the absence of DIA/LIF. (C) Differentiated colony induced by incubation with 10⁻⁶ M retinoic acid for 5 days in the presence of 10 i.u. ml⁻¹ DIA/LIF. (D) Differentiated colony induced by incubation with 5 mM 3-methoxybenzamide for 4 days in the presence of 100 i.u. ml⁻¹ DIA/LIF. Bar, 25 μm.

(Fig. 2A), although seven others did contain varying proportions of euploid cells. The karyotype of EFC-1 appeared stable, as it was still euploid after several further passages (Table 3).

The ES cells derived in the absence of feeders remained dependent on the continuous presence of DIA/LIF to suppress differentiation (Fig. 3). On withdrawal of DIA/LIF from the culture medium they rapidly and irreversibly differentiated into large flattened cells (Fig. 3B). Differentiation could also be induced in the presence of DIA/LIF. This was achieved, as described for other established ES cells (Smith and Hooper, 1987; Smith, 1990), by exposure to either retinoic acid, which yields predominantly fibroblastic cells (Fig. 3C), or to 3-methoxybenzamide, which produces flattened epithelioid cells (Fig. 3D). EFC-1 cells were also induced to differentiate via aggregation (Martin and Evans, 1975; Doetschman et al. 1985). Under such conditions cystic embryoid bodies formed which contained a variety of differentiated tissues including yolk sac, cardiac muscle and blood islands (data not shown). The in vitro differentiation properties of ES cells isolated using DIA/LIF are thus similar to those of ES cells derived on feeders.

Production of chimaeras from ES cells derived with DIA/LIF

The identity of ES cells is definitively established by their ability to form chimaeras. The production of chimaeras also rigorously assesses the developmental potential of the cells and subsequently their ability to form functional gametes. Blastocyst injections were therefore performed to confirm the identity of the cell lines isolated in this study and to determine whether or not they had the capacity for germ-line transmission.

Four series of injections were carried out with different cell lines using recipient blastocysts from outbred albinos. The data are presented in Table 4. Coat colour mosaicism was used to detect chimaeric animals. This may not identify chimaeras with relatively minor ES cell contributions, particularly as small patches of sandy fur are not always obvious on an albino background. Nonetheless, overt coat colour chimaeras were obtained with all four cell lines. The female, substantially tetraploid line, LF1, and the trisomic line, JN5, produced chimaeras at relatively low frequency, whereas approximately one third of pups were chimaeric following injection of the diploid lines JN7 and EFC-1. All the chimaeras developed normally into...
Table 4. Production of chimaeras from ES cells derived in DIA/LIF

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Blastocysts injected</th>
<th>Progeny born</th>
<th>Total chimaeras</th>
<th>Male chimaeras</th>
<th>Germ-line chimaeras</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF1</td>
<td>36</td>
<td>11*</td>
<td>2</td>
<td>0</td>
<td>N.D.#</td>
</tr>
<tr>
<td>JN5</td>
<td>22</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>JN7</td>
<td>29</td>
<td>17</td>
<td>5</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>EFC-1</td>
<td>113</td>
<td>56</td>
<td>19</td>
<td>15</td>
<td>4</td>
</tr>
</tbody>
</table>

* An additional 8 offspring were eaten at birth before chimaerism could be determined.
# Not determined.

Blastocyst injections were performed with ES cells between passages 4 and 10. Chimaeras were detected by coat colour pigmentation and phenotypic males were test-bred with PO albino females.

Fig. 4. EFC-1 germ-line chimaera and chinchilla offspring
EFC-1 male chimaera and chinchilla offspring from cross with an albino PO female.

Discussion

Embryonic stem cells provide a powerful system for the experimental analysis of mammalian development. Their genetic manipulation allows the introduction of defined modifications into the mouse germ-line (reviewed Hooper, 1990), whilst in culture they constitute both an assay system for, and a potential source of, embryonic regulatory factors (Heath and Smith, 1988; Rathjen et al. 1990a,b). However, a significant disadvantage to the use of ES cells has been the requirement to employ feeder layers of embryonic fibroblasts to support the isolation and maintenance of pluripotential stem cells. The findings reported in this paper demonstrate that the regulatory factor DIA/LIF can substitute for the essential function of feeders and enable the derivation and propagation of germ-line competent ES cell lines with DIA/LIF.

Previous work has shown that the differentiation of ES cells in vitro can be reversibly inhibited by DIA/LIF (Smith and Hooper, 1987; Smith et al. 1988; Williams et al. 1988). It has also been found that ES cells cultured in the absence of feeders using BRL cell-conditioned medium retain the ability to form germ-line chimaeras (Hooper et al. 1987). In addition to DIA/LIF, however, BRL cells secrete other regulatory factors such as insulin-like growth factor-II (Dulak and Temin, 1973) and transforming growth factor-β (Massague et al. 1985). The data presented here on culture of E14 cells sampled in culture carried a chromosomal translocation, T6;9 (Fig. 2B). Cytogenetic examination confirmed that the translocation was transmitted to 50% of progeny, indicative of complete heterozygosity for the translocation in the germ-line (Searle et al. 1971). According to the most recent listings (Beechey, 1990), no reciprocal translocation between mouse chromosomes 6 and 9 has previously been described. The incidence of spontaneous or induced translocations or other rearrangements in ES lines and subsequent transmission through the germ-line could prove useful for a variety of chromosomal studies, such as work on chromosomal imprinting (Cattanach, 1986).

These results establish that it is possible to isolate and maintain pluripotential germ-line competent ES cell lines with DIA/LIF.

healthy adults. It is noteworthy that the harbouring of chromosomal abnormalities may reduce but does not necessarily eliminate the ability of ES cells to contribute to normal development.

The litters obtained after injection of EFC-1 showed a distortion of the sex ratio in favour of males (Table 4). This is diagnostic of colonisation of the germ-line by ES cells (Bradley, 1987), and reflects the conversion of female hosts to phenotypic males by XY ES cell progeny in the germ cell lineage. Consistent with this, the majority (15/19) of EFC-1 chimaeras were male. Four of these animals produced chinchilla offspring on mating with albino females, demonstrating transmission of the ES cell genotype (Fig. 4). Three of the males gave only chinchilla offspring, indicating that they were probably sex-converted female hosts, whilst the fourth male had a mosaic germ-line and produced both chinchilla and albino pups. One JN7 chimaera also showed 100% transmission of the chinchilla allele. This result was unexpected as the majority of JN7 cells
(Table 1) establish that purified DIA/LIF can sustain germ-line competent cells for extended periods. DIA/LIF is thus necessary and sufficient for the complete maintenance of ES cell pluripotentiality in the absence of feeders.

Feeder cells have been shown to express DIA/LIF in two forms, diffusible and matrix-localised (Rathjen et al. 1990a,b). The production of DIA/LIF presumably underlies their capacity to maintain undifferentiated ES cells. This study has demonstrated that DIA/LIF is also the key regulatory factor required for the initial isolation of ES cells and that other feeder functions are not essential. Direct culture of implantation-delayed blastocysts in media supplemented with human or murine DIA/LIF resulted in the generation of primary stem cell colonies with a comparable frequency (1/3) to that observed on feeders (Robertson, 1987). The colonies could be expanded into permanent cell lines with morphological and differentiation characteristics indistinguishable from those of ES cells derived on feeders. These cells remained dependent on DIA/LIF for maintenance of the stem cell phenotype. Interestingly, differentiation was not completely suppressed for all of the lines and in some cases significant numbers of differentiated cells persisted during continuous culture. This variability in the extent to which differentiation is inhibited has also been observed with ES cells originally isolated on feeders (AGS, unpublished data). It indicates that there may be a degree of heterogeneity between different ES isolates and suggests that there are additional regulatory factors that can inhibit or induce differentiation.

Only one of the fifteen lines established had an entirely normal karyotype (Table 3). The others showed varying degrees of chromosomal abnormality. It is not clear how this would compare with a random selection of ES lines derived on feeders, as comprehensive karyotype data on the latter is not readily available. Published data from one laboratory (Robertson et al. 1983b; Robertson and Bradley, 1986) indicates that the majority (27 out of 35) of cell lines derived on feeders are initially euploid. These data suggest that there may be some advantages to the use of feeders. However, this success could also be due to the mouse strains used and/or to the particular culture environment. Other workers have reported that not all ES lines differentiate normally and relatively few exhibit high levels of germ-line transmission (Martin et al. 1987; Frohman and Martin, 1989). The poor frequency of the latter is generally ascribed to the presence of chromosomal aberrations. This is borne out by cyogenetic analysis of several feeder-maintained ES cells, which has revealed a high proportion of abnormalities (EPE, unpublished data). The incidence of such karyotype defects suggests that general culture conditions for ES cell isolation might be further optimised.

The argument that DIA/LIF is the crucial regulatory factor, however, is substantiated by the isolation of the stable euploid line EFC-1 which forms viable chimaeras and can be transmitted through the germ-line. Although higher frequencies of chimaera formation and germ-line transmission have recently been obtained using strain C57BL/6 blastocysts as recipients (Schwartzberg et al. 1989), the figures for EFC-1 are comparable to those previously reported for injection of feeder-derived ES cells into outbred hosts (Bradley et al. 1984; Bradley, 1987).

The successful isolation of ES cells in the absence of feeders indicates that inner cell mass cells may be directly responsive to DIA/LIF. Such a conclusion is supported by the observation that immunosurgically isolated ICMs exhibit enhanced growth and viability in defined media in the presence of DIA/LIF (JN and AGS, unpublished data). This is consistent with the proposition that DIA/LIF is an important regulatory factor in normal early embryonic development (Smith and Hooper, 1987; Rathjen et al. 1990a,b).

The demonstration that the essential role of feeder layers in the culture of ES cells is inhibition of differentiation and that this function can be fulfilled by DIA/LIF marks a significant advance in the characterisation of ES cell culture. This will simplify the experimental and genetic manipulation of ES cells and may facilitate their isolation from other species. Ultimately, it paves the way towards a complete definition of the growth and differentiation requirements of ES cells and by implication of pluripotential embryo cells.

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References


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