CULTURE AND DIFFERENTIATION OF EMBRYONIC STEM CELLS

Austin G. Smith

AFRC Centre for Genome Research, University of Edinburgh, King’s Buildings, West Mains Road, Edinburgh EH9 3JQ, UK

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 inaccessibility 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

CO₂ incubator, model B 5060 EC/CO₂, Heraeus
Laminar flow hood, Envair²
Bench top centrifuge, no. 41135-305, MSE³
Inverted microscope, Olympus⁴
Stereomicroscope⁵
Filter housing, no. YF55 040 00, Millipore⁶
Dispensing pressure vessel, no. XX67 00P 05⁷
Vortex, no. 330/0055/00, BDH⁸

B. Chemicals

Phosphate buffered saline (Dulbecco A) tablets, no. BR014G, Oxoid⁹
Trypsin 1:250, no. 0125-13-1, Difco
Dulbecco’s modified Eagle’s medium (DMEM) (powder), no. 10-331, Flow
Ham’s F12 medium (powder), no. 10-431
Sodium bicarbonate, no. S5761, Sigma
Insulin (bovine), no. I6634
Transferrin (human), no. 1073 974, Boehringer Mannheim
Sodium selenite, no. S9133
HEPES (1 M solution, pH 7.4), no. 16-884-49
Gelatin, no. G9391
EDTA (disodium salt), no. ED2SS
Calcium chloride, no. 10070 3HH
BES, no. B6266
Analar water, no. 10292 3C or Millipore-filtered water (Milli-Q-plus)
2-Mercaptoethanol, no. M7522
All-trans-retinoic acid, no. 22,301-8, Aldrich
3-Methoxybenzamide, no. M1,005-0
Dimethyl sulphoxide, no. D/4121/08, FSA
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)].

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, pCl0-6R, 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
Tissue culture plasticware (various), Nunc
Millidisk cartridge filters (0.2 μm), no. MCGL 10503
Micropipettes (various), Gilson
Micropipette tips, nos. LL 1030-800 and LL 1040-800, Laser
Syringe filters (Flowpore, 0.2 μm), no. 64-001-04
Hemacytometer, no. 403/0061/01
Bottle-top filters, no. 7111

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