Haematopoietic and lymphoid cell culture

Edited by

Margaret J. Dallman

and

Jonathan R. Lamb

PUBLISHED BY THE PRESS SYNDICATE OF THE UNIVERSITY OF CAMBRIDGE
The Pitt Building, Trumpington Street, Cambridge, United Kingdom

CAMBRIDGE UNIVERSITY PRESS
The Edinburgh Building, Cambridge CB2 2RU, UK http://www.cup.cam.ac.uk
40 West 20th Street, New York, NY 10011-4211, USA http://www.cup.org
10 Stamford Road, Oakleigh, Melbourne 3166, Australia
Ruiz de Alarcón 13, 28014 Madrid, Spain

First published 2000

Printed in the United Kingdom at the University Press, Cambridge

Typeface Bembo (Monotype) 10½/13pt. *System* QuarkXPress® [SE]

A catalogue record for this book is available from the British Library

Library of Congress Cataloguing in Publication data

Haematopoietic and lymphoid cell culture / editors, Maggie Dallman and
Jonathan Lamb.
p. cm. – (Handbooks in practical animal cell biology)
ISBN 0 521 62043 0. – ISBN 0 521 62969 1 (pbk.)
1. Hematopoietic stem cells Handbooks, manuals, etc.
2. Immunocompetent cells Handbooks, manuals, etc. 3. Cell culture
Handbooks, manuals, etc. I. Dallman, Maggie, 1957– . II. Lamb,
Jonathan R. III. Series.
QP92.H34 2000
612.4′1–dc21 99-38930 CIP

ISBN 0 521 62043 0 hardback
ISBN 0 521 62969 1 paperback

Contents

1

Haematopoietic differentiation of embryonic stem cells

John Ansell and Nicholas Hole

Introduction

All of the blood cell lineages differentiate from a pool of haematopoietic stem cells (HSC). These cells, although in a very small minority in the bone marrow of adult mammals, are highly pluripotent, not only capable of differentiating into all of the mature cell types (platelets, mast cells, neutrophils, monocyte/macrophages, eosinophils, erythrocytes, thymocytes and B cells) of the haematopoietic system but also of 'self-renewing', maintaining and if necessary expanding their numbers throughout life. HSC also provide the long-term repopulating ability of bone marrow after its transplantation, say, into an irradiated or genetically compromised host. Thus bone marrow transplantation has been the preferred and sometimes the only option for patients undergoing intensive radio- and/or chemotherapy not only for aggressive leukaemia and related cancers but also in the treatment of blood disorders. However, the extreme difficulties in tracing matched donors in sufficient numbers have led to a search for alternative sources of HSC. In the last five years, two alternatives have been developed: peripheral blood stem cells and umbilical cord blood stem cells. In the former the very small numbers of HSC found in adults' peripheral blood can be increased by 'mobilisation' treatments and collected from a blood donation. In the latter there are proportionately higher numbers of stem cells in fetal blood, which can be collected from the umbilical cord at delivery. In both cases the stem cells have to be enriched from the blood and their numbers expanded before transplantation. There is increasing evidence that the expansion of stem cell numbers using growth factors and cytokines reduces their self-renewal properties and thus their potential for long-term reconstitution of the blood (Holyoake *et al.*, 1997).

These developments have great potential to provide a much more readily available source of transplantable HSC and there is a need to exploit their development rapidly.

However, studies of the physiological and molecular/genetic control of HSC proliferation and differentiation have been hampered by several factors:

1 Their paucity of numbers *in vivo* is compounded by an imprecise definition of their phenotype, making specific isolation of HSC difficult.
2 Derivation of haematopoietic cell lines *in vitro* is dependent upon (usually retroviral) transformation. Those lines that have been developed have limited pluripotency and their maintenance is usually only possible in the presence of cytokines and/or growth factors.
3 Genetic manipulation of HSC is possible but this is typically reliant on retroviral infection.

The lack of detailed knowledge of the mechanisms controlling stem cell differentiation and self-renewal profoundly affects our ability to expand HSC numbers without their concomitant differentiation and reduction in pluripotentiality although clearly this HSE expansion process must occur at some point during embryonic development.

One class of stem cells that can be maintained and expanded *in vitro* without losing *in vivo* pluripotency is murine embryonic stem (ES) cells. These cells are derived from the inner cell mass of early (3.5 day) mouse blastocysts and can be maintained indefinitely as undifferentiated cell lines *in vitro* in the presence of a polypeptide cytokine – leukemia inhibitory factor (LIF) (Smith *et al.*, 1988). After their introduction back into pre-implantation embryos, ES cells have the capacity to contribute to all tissue systems and, crucially, to the germ line of the resulting animal. It has been formally demonstrated that ES cells can generate pluripotent HSC capable of long-term reconstitution (Forrester *et al.*, 1991). This was achieved using techniques of aggregation of ES cells with tetraploid host embryos. Mice derived by this process are entirely from the ES cell contribution to the original aggregation, tetraploid cells only having contributed to extra-embryonic structures. Although peri-natal death is the most likely outcome of this procedure, Forrester *et al.* (1991) demonstrated that the fetal livers of such mice contained HSC capable of entirely repopulating the haematopoietic system of irradiated secondary recipients. All of the cell types of the blood of the reconstituted host were shown to have been ES-derived.

Importantly for haematologists ES cells also have the capacity to differentiate *in vitro* into a variety of different cell types, including those of the haematopoietic system, albeit in a relatively disorganised way. These properties of ES cells have excited the interest of stem cell biologists, not only because they facilitate the opportunity to study the 'rules' of stem cell growth and self-maintenance, but also because of the ease with which ES cells can

be genetically manipulated prior to their re-introduction *in vivo*. Through this process it becomes possible to generate a range of animal models of human disease and malignancy.

Thus the ability to control and direct the differentiation of ES cells into haematopoietic lineages could have several advantages:

1 As a potential source of long-term haematopoietic repopulating cells to be used for transplantation into compromised hosts.
2 As an *in vitro* test system for protocols designed to expand stem cell numbers.
3 As a model system in which the effects of transgenic modifications could be screened prior to their introduction into the germ-line.
4 As a system in which the molecular events controlling the differentiation and maintenance of HSC could be delineated.

As mentioned above, when LIF is removed from the culture medium ES cells will differentiate, giving rise to various endodermal, mesenchymal and neuronal cells. As part of this process the differentiation of haematopoietic cells is now well established and erythrocytes, macrophages, lymphocytes, neutrophils, and mast cells have all been observed during ES cell differentiation *in vitro* (Doetschman *et al.*, 1985; Wiles & Keller, 1991; Chen, Kosco & Staerz, 1992). Initially only long-term lymphopoiesis in mice in receipt of differentiated ES cells had been achieved (Chen *et al.*, 1992) suggesting that repopulating potential after ES cell differentiation was limited to the production of lymphoid progenitors. Subsequently we and others have demonstrated that multilineage repopulation of mice using ES-derived progenitors is possible (Palacios, Golunski & Samardis, 1995; Hole *et al.*, 1996*a*). The production *in vivo* of differentiated haematopoietic cells derived from pluripotential ES cells implies that *in vitro* self-renewing haematopoietic precursors must be present at some point following the withdrawal of LIF. The protocols for the differentiation and identification of such haematopoietic progenitors from ES cells are the subjects of this chapter.

General notes on methodologies and reagents

In all of the methodologies the water used is either 'Analar' grade (BDH) or Milli-Q (Millipore) purified.

ES cell culture and differentiation medium: the only difference between these two media is the batch of serum used and the absence of LIF in the latter. We are currently using fetal calf serum from Globepharm in the ES cell culture medium and from Boehringer in the differentiation

medium. Regardless of the source of serum, the potential supplies have to be batch-tested to check for the least differentiation effects in the case of ES cell culture and, for example, for maximum CFU-A yielding potential in differentiation cultures. Similarly the donor horse serum used in the CFU-A assay should be batch-tested before use (see Pragnell, Freshney & Wright, 1994)

Reagents

10× Glasgow's modified Eagles medium (GMEM)*
10× α-Modified Eagles medium (αMEM)*
Horse serum*
Sodium bicarbonate*
Non-essential amino acids*
Glutamine/pyruvate*
Chicken serum*
0.1 M 2-mercaptoethanol (BDH)
PBS tablets (Oxoid)
Collagenase (Difco)
Gelatin (porcine skin gelatin Type A) (Sigma)
Noble agar (ICN Flow)
Trypsin (ICN Flow)
EDTA disodium salt (Fischer Scientific UK)
Methyl cellulose; 15 centipoises viscosity (Metachem Diagnostics Ltd)

* From GIBCO–BRL

Embryonic stem cell isolation and culture

The derivation of ES cell lines and their routine culture has been described in extensive detail by Smith (1991) and will not be specifically dealt with in any detail in this chapter. These methodologies describe ES cell culture in the absence of feeder cells using LIF supplemented medium. Although some laboratories continue to grow ES cells on feeder layers these protocols are generally inconvenient for ES cell differentiation. Inadequate mitotic inactivation and the possibility of feeder cell inclusion into differentiating ES cell structures are particular problems.

ES cell culture

The ES cell lines that have been used in the protocols described here include EFC-1 (Nichols, Evans & Smith, 1990) and CGR8 (ECACC 95011018)

although the methods can equally be applied to most ES cell lines. In brief, ES cells are maintained in GMEM supplemented with 10% fetal calf serum, 1% non-essential amino acids, 2% L-glutamine/sodium pyruvate (from a stock solution containing a 1:1 volume ratio of 200 mM glutamine and 100 mM pyruvate), 1×10^{-4} M 2-mercaptoethanol and 0.2% sodium bicarbonate. To maintain ES cells in their undifferentiated state 100 U/ml of LIF (obtained as culture supernatant from Cos-7 cells transfected with a LIF gene-containing expression vector (Smith, 1991)) is added to the culture medium. 1 unit (U) of LIF is defined as that volume of transfected Cos-7 supernatant in 1 ml of medium giving rise to detectable inhibition of ES cell differentiation. Cells are incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air usually in 25 cm^2 flasks coated with 1% gelatin.

ES cell harvesting

ES cells are harvested when sub-confluent.

1 After aspiration of the spent culture medium the cells are washed gently with 5 ml PBS.
2 Cells are released into a single cell suspension by the addition of 1 ml of TVP (1% chick serum, 1% trypsin, 8.4×10^{-4} M EDTA) and incubation at 37 °C for 2 min.
3 The cell suspension is harvested after physical agitation, 9 ml of culture medium added and the suspension spun at 100*g* for 5 min.
4 After aspiration of the supernatant and resuspension in 10 ml culture medium the cells are counted in a haemocytometer.
5 A similar procedure is used should cells need to be frozen at this stage except that cells are harvested in 7 ml of medium spun and resuspended in 1 ml of 10% DMSO (v/v) in ES cell culture medium. 0.5 ml of suspension is then pipetted into a cryo-tube and frozen at −70 °C overnight prior to storage in liquid nitrogen.

From a sub-confluent 25 cm^2 flask, one should typically expect a yield of $\sim 5–9 \times 10^6$ cells.

Generation of undifferentiated ES cell aggregates

Studies of the differentiation of mesodermal lineages from ES cells have usually relied on the formation of aggregates of ES cells (see Wiles, 1993; Hole & Smith, 1994) prior to the formation of embryoid bodies (see below). Such aggregates have been formed by enzymatic digestion of monolayer

cultures (Schmidt, Bruyns & Snodgrass, 1991), direct formation in semi-solid medium (Keller *et al.*, 1993; Wiles, 1993) and in hanging drops (Hole & Smith, 1994). This last technique is the one currently in use in our laboratories for the differentiation of transplantable haematopoietic progenitors and will be described in some detail. After the removal of LIF, aggregates will differentiate *in situ* in methyl cellulose, in suspension culture or on stromal layers. A proportion of these aggregates form 'embryoid bodies' (EBs), complex structures with large fluid-filled cysts that have a superficial similarity to embryonic yolk sacs. These structures contain cells of a number of different lineages, such as muscle, endothelium and cells of the haematopoietic system: evidence for haematopoietic cells is often indicated by the presence of haemoglobinised blood islands.

The benefits of one differentiation system over another are a matter of some debate. Haematopoietic differentiation in suspension culture or in semi-solid medium may well be dependent on the formation of EBs (Doetschman *et al.*, 1985) and while some workers have claimed that formation of cystic structures may be a pre-requisite for complete differentiation (Chen *et al.*, 1992; Hooper, 1994), this may not be an absolute requirement in the case of haematopoietic differentiation (Doetschman *et al.*, 1985; Schmidt *et al.*, 1991). Differentiation in EBs can be followed for extended periods (>30 days), but two to three weeks is a more normal time period.

Hanging drop method

Although this method may be time-consuming (Fig. 1.1) it produces, prior to differentiation, ES cell aggregates of a uniform size. This may be important for subsequent differentiation into embryoid bodies since endogenously produced LIF may exert some residual autocrine or paracrine function for a time after removal of exogenous LIF.

1 Adjust the density of the cell harvest (see above) to $\sim 3 \times 10^4$ cells/ml with culture medium and add 100 U/ml of LIF.
2 Add 10 ml water to the base of a (square) bacteriological plate. Turn the lid upside down and using an Eppendorf multipipette, or its equivalent, dispense 10 μl drops of cell suspension (approx. 300 cells) on the now upturned underside of the lid taking care to keep sufficient distance between drops to avoid coalescence; approximately 100 drops can be placed on one lid.
3 With a smooth, swift action, replace the lid on its base and carefully place

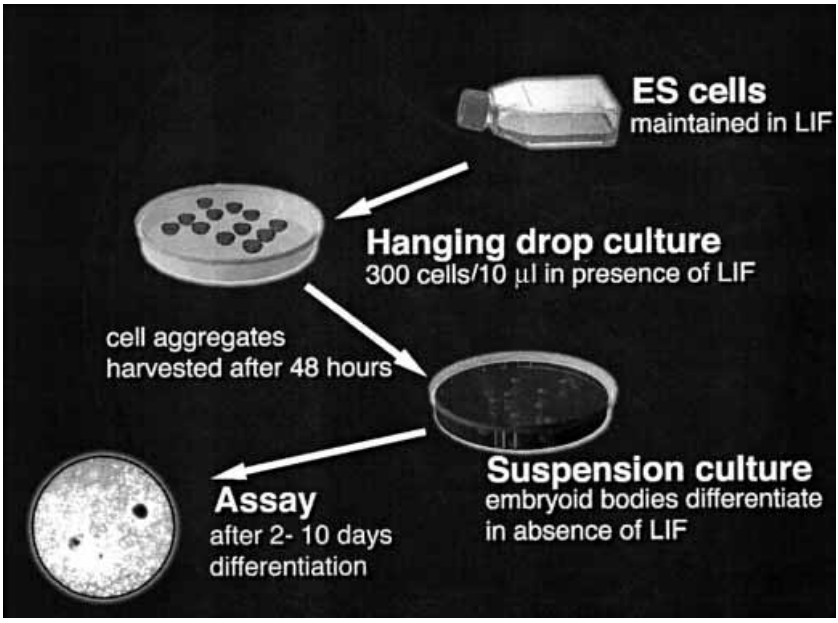

Fig. 1.1. Schematic diagram of haematopoietic differentiation of embryonal stem cells *in vitro*: hanging drop method.

the dish in a humidified incubator in an atmosphere of 7.5% CO_2 in air. Culture the hanging drops for 48 h.

4 After two days the ES cells will have settled in the meniscus of the hanging drop forming smooth, spherical aggregates of a uniform size.
5 With cell division these aggregates have relatively little structure and are sensitive to mechanical disruption but will now contain ~1500 cells, the majority of which should be in an undifferentiated state. They are therefore harvested with care by aspiration with a wide bore pipette and placed in a 15 ml centrifuge tube and centrifuged at 80*g* for 3 min.
6 Aspirate the supernatant from the aggregates and wash twice in 5 ml differentiation medium.

High density culture

A more rapid method for generating large numbers of aggregates is to culture ES cells at high density in non-gelatinised flasks. The ES cells form aggregates that are only loosely attached to the substrate and these can be harvested directly. Aggregates prepared in this way may vary in size and their subsequent differentiation is also variable.

1 Seed a 25 cm^2 non-gelatinised tissue culture flask with 2×10^6 ES cells in 10 ml of pre-warmed culture medium in the presence of LIF.
2 Culture for two days in humidified 7.5% CO_2 incubator.
3 Depending on the growth rate of the ES cell line used, the medium may have to be changed after one day. If so, simply carefully replace spent medium with fresh culture medium and LIF. In replacing the medium, be careful not to disturb the attached cells at this point.
4 The aggregates are then harvested directly in a similar way to that described above.

Formation of embryoid bodies (EBs)

Differentiation in suspension culture

1 Re-suspend ES cell aggregates formed as described above at a concentration of ~100 aggregates/ml in differentiation medium containing 50 units/ml of penicillin and 50 μg/ml of streptomycin.
2 The suspension is cultured in 9 cm microbial Petri dishes in a humidified atmosphere of 7.5% CO_2 in air. The medium should be changed every two days by allowing EBs to settle and aspirating supernatant.
3 The aggregates do not attach to the Petri dishes, but as the EBs begin to differentiate they adhere to the substrate, and can be detached by gentle aspiration. The EBs that develop can form very large cystic structures (up to ~6 mm in size). (See Fig. 1.2).

Differentiation in semi-solid medium

Suspension culture of EBs is appropriate where the haematopoietic cells under study are immobile or only poorly motile (such as HSC or red blood cells [RBC]). However, where motile haematopoietic cells are being investigated, identifying which EBs are producing (for example) macrophages may require a method that restricts dispersion of EBs and their progeny. One approach is to culture aggregates in semi-solid medium, although the difficulties of changing the medium in this system militates against extended (>15 days) culture. The semi-solid medium of choice in our laboratory contains 0.3% Noble agar.

1 Prepare 0.3% Noble agar by mixing equal volumes of freshly prepared 0.6% (2×) agar stock (2.4 g of agar boiled in 200 ml of tissue culture grade water) which has been cooled to 56°C in a water bath and 2× differentiation medium pre-warmed to 37°C.

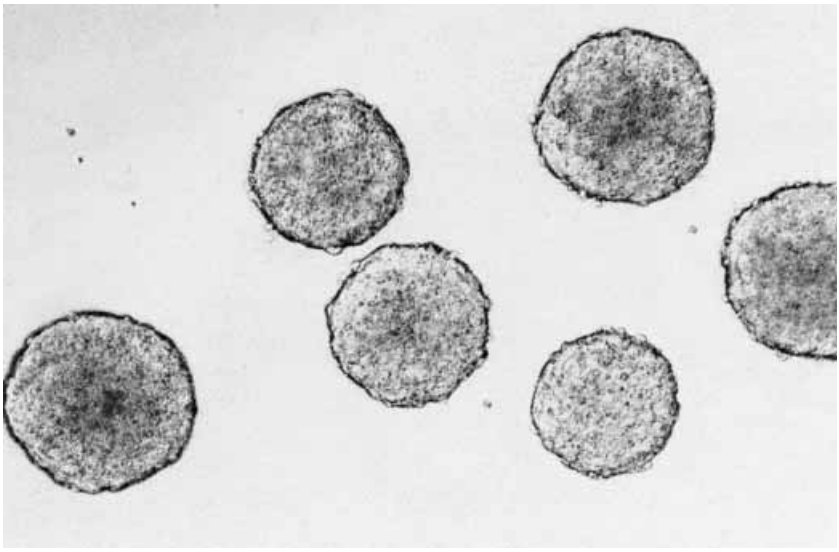

Fig. 1.2. Embryoid bodies after 2 days of culture in the absence of LIF.

2 Stir the mixture and allow to equilibrate at 37 °C.
3 Approx. 100–500 ES cell aggregates should be re-suspended in 10 ml agar medium, transferred to a 9 cm microbial Petri dish and cultured as above.

If necessary it is possible to 'feed' the EBs by gently overlaying the culture with 5 ml fresh differentiation medium.

Direct differentiation in methyl cellulose

Direct plating of ES cells into a medium containing methyl cellulose allows ES cells to form EBs and undergo haematopoietic differentiation without the prior formation of ES cell aggregates. This method is quicker than those described above and it may be more appropriate for other ES cell isolates, but in our hands it does not improve differentiation and is still subject to the problems of culture in semi-solid medium.

1 Methyl cellulose medium is prepared by mixing an equal volume of 2× methyl cellulose stock (3.6 g of methyl cellulose dissolved in 200 ml of gently boiling tissue culture grade water and then allowed to to cool in a 37 °C water bath) with pre-warmed 2× differentiation medium.
2 The medium is stirred and allowed to equilibrate.

3 Approx. 10^3 cells/ml of freshly harvested ES cells are cultured in 2 ml methyl cellulose medium in 4 cm microbial Petri dishes.
4 Culture in a humidified 7.5% CO_2 in air atmosphere.

Discrete colonies of ES cells and subsequent EBs will begin to appear in the methyl cellulose medium after approximately three days.

This method is an adaptation of Wiles & Keller (1991). Their basic differentiation medium was Iscove's modified Dulbecco's medium (IDDM) supplemented with 4.5×10^{-4} M monothioglycerol and 10% fetal calf serum (FCS).

Any of the methodologies described above lend themselves to the inclusion of growth factors and/or cytokines in the medium to study the requirements, for example, for haematopoietic stem cell proliferation and differentiation or to alter the morphogenetic microenvironment generally.

Several groups have reported attempts to differentiate ES cells on bone-marrow-derived stromal cell substrates but the inevitable result of these experiments was preferential differentiation into cells of macrophage and monocyte lineages. This was thought to be due to the secretion of macrophage colony stimulating factor (M-CSF). More recently a Japanese group (Nakano, Kodama & Honjo, 1994) have used the OP9 stromal cell line, which was developed from an osteopetrotic mouse mutant [(C57BL/6× C3H)F2-op/op], which constituitively lacks functional M-CSF secretion. In these experiments ES cells were seeded directly onto the stromal layer in suspension culture. After passage the ES cells were shown to have differentiatied into a variety of cell types of the lymphohaematopoietic system with the formation of multilineage colonies. This system was characterised by the absence of embryoid body-like structures.

Analysis of differentiated progeny

Obviously once haematopoietic differentiation has been achieved by any of the above techniques many forms of analysis are possible. These include histological examination, flow cytometric analysis, gene expression studies, analyses of haematopoietic colony-forming potential and studies of the ability of the differentiated progeny to reconstitute the haematopoietic system of, say, irradiated recipient animals.

Histology

Simple histological analysis of cystic EBs has been used to define the appearance of blood islands, neuroepithelium, mesoderm and visceral and parietal

endoderm (Doetschman *et al.*, 1985; Hooper, 1994) and some groups have claimed that distinct areas of the embryoid bodies allow development of lymphoid cells in particular (Chen *et al.*, 1992). Erythroid cells were the first haematopoietic cell type to be described in differentiating embryoid bodies (Doetschman *et al.*, 1985) using light microscopy to observe haemoglobinisation directly. The red colour associated with erythropoiesis appears in EBs from 5 to 14 days after the initiation of differentiation. Quantitation of this phenomenon is a non-destructive and rapid means of determining the effects of culture conditions on haematopoietic development but does not in itself convey much information about other haematopoietic lineages.

Flow cytometric analysis

Flow cytometric analyses can be used to follow the differentiation of haematopoietic precursors and their progeny during EB differentiation. Although in mice a precise cell surface phenotype of HSCs has yet to be defined, most of the later lineages can be distinguished by the expression of specific antigens. The presence of B cells (B220 + ve), erythroid lineage cells (TER 119 + ve) and myeloid (Mac-1 + ve) cells have all been detected by flow cytometry, although in some laboratories only small numbers of cells expressing the haematopoietic lineage marker CD45 have been seen and those cells expressing neutrophil, macrophage and B cell markers have not been detected (Schmidt *et al.*, 1991). Problems arising with flow cytometric analyses include:

1 Embryoid bodies contain very few haematopoietic cells at least at early stages of differentiation and these may often be at the limits of flow cytometric detection.
2 The antigens that define lineages in the context of normal haematopoiesis may be expressed by non-haematopoietic lineages within the embryoid bodies. For example, ES cells possess low-level mRNA transcripts for Thy-1, CD44 and Ly-6 (Schmidt *et al.*, 1991; Hole *et al.*, 1996*a*,*b*). The appearance of these markers on the surface of differentiated progeny may thus not define them as haematopoietic.
3 During the preparation of single-cell suspensions from EBs, several groups commonly use enzymatic digestion protocols. This kind of treatment may alter the cell surface expression of markers by enzymatic degradation.

We have used two methods to derive single-cell suspensions from EBs to circumvent problem (3):

Homogenisation

1 EBs are harvested and washed twice in PBS by allowing the bodies to settle and aspirating the supernatant.
2 They are then resuspended in 1–2 ml of 1 mM EDTA in PBS and left at room temperature for 5 min.
3 After transfer to a glass or Teflon homogeniser, 5 strokes of the plunger will disrupt the EBs.
4 The supension is transferred to a 15 ml centrifuge tube and left to allow cellular clumps to settle out.
5 The single-cell suspension in the supernatant is harvested and washed in 10 ml PBS containing 1% heat-inactivated fetal calf serum by centrifugation at 100*g* for 5 min.
6 After re-suspension the cell count and viability is checked by trypan blue dye exclusion. The cell yield should be $1–5 \times 10^3$ cells/embryoid body, depending on time of differentiation, with a viability of 70–80%.

Enzyme digestion

1 Embryoid bodies are harvested and washed twice in PBS by allowing the bodies to settle and aspirating the supernatant.
2 Resuspend in 2 ml of TVP, transfer to a 15 ml centrifuge tube and incubate for 20 min at 37 °C on a roller.
3 Break up the remaining EB structures by gently pipetting through a Pasteur pipette.
4 Leave the suspension to settle for 5 min.
5 The supernatant containing a single-cell suspension is then harvested leaving behind any remaining clumps and non-cellular proteinaceous material, transferred to 10 ml of culture medium and centrifuged at 100*g* for 5 min.
6 Repeat the wash step in PBS containing 1% heat-inactivated PBS.
7 Cell counts and viability are checked by trypan blue exclusion.

Viability using this procedure should be between 80 and 90%. An alternative to TVP is to use Dispase (Boehringer Mannheim). EBs are incubated in PBS containing 1 unit/ml of Dispase for one hour with gentle agitation, aspirated several times through a 23-gauge needle and then washed as before. This has been our method of choice for repopulation studies (see below).

Gene expression studies

An alternative to flow cytometric analyses for many laboratories has been to follow the patterns of temporal and/or lineage restricted expression of mRNA transcripts in differentiating EBs using reverse transcriptase–polymerase chain reaction (RT-PCR) and Northern blotting. A detailed description of the methodologies involved and the data obtained through this approach is beyond the scope of this chapter and has been reviewed elsewhere (Hole & Ansell, 1996). Although this kind of approach does allow studies of developmental regulation of EB differentiation it is invasive and is dependent on the preparation of mRNA from bulk cultures. In the future, *in situ* expression analysis in individual EBs and visualisation with, say, confocal microscopy, may allow studies of the simultaneous expression of genes involved in haematopoietic differentiation both temporally and spatially. Thus it may be possible to relate gene expression in haematopoietic cells within EBs with co-ordinate expression in stromal elements in the vicinity. Attempts are being made to combine flow cytometric analyses with gene expression studies (Ansell & Hole, unpublished) by using ES cells made transgenic for fluorescent intracellular markers such as β-galactosidase or Green Fluorescent Protein, driven by the controlling elements of genes known to be expressed early during haematopoietic differentiation. These cells can then be sorted from bulk cultures and subjected to further molecular/biochemical analyses obviating the problems (described above) inherent in conventional flow cytometric analyses.

Analysis of colony forming potential

Characterisation of haematopoietic progenitors has been possible by the use of *in vitro* colony forming assays. Several of these have been used to confirm that the programme of ES cell differentiation follows the normal route of haematopoietic development and the expected spectrum of progenitors and cells from the multipotential to the terminally differentiated has been detected. Mixed-cell, erythroid and granulocyte/macrophage colony forming units have all been detected, first appearing at or around day 4 of differentiation, before the appearance of markers of their respective terminally differentiated progeny and thereafter decreasing in number as differentiation procedes. These data indicate that the appearance of the most primitive haematopoietic progenitors may be an early and transient event during embryoid body differentiation. Although there are no colony

forming unit assays that will detect the highly pluripotent HSCs that will effect long-term repopulation, there are several that can be used to detect multipotent progenitors. We have used one of these, the CFU-A assay (Pragnell *et al.*, 1994), which detects a primitive progenitor that has similar cycling characteristics to the CFU-S found in normal and regenerating marrow and responds to CFU-S-specific regulators, to assay for the earliest appearance of 'stem cells' in differentiating EBs.

CFU-A (colony forming units type A) assay

The *in vitro* CFU-A assay was set up essentially as described previously (Pragnell *et al.*, 1994). Briefly:

1 A 1 ml feeder layer consisting of 6% Noble agar in αMEM, containing 10% donor horse serum and containing conditioned medium as a source of cytokines is established in a 3 cm diameter tissue culture Petri dish. The feeder layer contains 10% conditioned medium from the L-929 fibroblast cell line (a crude source of CSF-1 [ECACC85011425]) and 5% conditioned medium from the AF1-19T cell line (a crude source of GM-CSF. The AF1-19T cell line is an NRK rat fibroblast cell line transformed with a malignant histiocytosis sarcoma virus (Franz *et al.*, 1985)). These cell lines are grown in roller bottles with modified Eagles medium (MEM) and 10% foetal calf serum to half-confluence. Conditioned medium is removed, filtered and stored at $-20\,^{\circ}C$.
2 EBs either intact (50–150) or homogenised (see above – 10^5cells/plate) are added to 3% Noble agar in αMEM supplemented with 10% horse serum to form an upperlayer.
3 The dishes are incubated for 11 days in a humidified atmosphere of 10% CO_2 and 5%O_2.
4 In this assay CFU-A are scored as macroscopic colonies of >1 mm diameter (Fig. 1.3).
5 The self-renewal potential of these colonies may be checked by 'picking' them after 7 days of culture. After disaggregating by vigorous pipetting in 100 ml of αMEM, individual CFU-A cell suspensions are then re-plated in the CFU-A assay.

The CFU-A assay and others that can detect primitive multipotential haematopoietic progenitors are useful, quick and easy assays for the early detection of haematopoiesis *in vitro*. They are however unable to define the

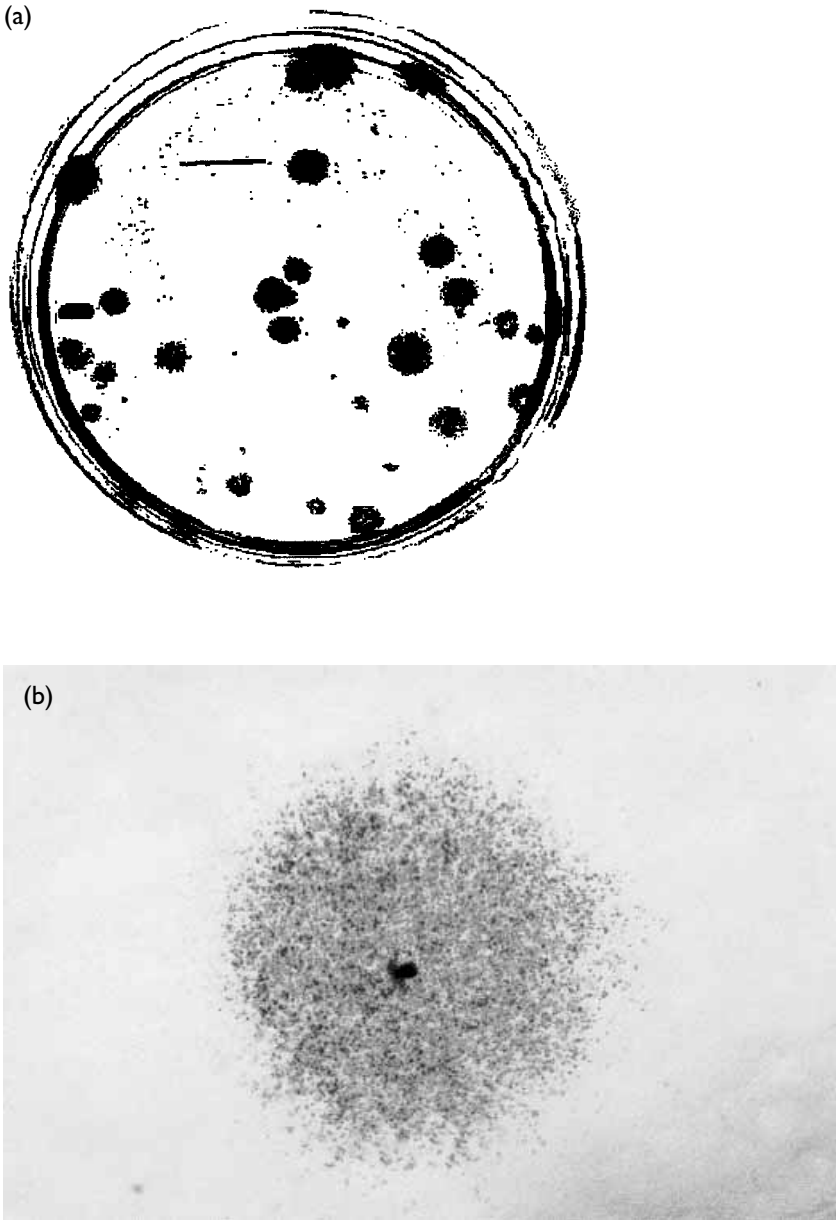

Fig. 1.3(a). Petri dish containing large CFU-A colonies clearly visible to the naked eye. (b). Close-up of a single CFU-A generated from an intact embryoid body. The remains of the three-dimensional structure of the body are at the centre of the colony.

true long-term repopulating HSCs. *In vitro* functional detection of lymphoid precursors is also difficult. In order to overcome these problems, adoptive transfer into irradiated or otherwise haematopoietically-compromised animals is necessary.

Reconstitution of recipient animals with ES-derived haematopoietic progenitors

Multilineage long-term reconstitution of recipients with ES-derived haematopoietic progenitors has been reported by Palacios *et al.* (1995) and ourselves (Hole *et al.*, 1996*a*). In the former paper, ES-cell to haematopoietic progenitor cell differentiation was achieved in culture conditions using a stromal layer supplemented with an undefined cytokine cocktail. In our system, having identified the time-point of first appearance of haematopoietic cells using the CFU-A assay, we reasoned that repopulating cells may be present 24 hours earlier. Thus EBs after 4 days of differentiation (without added cytokines) were disaggregated by the Dispase method (see above) and injected (along with limiting doses of differentially marked carrier spleen cells) intravenously into mice irradiated with 10.5 Gy of γ-irradiation. Long-term (>2 years) multi-lineage repopulation with ES-derived progenitors was achieved with this strategy albeit at low levels ($<10\%$ of peripheral blood). Intriguingly we were also able to demonstrate that injection of embryoid body cell suspensions after 5 days of differentiation promoted long-term survival of the irradiated recipients without concomitant evidence of ES-cell derived repopulation (Hole *et al.*, 1996*a*).

Conclusions/prospectives

The potential of utilising totipotential stem cells as an unlimited source of haematopoietic stem cells (and possibly others) is only now becoming realised. The increasing definition of the programme of *in vitro* differentiation of embryonal stem cells and primordial germ cells will allow more detailed analysis of the haematopoietic process. Although the work described here refers only to embryonal stem cells derived from mouse, the isolation of these cells from other species, including man, would allow the development of new approaches to the detection and possible therapy of haematopoietic disease.

Acknowledgements

Work described in this chapter has been supported in part by grants from the Leukemia Research Fund, Wellcome Trust and Melville Trust for Cancer Research. The authors acknowledge the help and advice of their colleagues Dr Gerry Graham and Dr Austin Smith and the technical assistance of Janice Murray, Helen Taylor and Kay Samuel.

References

Chen, U., Kosco, M. & Staerz, U. (1992). Establishment and characterisation of lymphoid and myeloid mixed-cell populations from mouse late embryoid bodies, 'embryonic-stem-cell fetuses'. *Proc. Natl. Acad. Sci. USA*, **89**, 2541–5.

Doetschman, T., Eisetter, H., Katz, M., Schmidt, W. & Kemler, R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.*, **87**, 27–45.

Forrester, L. M., Bernstein, A., Rossant, J. & Nagy, A. (1991). Long-term reconstitution of the mouse hematopoietic system by embryonic stem cell-derived fetal liver. *Proc. Natl. Acad. Sci. (USA)*, **89**, 7514–17.

Franz. T., Lohler, J., Fusco, A., Pragnell, I., Nobis, P., Padua, R. & Ostertag, W. (1985). Transformation of mononuclear phagocytes in vivo and malignant histiocytosis caused by a novel murine spleen focusing virus. *Nature*, **315**, 149–51.

Hole, N. & Ansell, J. D. (1996). In vitro differentiation of embryonal stem cells as a tool for the study of haematopoiesis. In *Weir's Handbook of Experimental Immunology*, vol. IV, 5th edn, ed. L. A. Herzenberg, D. Weir, L. A. Herzenberg & C. C. Blackwell, pp. 147.1–147.5. Oxford: Blackwell Science.

Hole, N. & Smith, A. G. (1994). Embryonic stem cells and hematopoiesis. In *Culture of Hematopoietic Cells*, ed. R. I. Freshney, I. B. Pragnell & M. G. Freshney, pp. 235–49. New York: Wiley-Liss.

Hole, N., Graham. G. J., Menzel, U. & Ansell, J. D. (1996*a*). A limited temporal window for the derivation of multilineage repopulating progenitors during embryonal stem cell differentiation. *Blood*, **88**, 1266–76.

Hole, N., Leung, R., Doostdar, L., Menzel, U., Samuel, K., Murray, J., Taylor, H., Graham, G. & Ansell, J. D. (1996*b*). Haematopoietic differentiation of embryonal stem cells in vitro. In *Gene Technology*, ed. A. Zander, pp. 3–17. Springer Verlag.

Holyoake, T. L., Alcorn, M. J., Richmond, L., Farrell, E., Pearson, C., Green, R., Dunlop, D. J., Fitzsimons, E., Pragnell, I. B. & Franklin, I. M. (1997). CD34 +ve PBPC expanded ex vivo may not provide durable engraftment following myelo-ablative chemo-radiotherapy regimes. *Bone Marrow Transplant.*, **19**, 1095–101.

Hooper, M. L. (1994). *Embryonal Stem Cells*. Chur: Harwood Academic.

Keller, G., Kennedy M., Papayannopouloum, T. & Wiles, M. V. (1993). Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol. Cell. Biol.*, **13**, 473–86.

Nakano, T., Kodama, H. & Honjo, T. (1994). Generation of lymphohematopoietic cells from embryonic cells in culture. *Science*, **265**, 1098–101.

Nichols, J., Evans, E. P. Smith, A. G. (1990). Establishment of germ-line competent embryonic stem (ES) cells using differentiation inhibiting activity (DIA). *Development*, **110**, 1341–8.

Palacios, R., Golunski, E. & Samardis, J. (1995). In vitro generation of haematopoietic stem cells from an embryonic stem cell line. *Proc. Natl. Acad. Sci. USA*, **92**, 7530–4.

Pragnell, I. B., Freshney, M. G. & Wright, E. G. (1994). CFU-A assay for measurement of murine and human early progenitors. In *Culture of Haematopoietic Cells*, ed. R. I. Freshney, I. B. Pragnell & M. G. Freshney, pp. 67–79. New York: Wiley-Liss.

Schmidt, R. M., Bruyns, E. & Snodgrass, H. R. (1991). Hematopoietic development of embryonic stem cells in vitro: cytokine and receptor expression. *Genes Develop.*, **5**, 728–40.

Smith, A. G. (1991). Culture and differentiation of embryonic stem cells. *J. Tiss. Cult. Meth.*, **13**, 89–94.

Smith, A. G., Heath, J. K., Donaldson, D. D. *et al.* (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature*, **336**, 688–90.

Wiles, M. V. (1993). Embryonic stem cell differentiation in vitro. *Meth. Enzymol.*, **225**, 900–18.

Wiles, M. V. & Keller, G. (1991). Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* **111**, 259–67.