Haematopoietic Differentiation of Embryonal Stem Cells.

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Dedicated to my Father and Mother.

Declaration.

I declare that the composition of this thesis and the work presented therein was carried out by myself, except where indicated.

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Contents

Abstract

The molecular events which control haematopoietic development, stem cell commitment and self-maintenance are poorly defined and understood. Although many advances have been made in recent years with improved stem cell purification and culturing techniques, the various limitations attached to these techniques have constrained such study. The use of murine totipotential embryonic stem (ES) cells have to an extent surmounted some of these limitations. In the appropriate culture conditions ES cells and their differentiated 3-dimensional aggregates, embryoid bodies, are able to give rise to all haematopoietic lineages and have consequently proved particularly useful for studying gene expression and regulation. Furthermore the ability to ablate gene function by the generation of null alleles (gene knockouts) allows the examination of function, directly.

Exploiting the ability of ES cells to differentiate toward haematopoietic lineages, the project sought to investigate gene expression with a view to identifying novel genes/proteins involved in the early stages of ES cell commitment to haematopoietic lineages. In the culture system used, it has been shown that under standard differentiation conditions 30-50% of embryoid bodies are able to give rise to haematopoietic colonies. This level of haematopoietic commitment was modulated with the addition of chemical inducers to the differentiation environment of embryoid bodies. Using the CFU-A assay to assess haematopoietic commitment and differentiation, we have found that the number of CFU-A colonies can be increased 2-fold after treatment of embryoid bodies with 1% DMSO during the first 48 hours of differentiation, and conversely reduced to below 5% after treatment with 10^{-8} M all *trans*-retinoic acid (atRA) for the same period.

Lineage commitment in untreated, DMSO and atRA treated embryoid bodies was further investigated using reverse transcription-polymerase chain reaction techniques. The mesoderm marker *brachyury* was found to be expressed in both DMSO and atRA treated samples to similar extents, suggesting neither compound to be exclusively exerting its effect by enhancing or inhibiting mesoderm commitment. However, the level of transcripts of various haematopoietic markers such as β-globin, PU-1 and CD45 were found to be raised in DMSO treated embryoid bodies compared to either untreated or atRA treated samples.

Employing the opposing effects of DMSO and atRA, 2-dimensional protein gel electrophoresis of L-[^{35}S]-methionine pulse labelled embryoid bodies was used as a screening method for the identification of proteins that could be involved in either ES cell haematopoietic commitment, or haematopoietic progenitor cell differentiation. After analysis of the gels several proteins were found to display an expression profile mirroring the CFU-A assay results. That is their expression level was elevated and diminished after DMSO and atRA treatment respectively. These proteins therefore serve as strong candidates for further identification and analysis for future studies. Furthermore the contrasting effects of the two compounds are being investigated for differential cDNA library screening.

It has been clearly demonstrated, that the haematopoietic commitment of ES cells and their differentiated aggregates can be successfully modulated by the addition of chemical inducers. Consequently these effects will prove a useful means of screening for genes and proteins involved in haematopoietic commitment and differentiation.

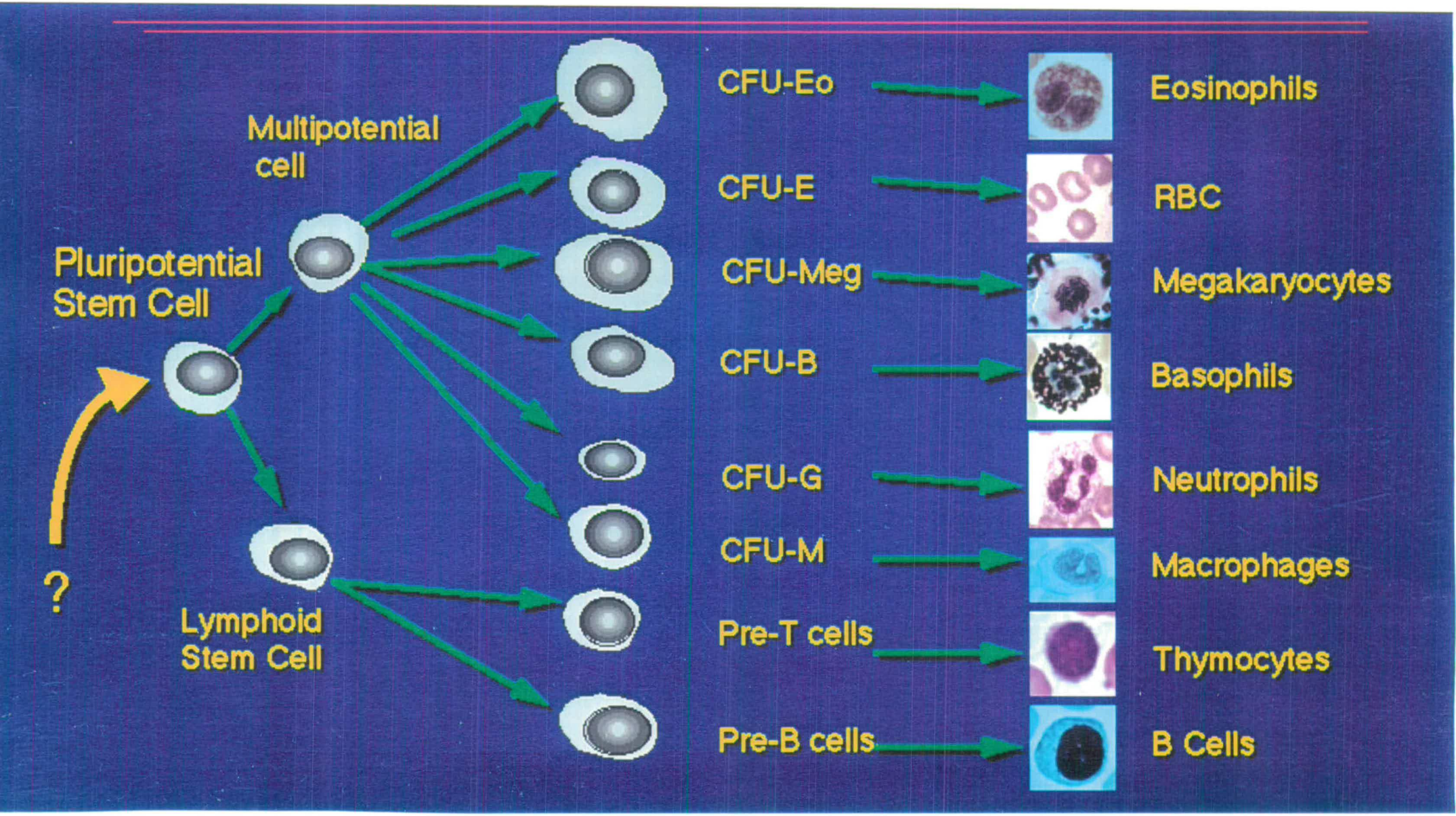


Figure 1.1 The haematopoietic cascade (figure courtesy of Dr. N. Hole)

1. Introduction.

1.1 Haematopoiesis.

Haematopoiesis is the unidirectional process whereby pluripotential haematopoietic stem cells (HSCs) differentiate to give multipotent progenitors, which in turn commit to and terminally differentiate towards one of the lineages in the lymphohaematopoietic system (Figure 1.1). Crossing interdisciplinary boundaries from immunology and haematology to developmental biology, haematopoiesis demonstrates basic biological principles such as homeostasis and biological plasticity; exemplifies a complete model intergrating stem cell biology, cellular commitment, differentiation, growth factor biology, cellular signalling, transcriptional control, cell death and survival; and provides insights not only into normal but disease and malignant states.

Although much remains elusive about this intricate process, numerous advances have been made in recent years through improved tissue culture and molecular biological techniques. An overview of some of these findings is outlined in the following sections.

1.1.1 Haematopoietic stem cells.

Haematopoietic stem cells (HSCs) are defined as the population of cells with the dual capacity of self-maintenance, and differentiation into an expanded population of progenitor cells. It has been proposed that HSCs persist throughout an animal's life, continually replenishing the terminally differentiated blood cell compartments which have limited lifespans (Keller and Snodgrass, 1990; Jordan *et al* 1990). It is thought that during steady state most HSCs are in the G_0 phase of the cell cycle, and due to this quiesence are able to maintain their genetic integrity over long periods of time (Lajtha, 1979). While proliferating however, HSCs display a diminished stem cell activity (Fleming *et al*, 1993)

In the adult, HSCs reside in the bone marrow and have been estimated by various studies to constitute no more than 1 per 10000 of nucleated marrow cells in the mouse (Micklem *et al*, 1987; Harrison *et al*, 1988). Due to their rarity it has proved difficult to isolate and characterise these cells. Isolation would not only facilitate the study of the growth and differentiation of HSCs, but would also be beneficial for the purposes of gene therapy and

cell/organ transplantation. The definitive assay for the detection of HSCs utilises their unique ability to provide long-term repopulation of all lineages of the haematopoietic system, usually of lethally irradiated recipients (Till and McCulloch, 1968).

Using cell surface marker expression patterns Spangrude *et al* (1988) have shown that bone marrow cells which express high levels of stem cell antigen-1 (Sca-1), low levels of the cell surface differentiation antigen Thy-1 and display either low levels or complete absence of specific cell surface markers for erythroid (e.g. B220), myeloid (e.g. Mac-1) and lymphoid lineages (e.g. CD4CD8) (collectively reffered to as Lin), to be a rich source of stem cells. Although these cells were later shown to be more heterogeneous than previously thought, consisting not only of long- but also short-term repopulating cells (Smith *et al*, 1991a), Sca-1^{+} Thy-1^{lo} Lin^{-} cells still remain a useful starting population for further HSC enrichment.

Some enrichment techniques applied include: 1) separation of cells on the basis of size and density (Jones *et al*, 1990; Ploemacher and Brons, 1989); 2) low uptake of fluorescent dyes e.g. rhodamine 123 which is taken up by the mitochondrial membrane (Spangrude and Johnson , 1990) and Hoechst 33342 which binds DNA (Neben *et al*, 1991); 3) resistance to cytotoxic agents such as 5-fluorouracil which do not affect quiescent HSCs (Hodgson and Bradley, 1979); 4) adherence to plastic tissue culture flasks (Kiefer *et al*, 1991).

Although some of these procedures have yielded highly enriched populations of HSCs their separation from the more mature spleen colony forming cells (CFU-S) with only short-term repopulating ability has proved difficult (Jones *et al*, 1989). There are furthermore two types of CFU-S cells: those which form the characteristic nodules on the spleens of recipients 8 days after injection (CFU-S_8), and a slightly more primitive cell type which form nodules after 12 days (CFU-S_{12}) (Till and McCulloch, 1968)

Recently Morrison *et al* (1995) have identified a cell subset from the liver of day 13.5 p.c. foetuses which display typical characteristics of HSCs, that is they provide long-term multineage haematopoietic reconstitution when as few as 6 cells are injected into lethally irradiated recipients. However it has been argued that in principle it is never possible to isolate true stem cells unequivocally, since in order to be defined they must be exposed to experimental manipulation, such as those described, which may in turn affect the properties of the cells (Potten and Loeffler 1990).

Various models for the commitment and differentiation of HSCs have been proposed two of which predominate. In both models haematopoietic growth factors and cytokines play key roles. Although much of the evidence for these models has been derived from *in vitro* cultures some of the findings have been found to have physiological relevance. The first model suggests that self-maintenance and commitment of HSCs and multipotent stem cells is stochastic in nature and growth factors act to promote the survival and expansion of cells which become commited to a particular lineage (Ogawa, 1993). This hypothesis is thought to be supported by the fact that multilineages can be derived from colonies of single cell origin, and by findings which suggest that haematopoietic growth factors suppress apoptotic cell death (Kinoshita *et al*, 1995; Abrahamson *et al*, 1995).

The second model proposes a more instructive system in which stromal cells in the haematopoietic microenvironment direct every stage of haematopoietic cell development via cytokine release (Uchida *et al*, 1993). The bone marrow consists of a heterogenous population of cells. Excluding haematopoietic cells these comprise endothelial cells, fibroblasts and adipocytes amongst others, and are thought to influence and regulate the commitment of pluripotent and multipotent haematopoietic cells (Perkins and Fleischman, 1988). This model is supported by the results of long-term bone marrow culture (LT-BMC) experiments, first described by Dexter *et al* (1977), where growth of immature haematopoietic cells and the sustenance of the colonies thereafter, take place and have been shown to be dependent on direct physical contact with the stromal layer. This direct contact is thought to mimic the *in vivo* circumstances. However a recently isolated mouse stromal cell line has been shown to support haematopoietic growth without direct cell-cell contact via a putative diffusible growth factor(s) (Burroughs *et al*, 1994).

In an attempt to show the relevance of the microenvironment directed haematopoiesis model to the *in vivo* sitution Friedrich *et al* (1996) have isolated a large number of conditionally immortalised stromal cell lines from mouse bone marrow and foetal liver. These cell lines do not express haemtaopoietic related cell surface markers, but resemble epithelial and fibroblastic cells and preferentially support either lymphoid or myeloid differentiation of primitive haematopoietic cells respectively. Although there is mounting evidence in favour of the microenvironment directed model (Just *et al* 1991; Morrison and Weissman, 1994), both the stochastic and instructive systems probably contribute to *in vivo* haematopoiesis

1.1.2 Haematopoietic growth factors.

Haematopoietic growth factors are soluble molecules originally isolated from LT-BMC systems, some of which are now used clinically for the treatment of leukaemias and other disorders (reviewed in Sachs, 1996). A variety of cytokines and interleukins (IL) have since been isolated and shown to have a wide range of functions in the immunohaematopoietic system, from the maintenance of HSCs, e.g. stem cell factor (SCF) (Tsuji *et al*, 1991), to initiating an immune response e.g. interferon γ (IFN-γ).

Some cytokines can act in a lineage specific manner, such as erythropoietin (Epo) and granulocyte-colony stimulating factor (G-CSF), which support the growth of erythroid and granulocytic lineages respectively. The great majority of cytokines however, display functional pleiotropy such as IL-3 and IL-6. The former supports the growth of erythroid, myeloid and megakaryocytic lineages while the latter not only induces the maturation of B-cells into antibody producing plasma cells but effects T-cell and monocytic growth. One of the consequences of pleiotropy is functional overlap and hence redundancy. For instance several other factors such as IL-2 and IL-4 are also able to induce antibody production in B-cells. Redundancy in the system is particularly well demonstrated with gene 'knock-out' experiments in mice, where the introduction of mutations into a specific gene can render that gene disfunctional. For example in the case of IL-2 which was thought to be essential for T-cell development, the knock-out mice have in fact proved to be normal with this respect (Schorle, 1991).

Despite this redundancy there does appear to be a hierachy for cytokines and some do play a more crucial role than others. In the case of SCF which is thought to be involved in the cycling of stem cells, mice harboring a homozygous mutation in the gene suffer from severe anaemia detectable in foetuses from as early as 13 days gestation (Chui *et al* 1976), and the cytokine continues to play an important role in foetal and adult erythropopiesis (Chui *et al* 1978).

Another emerging characteristic of cytokines is their pleiotropy not only in the immunohaematopoietic system but in multiple cell types. Cytokines such as leukaemia inhibitory factor (LIF) and IL-6 have been found to exert an influence on a variety of cell types. For instance, LIF can stimulate the differentiation of sensory neurones from mouse neural crest cultures (Murphy *et al*, 1991), and both LIF and IL-6 have been found to stimulate the expression of acute phase plasma proteins from hepatocytes (Baumann and Schendel, 1991).

Not all cytokines support the proliferation of haematopoietic cells. Some, such as macrophage inflammatory factor-1α (MIP-1α) (Dunlop *et al*, 1992), transforming growth factor β (TGF-β) (Ottman and Pelus, 1988) and more recently IFN-γ (Selleri *et al* 1996) have all been shown to have inhibitory effects. Although the effects of TGF-β are likely to be more complex since mice bearing a knock-out mutation for this gene display impaired haematopoiesis and vasculogenesis (Dickinson *et al*, 1995). These findings will be discussed in more detail in section 1.2.

1.1.3 Haematopoietic growth factor receptors.

Haematopoietic growth factor receptors can be classified into several different families. The majority of haematopoietic cytokines, and some non-haematopoietic factors such as prolactin and growth hormone, exert their effects through a distinct family of membrane bound receptors termed the cytokine receptor superfamily (reviewed in Bazan, 1990). This family is further sub-divided into Types I and II according to their structural characteristics. One of the main features of cytokine/receptor association is the subsequent dimerisation of the receptor which appears to be a key step in the initiation of signal transduction. In the case of cytokine receptors which consist of a single chain such as those for Epo and G-CSF, two receptors homodimerise in response to ligand. Most receptors however share a common membrane bound chain which heterodimerises with single or multiple ligand specific chains, such as the common β (β_c) chain which heterodimerises with the IL-3, IL-5 and GM-CSF receptors upon ligand binding.

Another family of receptors which a growing number of haematopoietic cytokines have been found to exert their effects through, e.g. macrophage colony stimulating factor (M-CSF) and SCF (Sherr *et al*, 1985; Chabot *et al*, 1988), is the receptor tyrosine kinase receptor superfamily. The membrane bound tyrosine kinase receptors are a large family of proteins which mediate a variety of cellular signals in response to growth factor ligands. Many of these are also retrovirally encoded and can act as transforming oncogenes. Some members of this family, such as c-kit the receptor for SCF, have been shown to play an important role in murine haematopoiesis. In fact one characteristic feature of populations of haematopoietic cells containing HSCs is c-kit expression (Ikuta and Weissman, 1992).

The extracellular amino terminus of receptor tyrosine kinases consists of five immunoglobulin domains, thought to be essential for ligand binding while the intracellular segment is a highly conserved catalytic kinase. Like cytokine receptors,

receptor tyrosine kinases dimerise upon ligand binding followed by autophosphorylation which is an essential step in the signal transduction cascade. Cytokine receptors which lack an intrinsic kinase activity achieve this phosphorylation step by associating with cytoplasmic tyrosine kinases such as the Janus kinases (Jaks) which phosphorylate the receptors after dimerisation.

1.1.4 Signal transduction in haematopoietic cells.

Signal transduction across the cytoplasm is dependent on a series of protein kinases most of which are tyrosine kinases although some such as the mitogen activated protein (MAP) kinases have the ability to phosphorylate other amino acid residues (Rossmando *et al*, 1992). To date eight distinct families of protein tyrosine kinases have been identified including the Src and Jak families, many of which have been implicated in haematopoietic cell signal transduction. Tyrosine kinases interact with and can become activated by both cytokine and tyrosine kinase receptors, while a particular type of receptor can be associated with members of more than one family of tyrosine kinases. The IL-2 receptor for instance has been found to associate with both Src (Torigoe *et al* 1992) and Jak family of kinases (Johnston *et al*, 1994).

After ligand binding and receptor dimerisation, it is thought protein kinases come in close contact with receptors and become activated. The signal is then relayed across the cytoplasm via a series of protein kinases and adaptor proteins until it results in the activation of transcription factors. Due to their complexity much of the detail of signal transduction pathways triggered by specific cytokines remains unclear. However advances have been made and the MAP kinase pathway which is triggered by some receptors including the M-CSF receptor, c-fms is now reasonably well elucidated (Lioubin *et al*, 1994).

Tyrosine phosphorylation, which sets in motion the process of cell growth and differentiation must be regulated in order to avoid aberrant cell growth. One way in which this is achieved is through the opposing catalytic activity of protein tyrosine phosphatases. These are a growing family of proteins which can be either membrane bound, like CD45, or intracellular enzymes such as haematopoietic cellular phosphatase (HCP). Recent data suggests that intracellular phosphatases associate with receptor complexes in a similar way to kinases and negatively regulate activated receptors (Yi *et al*, 1993).

The role of HCP in the regulation of haematopoiesis is further emphasised by the spontaneous occurence of autosomal recessive mutations in the HCP gene in the C57BL/6J strain of mice. Homozygous *motheaten viable* (me^v) and *motheaten* (*me*) mice display reduced and no HCP activity respectively (Shultz *et al*, 1993). In the case of the *motheaten* mutation there is an increased level of a variety of haematopoietic lineages including erythrocytes, monocytes/macrophages and granulocytes and the pups die within 3-9 weeks after birth from systemic autoimmune disease.

1.1.5 Haematopoietic transcription factors.

As mentioned signal transduction culminates in the activation of transcription factors which bind to their cognate DNA recognition sequences or response elements. Transcription factors therefore play a central role in lineage commitment and thus much effort has been expended in identifying transcription factors that are involved in haematopoietic commitment. Consequently a number of transcription factors belonging to different families have been shown to be involved in various stages of haematopoietic commitment and differentiation.

Gene 'knock-out' mutation studies for example have demonstrated that the zinc-finger transcription factors GATA-1 and 2 have an important role in haematopoiesis. These factors belong to the highly conserved GATA family of transcription proteins, six of which have been identified and found to be expressed in a wide range of tissues (Laverriere *et al*, 1994). Mice with a null mutation in GATA-1 die of severe anaemia *in utero* (Pevney *et al*, 1991). Further *in vitro* colony forming assay studies of haematopoietic tissue samples from these mice have revealed that the main lineage affected by the GATA-1^{-}/$^{-}$ mutation is the erythroid lineage where maturation beyond the pro-erythroid stage does not take place (Pevney *et al*, 1995). Lack of GATA-2 however appears to affect a wider range of lineages including the lymphoid and myeloid compartments (Tsai *et al*, 1994). The role of these transcription factors is discussed in more detail in section 2.

The major family of transcription factors which appear to be specifically involved in the commitment and maintenance of the lymphoid lineage are the Ikaros zinc-finger protein. Mice homozygous for a mutation in the DNA binding domain of the Ikaros protein, have normal erythroid and myeloid cells but lack T- and B-cell progenitors. Furthermore the thymus is grossly under developed and the peripheral lymph nodes absent (Georgopoulos

et al, 1994). Recent studies have also shown that mice heterozygous for the same Ikaros mutation die of leukaemia or lymphoma within six months of birth (Winandy *et al*, 1995), suggesting a role for Ikaros both in the commitment and differentiation of lymphoid progenitors, and in the homeostatic control of T-cell proliferation.

While the intentional disruption of certain genes has helped identify their putative roles in haematopoiesis, some transcription factors have been implicated as a consequence of chance mutations giving rise to aberrant haematopoietic cell growth. For instance the majority of acute myeloid leukaemias arise from chromosomal rearrangements that disrupt the *AML-1*. The disruption of *AML-1* in mice has recently confirmed its role in regulating commitment and differentiation of all lineages during definitive haematopoiesis (Okuda *et al*, 1996).

Similarly over 95% of promyelocytic leukaemias have been attributed to the chromosomal translocation t(15;17)(q22;q21). The point of translocation on chromosome 15 encodes the PML protein, while that of chromosome 17 encodes the retinoic acid receptor RARα, one of the three members of this family of retinoic acid receptors. RARα is a ligand binding transcription factor which has been implicated in neutrophilic differentiation (Tsai and Collins, 1993). PML protein contains various motifs typical of transcription factors including leucine zipper and zinc-finger motifs. Although the exact function of PML is yet to be established it has been suggested that under normal conditions it acts as a growth suppressor (Mu *et al.* 1994; Okuda *et al*, 1994)).

The identification of transcription factors and their related family members has proved an important step in elucidating haematopoietic commitment and differentiation. However the rate limiting step remains the identification of their target genes.

Due to the diversity of the components and processes involved in haematopoietic commitment a complete picture of a set of events contributing to any specific pathway has yet to be achieved. Gradually however various findings can be pieced together to yield a detailed understanding of how this complex process takes place. For example the newly identified Stats (signal transducers and activator of transcription) family of transciption factors have been found to mainly associate with and be phosphorylated by the Janus kinases. Stat 4, the expression of which is resticted to the myeloid lineage has been shown to be phosphorylated in response to IL-12 (Jacobson *et al*, 1995) and the IL-12 receptor appears to activate Jak2 and Tyk2 (Bacon *et al*, 1995). The pathway remains to be elucidated however since target genes for Stat 4 have yet to be reported.

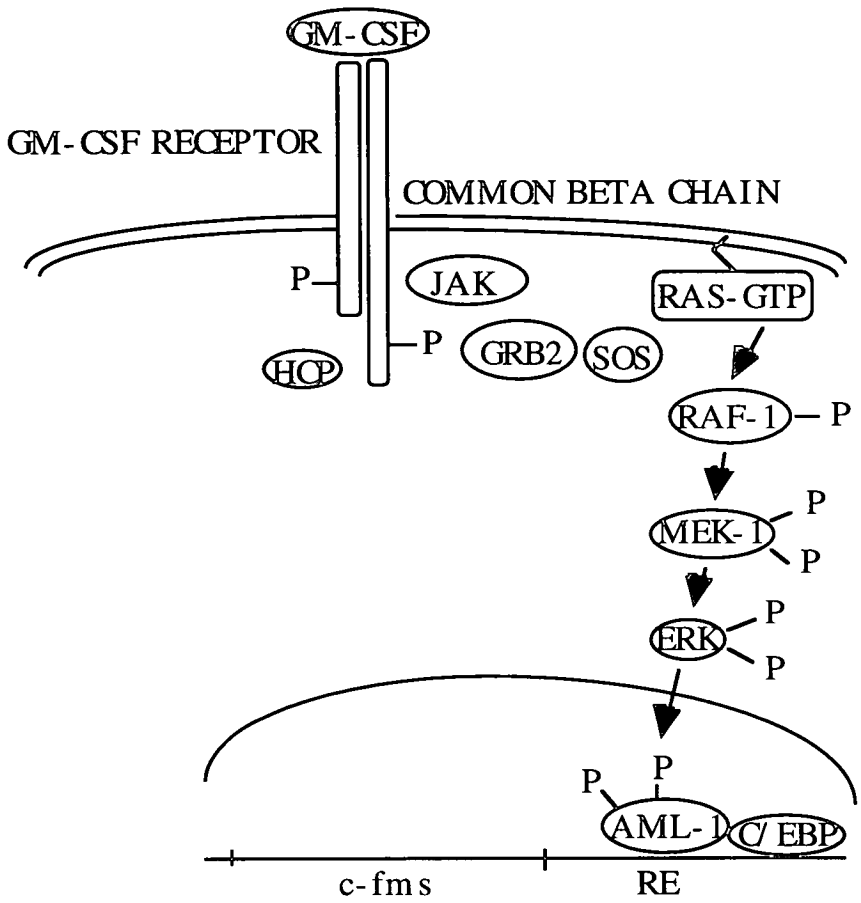


Figure1.2. Showing the cascade of events which may play a part in initiating transcription from the *c-fms* gene, the M-CSF receptor. After binding, GM-CSF causes the dimerisation of its receptor which results in the phosphorylation (P) of the receptor chains by a member of the Jak tyrosine kinase family (JAK). Ras-GTP may then become activated via accesory molecules such as Grb-2 and son of sevenless (SOS) which results in the phosphorylation of Raf. AML-1 is eventually activated by ERK after phosphorylation of 2 serine residues and binds to its response element (ER) on the *c-fms* promoter, possibly as a dimer with C/EBP.

Studies of erythropoiesis have similarly revealed that Epo upon binding to its receptor, can activate GATA-1, which in turn *trans*activates the expression of Epo-receptor and its own genes thus forming the basis of a self regulating positive feedback loop (Zon *et al*, 1991; Dalyot *et al*, 1993). In this example it is the signalling pathway regulating the activation of GATA-1 which is poorly understood.

More recently a clearer pathway for myeloid commitment has emerged (Figure 1.2). It has been shown that in mouse fibroblast and IL-3 dependent haematopoietic cell lines, GM-CSF (granulocyte/macrophage-colony stimulating factor) can directly stimulate the expression of c-fms (Helftenbein *et al*, 1996). The GM-CSF ligand/receptor interaction can set in motion phosphorylation reactions in more than one signalling pathway including the Jak/Stats and Raf/MAP-kinase pathway (Okuda *et al*, 1992). Protein studies have demonstrated that one of the kinases in the Raf/MAP-kinase pathway, extracellular signal-regulated kinase (ERK), directly phophorylates AML-1 on two serine residues, ERK itself being a substrate for another protein kinase, MEK-1 (Tanaka *et al*, 1996). Electrophoresis mobility shift assays have identified c-fms as one of the target genes for AML-1 (Zhang *et al*, 1996).

Once activated, AML-1 together with C/EBP (CCAAT enhancer binding protein) possibly as a heterodimer complex, has been shown to *trans*activate the expression of the myeloid specific receptor, c-fms. PU.1, another transcription factor implicated in myeloid commitment and differentiation has also been shown to *trans*activate the expression of *c-fms* (Zhang *et al*, 1994). Whether PU.1 is activated by the same cascade of events as AML-1 or by a distinct pathway is unclear. The model outlined here, demonstrates the manner in which insights into haematopoietic differentiation can be gained. No doubt pathways such as this will grow in complexity as more signalling molecules and target genes are identified.

1.2 Haematopoietic development.

Stem cells are produced and their fate determined during the very early stages of development. Due to the inability of adult tissue to produce cells of such paramount importance the ontogeny and commitment of stem cells during embryogenesis is of particular interest. Various methods and experimental systems have been employed for the study of haematopoietic development.

Basic principles of developmental biology have been particularly well elucidated in *Xenopus laevis* and the chick, due to the accessibility of the embryos. The mouse model, has proved an indispensable source for the study of haematopoiesis and much of the technical advances already mentioned in section 1.1 have greatly facilitated this process. There are many similarities between murine and human haematopoietic development, and most clinical advances made in the treatment of haematopoietic disorders originate from studies carried out in the mouse. For this reason the primary focus in the following sections will be on the haematopoietic development of the common house mouse *Mus musculus*. Since comparative studies have proved to be an important means of identifying evolutionary conserved genes thought to play an important role in any given system comparisons and parallels will be drawn from other models where appropriate.

The embryonic stage of haematopoiesis is referred to as 'primitive' and from the foetal to the adult stages as 'definitive'. Haematopoietic development is a compartmentalised process which in the mouse first appears in the yolk sac and following that, sequentially, in the aorta, gonad, mesonephros (AGM) region of the interembryonic mesoderm, liver, thymus, omentum, spleen and finally bone marrow. Although many questions remain unanswered and the detailed molecular and cellular events which direct this process are poorly understood a framework within which these issues can be addressed has emerged. Some aspects of this progress are outlined in the following sections.

1.2.1 Haematopoietic ontogeny.

In mammals after fertilisation and the cleavages which give rise to the blastula, the totipotential cells of the inner cell mass (ICM) separate to form the embryonic and extra-embryonic epiblasts. The embryonic epiblast gives rise to the ectoderm and endoderm. The region of the ectoderm referred to as the primitive streak, containing a population of cells thought to have retained their totipotentiality, invaginates and the cells migrate out to form the mesoderm layer. The expression of the putative transcription factor *brachyury* (Kispert *et al*, 1995), at around 6.5 days of gestation in the mouse in all mesodermal tissue and is thought to mark the beginning of mesoderm formation (Wilkinson *et al*, 1990).

At least five types of mesoderm are derived which go on to form various organs. The lateral plate mesoderm gives rise to the haematopoietic system as well as the blood vessels and heart. Transcripts for haematopoietic specific genes such as GATA-1 and α-globin have been detected at around 7 days post coitum (p.c.) in murine embryos (Rich *et al*,

1994). Once commitment has taken place haematopoietic and vascular progenitors migrate out to an extra-embryonic location on the yolk sac, the position of which is classified as ventral, and give rise to blood islands.

The derivation of mesoderm in birds is very similar that of mammals. After invagination of the primitive streak, the totipotential cells of the epiblast migrate into the area pellucida and a proportion of them give rise to the extra-embryonic splanchnoderm which is composed of mesoderm and endoderm. Haematopoietic progenitors then migrate out to the posterior area opaca and give rise to blood islands which are morphologically detectable by the 7 somite stage of chick embryogenesis. GATA-1 and 2 transcripts can be detected at the onset of gastrulation in the chick embryo. GATA-1 displays a more restricted expression pattern than GATA-2, being largely expressed in the posterior region of the embryonic mesoderm, whereas GATA-2 can be detected both in the anterior and posterior regions (Leonard *et al*, 1993).

Due to the accessibility of the *Xenopus* embryo a better understanding of gastrulation and hence haematopoietic initiation is being arrived at in this animal system. The *Xenopus* embryo is conventionally divided into two poles: the animal and the vegetal poles which give rise to ectoderm and endoderm respectively. The dorso-ventral axis of the embryo is established upon fertilisation, the dorsal region being opposite the point of sperm entry. As in the mammalian and avian systems *Xenopus* mesoderm is derived from the ectoderm. *Xenopus* embryo explant assays have shown that at the blastula stage, signalling molecules from the ectoderm induce the equatorial cells of the ectoderm which are believed to have maintained their totipotentiality, to give rise to mesoderm (reviewed by Kimelman *et al*, 1992). The expression of the *Xenopus* homologue to *brachyury*, *Xbra* in the marginal zone once again marks the beginning of mesoderm formation. The newly formed mesoderm is futher patterned dorso-ventrally by signalling molecules from the Spemann organiser and the most ventral region becomes commited to the haematopoietic lineage. Ventral blood islands in *Xenopus* are functionally and positionally equivalent to their mammalian and avian counterparts on the yolk sac.

Various signalling molecules have been implicated in the patterning of the *Xenopus* embryo including the fibroblast growth factor (FGF), Wnt and TGF-β families- the TGF-β family being further divided into the activin and bone morphogenetic protein (BMP) sub-families. Evidence from *Xenopus* embryo explant assays strongly suggests that such signalling molecules create gradients in the embryo, and exert their effects within tight threshold boundaries (Gurdon *et al*, 1995).

Activin and FGF have been shown to be particularly strong candidates for mesoderm induction in *Xenopus*. Explants of the animal pole of the *Xenopus* embryo have been found to give rise to mesoderm in response to both these molecules and conversely the disruption of their receptors has been found to prevent mesoderm induction (Amaya *et al*, 1991; Hemmati-Brivanlou and Melton, 1992), the disruption of the FGF receptor leading to defects in the ventral structures.

As in the chick, GATA-1 and 2 are expressed very early on in *Xenopus*. Both are expressed as early as stage 11 of *Xenopus* development, haematopoiesis not being evident until stage 32. At stage 11 GATA-1 expresson is restricted to the ventral region, and GATA-2 is detected throughout the animal pole. However after gastrulation GATA-2 expression also becomes restricted to the ventral and lateral regions (Kelley *et al*, 1994).

Using explant assays Walmsley *et al* (1994) have shown that GATA-2 expression and subsequently haematopoiesis, as detected by globin expression, is inhibited in the presense of activin and also in response to signals from the Spemann organiser. Therefore it has been suggested that in *Xenopus*, induction of haematopoietic tissue from mesoderm occurs by default, in the absence of inducers. However the injection of BMP-4 mRNA into the animal pole of the *Xenopus* embryo has been shown to both ventralise mesoderm (Dale *et al*, 1992) and induce erythropoietin expression (Maéno *et al*, 1994). Therefore BMP-4 has been suggested to be a haematopoietic inducer possibly acting synergistically with other factors. This apparent contradiction between the inductive ability of BMP-4 and the inhibiting activity of the Spemann organiser has recently become clearer. It has been shown that the putative Spemann organiser signal, noggin, binds BMP-4 with high affinity and inactivates it, which results in the dorsalisation of the mesoderm tissue (Zimmerman *et al*, 1996).

The families of signalling molecules mentioned are also expressed in avian and mammalian embryos and implicated in directing various stages of development from neural development (Mason *et al*, 1994), to limb bud patterning (Francis *et al*, 1994). In the chick embryo FGF-2 has been suggested to have an important role in inducing haematopoietic tissue. Gordon-Thomson and Fabian (1994) have shown that haematopoiesis, as detected by haemoglobinisation, can be inhibited in the pre-gastrulation chick embryo by culturing the blastoderm in the presence of polyclonal antibody raised against FGF-2. Furthermore this inhibition is shown to be effective only during a brief window of time with the anti-sera having little effect on erythropoiesis in later stages of embryogenesis.

In mice, the disruption of the BMP-4 gene has been shown to severely inhibit mesoderm and hence blood island formation. In fact foetuses fail to develop beyond 9.5 days p.c. and mesoderm markers such as *brachyury* fail to be expressed (Winnier *et al*, 1995). More recently, preliminary experiments with murine embryonic stem cell lines have suggested that BMP-4 may be more directly involved in inducing haematopoietic lineages (Johansson and Wiles, 1996).

1.2.2 Primitive Haematopoiesis.

The first physiological sign of haematopoiesis is the appearance of blood islands on the visceral yolk sac of most mammals and birds. In the mouse, blood islands begin to form at around 7.25 days p.c. and are fully manifest by day 8.5. Structurally blood islands are aggregates of blood cells encapsulated by a layer of endothelial cells, this structure being supported by the visceral yolk sac endoderm. At around 9 days p.c. angiogenesis begins and a network of capillaries between the blood islands forms which then become connected with the developing heart. By day 9.5 the heart begins to beat and circulation is established.

The close association of the haematopoietic cells with the endothelial and endodermal layers has been proposed to be essential for blood island formation, with the endothelial and the endodermal layers providing the necessary microenvironment for haematopoiesis and vasculogenesis. Several studies support this hypothesis. Murine bone marrow progenitors have for instance been found to have a much increased growth and proliferative potential when co-cultured with cell lines isolated from the yolk sac endoderm (Yoder *et al*, 1995). Similar results have been reported from experiments involving the co-culture of the endothelial layer with yolk sac HSCs (Auerbach *et al*, 1996). However using an *in vitro* culture system Palis *et al* (1995) have shown that visceral yolk sac explants from day 7.5 embryos can give rise to haematopoietic cells in the absence of the endodermal layer, but there is a failure in angiogenesis.

Various experiments with cytokines and their receptors have further demonstrated the importance of the interaction between the endoderm and endothelium with the haematopoietic or mesothelial layer. Mice carrying a homozygous mutation in the Flk-1 tyrosine kinase receptor for instance, fail to produce any mature endothelial cells in the yolk sac which is thought to result in the subsequent absence of haematopoietic progenitors and hence blood island formation (Shelaby *et al*, 1995). Similar results have been obtained

with TGF-β1 knock-out mice where the yolk sac of the homozygous mutant foetuses displays defective endothelial differentiation and a much reduced erythroid population (Dickinson *et al*, 1995). The mutation was found to have less of a direct effect on the endoderm. Taken together these findings suggest that normal blood island haematopoiesis is dependent on both a functional endoderm and endothelium.

Among the transcription factors which have been found to play an important role in primitive haematopoiesis is SCL/Tal1, a member of the basic helix-loop-helix family of transcription factors. Originally identified as one of the genes disrupted by a chromosomal translocation in a case of acute leukaemia, *scl* has been shown to be expressed in mouse blood islands at around 7.5 days p.c. with its expression remaining largely restricted to the haematopoietic lineages thereafter (Kallianpur *et al*, 1994). The disruption of the gene in mice has further shown that *scl* $^{-/-}$ embryos fail to develop beyond 9.5 days p.c. (Shivdasani *et al*, 1995; Robb *et al*, 1995). Detailed analysis of the embryos has shown a marked absence of blood island formation and haematopoiesis. The expression of other haematopoietic transcription factors such as GATA-1 and PU.1 were also affected, although GATA-2 was found to be present, highlighting the importance of the gene during primitive haematopoiesis. More recently studies with chimaeric mice, generated by the injection of *scl* $^{-/-}$ ES cells into wild type blastocysts, have implicated *scl* in the very early stages of stem cell commitment since the mutant cell line fails to contribute to any of the haematopoietic lineages of the adult mouse (Porcher *et al*, 1996).

Mammalian primitive haematopoiesis produces a limited range of blood cells. The haematopoietic cells in the yolk sac blood islands are mainly nucleated erythroblasts, expressing foetal haemoglobin, and do not undergo any further maturation. Small populations of monocytes and macrophages are also thought to be present. No other lineages have so far been identified *in situ*. However Palacios and Imhof (1993) have reported the ability of yolk sacs of 8-8.5 day old embryos to produce lymphoid precursors. In this study yolk sac cells were injected into various haematopoetic organs of sub-lethally irradiated CB17 severely combined immuno-deficient (SCID) mice followed by fluorescence-activated cell sorter (FACS) analysis of the haematopoietic tissues of the recipients. Both T- and B- cell presursors were detected. This study indicates that yolk sac HSCs are able to give rise to a wide range of haematopoietic cell types when placed in a conducive environment and in response to the appropriate growth factors. This is further demonstrated by *in vitro* colony forming assays in which yolk sac HSCs have been found to give rise to both lymphoid and myeloid lineages (Huang *et al*, 1994; Liu and Auerbach, 1991).

Erythropoiesis in the murine yolk sac blood islands is thought to be maintained by erythropoietin. Preliminary *in vitro* evidence suggests that embryonic erythroid cells like those of the foetus and adult are able to respond to Epo (Rich *et al*, 1994). Using blast-forming unit (CFU-B) and erythroid-colony forming unit (CFU-E) assays, which detect early and late erythroid progenitors respectively, a marked increase in erythroid colonies were obtained from disaggregated 7.5 and 8.5 day murine embryos after treatment with Epo (Rich 1992). This together with the detection of Epo-receptor and ligand transcripts, as early as day 6 of gestation in mouse embryos strongly suggests that primitive haematopoiesis is also controled by erythropoietin, which may be secreted by the endothelial and/or endodermal layers of the yolk sac blood islands (Yasuda *et al*, 1993).

In the *Xenopus* the ventral blood islands are encapsulated by the hepatic endoderm which is also postulated to support primitive haematopoiesis and erythropoiesis by secreting an Epo-like factor. Unlike mammalian blood island haematopoiesis however, *Xenopus* ventral blood island tissue has been shown to contain myeloid and lymphoid progenitors (Smith *et al*, 1989).

Avian primitive haematopoiesis is very similar to that of the murine system. However in the chick the entire yolk sac becomes vascularised and remaines haematopoietic almost for the duration of gestation. Murine yolk sac blood island haematopoiesis however continues until day 12 p.c. by which time definitive haematopoiesis is established.

1.2.3 Intra-embryonic haematopoiesis.

Until recently it was believed that murine haematopoietic progenitors from the yolk sac migrated to the liver where they established definitive haematopoiesis and thereafter relocated to their final destination, the bone marrow. This hypothesis was supported by *in vitro* murine embryo culture assays where day 7 p.c. embryos cultured in the absence of their yolk sac lacked circulating blood cells and failed to develop liver haematopoiesis (Moore and Metcalf, 1970). However recently a population of haematopoietic progenitors in the dorsal region of the intra-embryonic mesoderm, which gives rise to the aorta, gonads and mesonephros (AGM), have been identified which are thought to also contribute to definitive haematopoiesis (Medvinsky *et al*, 1993).

A similar population of cells in the corresponding region of the avian embryo have long been known to exist. The morphologically distinct foci of haematopoietic cells on the

ventral wall of the chick embryo aorta, (which are thought to have delineated from endothelial and/or mesenchymal components), have been shown to contribute to definitive haematopoiesis by intra-species (chick/quail) and intra-strain (chick/chick) embryo grafting assays (Dierterlen-Lievre, 1975). This population of cells has recently been further enriched and characterised and been shown to give rise to erythroid and myeloid cells *in vitro* (Cormier, 1993).

Similar embryo grafting experiments with *Xenopus* embryos have also demonstrated that haematopoietic progenitors originating from both the extra- and intra-embryonic mesoderm contribute to adult haematopoiesis (Kau and Turpen, 1983). More recently however, using two-colour FACS analysis Chen and Turpen (1995) have shown that definitive *Xenopus* haematopoiesis is predominantly derived from haematopoietic cells of the dorsal lip plate which is analogous to the mammalian AGM.

The murine AGM region derives from the splanchnopleura, which is the ventral segment of the lateral mesoderm and is continuous with the yolk sac. AGM haematopoietic progenitors are first detected at around day 9 p.c., which is approximately only 12 hours prior to the initiation of liver haematopoiesis, and persist until day 11. Unlike yolk sac HSCs, those from the AGM region give rise to a wider range of haematopoietic lineages *in vivo*. In contrast to the chick embryo, haematopoietic cells of the mouse AGM do not form foci and were thus demonstrated to be present by two similar experimental approaches.

In the first instance the splanchnopleura from day 8.5-9 p.c. embryos were surgically transplanted under the kidney capsule of SCID mice (Godin *et al*, 1993). Subsequent FACS analysis of the haematopoietic tissue of recipient mice showed the presence of donor derived immunoglobulin M (IgM), IgM secreting plasma cells and the B1a cell subset (CD5 B cells), which are among the most primitive B-cells. Further maturation of these cells was not however detected. In the second study day 9 embryos were dissected and AGM cells injected into lethally irradiated recipients (Medvinsky *et al*, 1993). Donor derived day 8 CFU-S colonies, which are among the more commited haematopoietic progenitors, were subsequently detected in the recipients. It was furthermore found that as the CFU-S population in the AGM region decreased, those of the liver increased in number.

In a later study similar experiments were performed but with the AGM of day 10 p.c. embryos (Müller *et al* , 1994). These were found to provide long term repopulation of lethally irradiated recipient mice. Moreover the day 10 p.c. AGM cells were found to contribute to erythroid, myeloid and lymphoid compartments, and subsequent secondary

and tertiary transplantations have proved to have consistent repopulating ability. In order to explain the emergence of the more mature CFU-S cell types before the primitive long term repopulating HSCs Müller *et al* have proposed a 'reverse haematopoietic hierachy' model in the embryo. However this apparent anomaly could also be explained in terms of the Potten and Loeffler's (1990) stem cell theory, where they propose various degrees of 'stemness' and commitment and that depending on the circumstances, cells may either become more commited to differentation or revert to a more pluripotential state.

In a recent study further dissection of the AGM region of day 10 mouse embryos, into their dorsal aorta and uro-genital ridge components, was carried out and each cell population assayed for their CFU-S forming ability (Medvinsky *et al*, 1996). It was subsequently found that although the frequency of CFU-S is higher in the dorsal aorta segment, the highest total number of CFU-S was obtained from the uro-genital portion. At around day 10 the uro-genital ridges are occupied by primordial germ cells (PGCs). PGCs are an ectodermally derived population of cells which can be specifically detected by alkali-phosphatase staining. Using this assay, PGCs are first detected at about 7 days p.c. At around day 8 they begin their migratory rout to the genital ridges via the hindgut epithelium. At approximately day 9 p.c. they arrive at the dorsal mesentry and proceed to the genital ridges which they colonise by day 11-11.5 p.c. and give rise to the germ layers in the prespective gonads.

The time of their arrival at the dorsal mesentry coincides with the appearance of haematopoietic progenitors in this region. Recent studies have further confirmed the haematopoietic potential of PGCs (Rich, 1995). *In vitro* colony forming assays have demonstrated that after exposure to cytokines such as IL-3 and LIF, PGCs are able to give rise to myeloid and erythroid colonies. Further analysis of the PGC population has shown that they express *c-kit* (Manova and Backvarova, 1991), and their *in vitro* proliferation and survival depend on SCF (Rich, 1995).

More recently clusters of cells associated with the dorsal aorta of 30-37 days gestation human embryos have been found, which test positive for haematopoietic markers and have also been shown to give rise to high numbers of clonogenic haematopoietic progenitors *in vitro* (Tavian *et al*, 1996). Thus there is increasing evidence to suggest that the AGM plays an evolutionary important role in haematopoietic development and makes a contribution to definitive haematopoiesis. New models for haematopoietic development have therefore been proposed which take this into account. But the degree and manner in

which the AGM makes this contribution, or how AGM haematopoiesisis is initiated and the factors which control this process remain to be defined.

1.2.4 Definitive haematopoiesis.

In the avian model, after para-aortic haematopoiesis, haematopoietic progenitors are detected in the thymus, spleen, bursa Fabricius and bone marrow in sequence. A similar sequence is seen in the mouse where after the AGM, haematopoiesis is observed in the liver, thymus, omentum, spleen and finally bone marrow which remains the main site of haematopoiesis throughout adult life.

At approximately day 9 p.c. the liver begins to form and by day 10.5 enucleated erythrocytes expressing adult haemoglobin, B-cells and macrophages are observed which mark the onset of definitive haematopoiesis (Barker, 1968). The thymus is also thought to be colonised by progenitors at around day 10.5 (Jotereau *et al*, 1986). Foetal liver haematopoiesis continues until around day 16 p.c. with the mid-stages being found to be particularly c-Kit dependent, since haematopoietic progenitors from the liver of day 12.5 p.c. mouse embryos fail to give rise to myeloid and erythroid lineages *in vitro* in the presence of anti-c-Kit monoclonal antibody (Ogawa *et al*, 1993a). This is not the case for haematopoietic liver progenitors from younger or older embryos. Although haematopoiesis shifts to other sites, the liver does not lose its haematopoietic potential totally. Thus if bone marrow haematopoiesis is diminished in the adult the liver can be found to supplement the system. More recently reconstitution of the haematopoietic system of lethally irradiated recipients with cells derived from the adult mouse liver have been reported (Taniguchi *et al*, 1996; Watanabe *et al*, 1996).

Haematopoiesis has also been observed in the omentum of day 13 p.c. mouse embryos. the omentum is a segment of connective tissue which supports the stomach and spleen. It has been found to contain lymphoid and erythroid progenitors and remains at least lymphopoietic throughout adult life in mice. After transplantation under the kidney capsule the omentum of day 13 mouse embryos has been found to reconstitute the haematopoietic system of SCID recipients (Solvason and Kearney, 1989). Haematopoietic progenitors do not arise *de novo* in the omentum but the tissue is thought to be colonised by cells from other sites possibly while *en route* to the spleen.

In mice the spleen plays an important role in foetal haematopoiesis and remains a site of erythropoiesis throughout adult life. However in humans the spleen does not contribute to foetal haematopoiesis significantly with studies showing that human foetal spleen is mostly occupied by mature erythrocytes and granulocytes (Wilkins *et al*, 1994).

Following their finding of haematopoietic activity in the AGM, Müller and Dzierzak (1993) have proposed a new model for the ontogeny of definitive haematopoiesis. Two waves of foetal liver colonisation by haematopoietic progenitors have been proposed: the first by yolk sac progenitors at approximately day 10 and the second by both yolk sac and AGM progenitors at around day 11 p.c., with the spleen and bone marrow being colonised by cells from the liver thereafter.

Recent findings however suggest that bone marrow and spleen are colonised by the same migrating population of progenitors which populate the liver. By analysing the cells in mouse embryo circulation Delassus and Cumano (1996) have found that the population of haematopoietic progenitors increases between day 10 and 12 of gestation followed by a significant decrease at day 13. A second increase between day 14 and 16 is observed, however the progenitors in this wave are more committed. Furthermore they detect the presence of HSCs in the bone marrow at day 15 p.c., 24 hours earlier than previously reported. Further studies are however required to confirm these findings.

1.3 Methods for studying haematopoiesis.

As already discussed in section 1.1.1, the direct study and manipulation of HSCs has proved difficult due to the obscure phenotype and rarity of this cell type. In order to circumvent these limitations therefore various techniques and methods have been devised and adopted for the study of HSCs and haematopoiesis. Some of the methods currently used by investigators will be discussed in the following sections.

1.3.1 Techniques used for the study of HSCs.

In order to be able to study the molecular events which control haematopoiesis, candidate genes must first be identified. As previously pointed out, naturally occuring mutations such as those in the *c-kit* and *HCP* genes, and chromosomal translocations which give rise to leukaemias, such as those disrupting the *AML-1* and *PML* genes have been useful sources of

information. However new appoaches must be devised if novel genes, involved in the haematopoietic process are to be identified. Various PCR (Brady *et al*, 1990; Guimarães *et al*, 1995) and subtraction library based techniques (Beadling *et al*, 1993; Jonk *et al*, 1994), have been described. In order to be useful however, such techniques require an accessible cell type.

Improved tissue culture techniques have greatly aided the *in vitro* maintenance of HSCs for limited periods of time. These techniques have been further facilitated by the identification and purification of haematopoietic growth factors which have been used either in concert with stromal cell lines (Szilvassy *et al*, 1996), or added directly to haematopoietic cell cultures (Sutherland *et al*, 1993) for the support of long term repopulating HSCs, and/or for encouraging the differentiation of HSCs toward particular lineages (Gabbianelli *et al*, 1995). Attempts have also been made to establish culture conditions which can support HSC maintenance and differentiation for prolonged periods of time which can then be used to purify and characterise HSC both phenotypically and genotypically (Sutherland *et al*, 1990). The use of such culture conditions however will always be limited by the abundance and availability of the bone marrow source, and the need to maintain the haematopoietic progenitors on stromal feeder layers, makes their genetic manipulation particularly complex since such cultures give rise to a heterogeneous cell population.

Devising techniques for the enrichment of HSCs, some of which were mentioned in section 1.1.1, have been particularly important for studying the properties of HSCs. One technique which has proved particularly useful has been the genetic marking of haematopoietic progenitor cells with retroviruses, by co-culture with infected cell lines (Capel *et al*, 1990; Jordan and Lemiscka, 1990). The molecular marking does not appear to alter the behaviour of the stem cell populations which have been found to provide long-term repopulation, contributing to all blood cell compartments of haematopoietic deficient recipients.

Although retroviral marking allows the behaviour of HSCs to be assessed *in vivo*, it is not particularly informative with respect to the phenotypic and genotypic characteristics of HSCs. Thus Jordan *et al* (1990a) have devised a method where retroviral marking is coupled with cell surface antigen and antibody panning enrichment techniques, resulting in the identification of a highly enriched foetal liver stem cell population.

Enriched HSC populations can be useful for molecular analysis. It is possible for instance, to study the effects of gene over-expression in such populations. However due to

experimental limitations complete inhibition of gene expression, which is more informative about gene function, has proved somewhat more difficult to achieve, either by transfection with antisense oligonucleotides or with retroviruses harbouring dominant negative transgenes. The function of transcription factors can be sequestered more effectively with competitive binding oligonucleotides (Voso *et al*, 1994). But molecular analysis of this kind carried out with isolated cell populations, does not take the effects of the haematopoietic microenvironment into consideration.

Leukaemic cell lines such as human leukaemia (HL)-60 and murine erythroid leukaemia (MEL) cell lines have also been useful sources of study since they can be maintained in culture indefinitly, but are not particularly informative with respect to the study of stem cell dynamics. One cell line which has proved useful for studying stem cells however, is the FDCP-mix A4 population. This cell line, isolated from murine LT-BMC cultures, has the capacity to differentiate to multiple haematopoietic lineages in the presence of stromal cells or cytokines, and is also capable of self-maintenance in response IL-3 (Spooncer *et al*, 1986; Heyworth *et al*, 1995). The potential of the cell line to reconstitute the haematopoietic system of recipients has, however not been studied.

Other investigators have attempted to establish immortalised multipotent haematopoietic cell lines with the use of viral or retroviral vectors, harboring genes which may play key roles in haematopoiesis. One such cell line was established from the bone marrow of mice transgenic for a GATA-1 driven temperature sensitive Simian virus (SV)-40 construct (Cairns *et al*, 1994). These cells have been reported to express myeloid specific cell surface markers and erythroid specific genes after exposure to Epo or IL-3. However detailed analysis of the multipotentiality of these cells with *in vitro* assays were not reported.

Tsai *et al* (1994a) have taken a similar approach in transfecting murine bone marrow cells, obtained 5 days after treatment with 5-fluorouracil, with a retroviral vector construct carrying a dominant negative RARα gene. Using this approach an SCF dependent cell line was established and found to have erythroid, myeloid and lymphoid potential *in vitro*. Retroviral transfection of HSCs is difficult to achieve not only because of their small numbers, but also since they are a quiesent population with low replicative rate, which hinders retroviral integration. Thus in this experiment it is difficult to assess what proportion of the targeted cells were 'true' HSCs or more committed progenitors. Furthermore the cell types derived from both strategies described, may be biased by the

use of vectors carrying genes known to be involved in the differentiation of particular haematopoietic lineages.

The *in vivo* potential of neither of the above two cell types have been assessed. However one immortalised cell line has been established, from day 12 p.c. foetal liver cells, transformed by a rearranged retroviral vector carrying the IL-3 gene (Wong *et al*, 1994), and has been shown to have the ability to reconstitute the haematopoietic system of lethally irradiated recipients in primary and secondary transplantation experiments. Moreover the recipients did not develop any malignancies or blood related abnormalities.

Despite these findings however it is difficult to extrapolate the behaviour of transformed cell lines to normal haematopoietic cells, and their use sheds very little light on processes involved in haematopoietic development. In recent years, however, the derivation and use of embryonic stem cell lines has changed the course of haematopoietic research. Not only are they relatively accessible and easily manipulated, but their use circumvents many of the limitations outlined above.

1.3.2 Embryonic stem cells.

Totipotential embryonic stem (ES) cells are currently the only untransformed stem cell population in culture. The first indication that such a cell line could be obtained *in vitro* came with the observation that when embryos of up to mid-gastrulation stage, or their dissected totipotent epiblast cells were transplanted into a well vascularised, non-uterine position such as the testes, the host developed tumours (Stevens, 1970). These teratocarcinomas of donor origin were found to contain an array of tissue types including skin, hair and bone, but no organised structures requiring the interaction of various tissue types for their development, such as kidney and heart. A high proportion of the cells in the tumours were also found to be proliferating undifferentiated cells referred to as embryonic carcinoma (EC) cells. When re-transplanted into hosts EC cells were found to once again give rise to teratocarcinomas and more undifferentiated EC cells. After several transplantations, *in vitro* EC cell lines were established from tumours, which could be maintained in an undifferentiated state either in the presence or absence of fibroblast feeder layers.

In certain inbred strains of mice teratocarcinomas of germ cell origin arise spontaneously in the gonads. Human EC cell lines have also been isolated from spontaneous germ cell

tumours (Thompson *et al*, 1984), however similar transplantation experiments as those performed in mice have failed to produce EC cells in other species.

In vitro, EC cells were found to give rise to all cell types including haematopoietic cells (Cudennec and Nicolas, 1977; Miwa *et al*, 1991). Furthermore, chimaeric mice were generated from EC cells after their injection into pre-implantation blastocysts and were found to be able to contribute to all three primary tissues (ectoderm, endoderm and mesoderm) and at low frequencies to the germ layer (Stewart and Mintz, 1981). These chimaeras however were found to be highly prone to developing tumours of EC cell origin (Rossant and McBurney, 1982). It therefore became desirable to establish a totipotent cell line which could contribute to the germ layers of chimaeric animals at higher frequencies, which is essential for detailed study of developmental and molecular processes.

Embryonic stem cell lines were thus established from 3.5 day p.c. pre-implantation male embryos from the 129 and C57B2 strain of mice (Evans and Kaufman, 1981; Martin, 1981). In brief the process involves the attachment of embryos to culture dishes which mimics the process of implantation, causing the trophectoderm to spread into a monolayer exposing the ICM. The ICM then proliferates to form a cluster which is disaggregated with trypsin. These totipotent cells were subsequently cultured on fibroblast feeder layers without which ES cells terminally differentiate.

Further analysis of the supernatant of the feeder layer showed the undifferentiated state of the cells was maintained by the glycoprotein leukaemia inhibitory factor (LIF) (Williams *et al*, 1988), also referred to as differentiation inhibiting activity (Smith *et al*, 1988), and that ES cells could be prevented from differentiating with the addition of recombinant LIF. The latter technique is preferred since it provides a homogeneous population of cells for experimentation.

Like EC cell lines, when introduced into hosts ES cells can give rise to teratocarcinomas, and can also be used to produce chimaeric mice. ES cells however contribute to the germ layer at a much higher frequency than EC cells, making them an extremely useful tool for transgenic and 'knock-out' mutation studies. Using compromised tetraploid host blastocysts Nagy *et al* (1990) have furthermore shown that mice entirely derived from ES cells can be generated.

Several examples of gene 'knockout' mice generated from ES cells, have already been mentioned. In brief the technique involves the transfection of a vector construct with a

selectable marker, harboring a mutant copy of the gene of interest into ES cells. Upon entry into the nucleus the mutated gene recombines with its homologue. The recombinant cells are selected and either combined with or injected into pre-implantation host embryos and the resulting chimaeras crossed to produce homozygous mutants. Due to their ability to differentiate to all cell types ES cells have been used extensively *in vitro*, particularly for the study of haematopoiesis, discussed in the next section.

Both EC and ES cells terminally differentiate by the removal of LIF, but they can also be induced to differentiate by the addition of chemicals such as sodium butyrate (Kosaka *et al*, 1994) and 6-thioguanine (Edwards *et al*, 1983). The most commonly used exogenously added inducers of differentiation however are retinoic acid and (RA) and dimethyl sulphoxide (DMSO). DMSO has been found to induce cardiac and skeletal muscle in EC and ES cells (Edwards *et al*, 1983; Dinsmore *et al*, 1996). At lower concentrations RA has also been found to induce cardiac and skeletal muscle cells from EC cells, whereas at higher concentrations of approximately 10^{-7} M it induces neural and glial lineages (Edwards *et al*, 1983). RA also has been found to induce neural and skeletal muscle tissues from ES cells, but has been found to inhibit cardiac muscle differentiation (Wobus *et al*, 1994; Bain *et al*, 1995). ES cells in other species such as rat, hamster and pig have also been reported, however the only other cell line which has been shown to give rise to chimaeric animals is the avian ES cell line (Pain *et al*, 1996). Recently sheep ICM cells have also been isolated which is a major step forward in establishing ES cells from this species (Karasiewics *et al*, 1996).

1.3.3 Haematopoietic differentiation of embryonic stem cells, *in vitro*.

Totipotential embryonic stem cells are a relatively accessible cell type which can, not only be easily manipulated, but also give rise to all eight lineages of the lymphohaematopoietic system *in vitro*, with high reproducibility. Most studies of haematopoietic differentiation of ES cells have been carried out with their cystic aggregates, referred to as embryoid bodies. Such structures are thought to mimic the *in vivo* microenvironment that are able to support haematopoiesis. Embryonic stem cells are highly adhesive in nature, interacting with one another and other cell types. This property is exploited *in vitro* to generate embryoid bodies which is generally achieved in one of two ways.

If LIF, or fibroblast feeder layers, are removed and ES cells cultured in suspension or media containing methyl cellulose in non-tissue culture grade dishes, the cells adhere to the surface of the dish and to one another, thus forming three dimensional embryoid bodies composed of differentiated cells. This method has been adopted in haematopoietic studies by growing ES cells on stromal cell lines known to support haematopoietic growth and differentiation. Under such conditions the resulting embryoid bodies are surrounded by differentiated haematopoietic cells. Alternatively ES cells can be grown in 'hanging drop' suspension cultures in the presence of LIF for approximately 48 hours during which time they form aggregates. Once formed, embryoid bodies are allowed to differentiate in suspension culture. The advantage with the 'hanging-drop' culture method is that it generates embryoid bodies of approximately uniform size, and unlike the methyl cellulose method where embryoid body formation and differentiation occur simultaneously, it provides a synchronised culture system where all aggregates begin differentiation at time zero.

The ability of ES cells/embryoid bodies to differentiate towards haematopoietic lineages *in vitro* was first reported by Doetschman and co-workers (1985) who found that as well as giving rise to cardiac muscle, blood islands also formed in these cysts. Analysis of these blood islands revealed that they were pockets of nucleated erythrocytes, expressing embryonic haemoglobin, encapsulated by endolethial cells. Detailed analysis of these blood islands has shown that they form a vascular network, which although not as ordered as those of yolk sac blood islands, nevertheless make contact with the surrounding endothelial layer and have been reported to contain blood cells in the lumen (Wang *et al*, 1992).

Most ES culture systems are supplemented with serum, (typically from bovine calf), and embryoid bodies are able to give rise to haematopoietic cells in these conditions without additional growth factors. However it has been found that embryoid body differentiation towards particular lineages can be encouraged with certain cytokines (Wiles and Keller, 1991; Bigas *et al*, 1995). For instance the addition of Epo increases the number of erythropoietic cells, whereas recombinant IL-3 increases macrophage and mast cell numbers, while GM-CSF has been reported to have little effect.

The pattern of haematopoietic gene expression in embryoid bodies has also been studied extensively (Schmitt *et al*, 1991, McClanahan *et al*, 1993). A range of haematopoietic cell specific genes have been found to be expressed in embryoid bodies including cell surface markers, e.g. Ly6A/E (Sca-1) and CD45, haematopoietic cytokine receptors, e.g. c-kit and

Epo-receptor, and transcription factors e.g. GATA-1. Furthermore this pattern of gene expression has been reported to be temporally ordered with mesoderm specific genes, such as *brachyury*, and genes associated with primitive haematopoietic cells being expressed first, followed by those indicative of more committed cell types.

Studies of haematopoietic cell types which arise in embryoid bodies have moreover confirmed these reports (Keller *et al*, 1993). Nucleated erythroid cells are the first to be observed in embryoid bodies followed by macrophages, enucleated erythroid cells, neutrophils, multilineage precursors and finally mast cells. This cell differentiation sequence, closely resembles that of normal murine haematopoietic development. Lymphoid precursors with the ability to rearrange T- and B- cell receptors, and reconstitute the lymphoid compartment of sublethally irradiated SCID mice, have also been observed in embryoid bodies (Gutierrez-Ramos *et al*, 1992; Potocnik *et al*, 1994).

Although progenitor and terminally differentiated haematopoietic cells have been derived *in vitro*, whether ES cells are able to give rise to self-maintaining haematopoietic stem cells has been the subject of some debate. Colony forming assays have demonstrated that ES cells are able to give rise to primitive haematopoietic cells *in vitro* (Bigas *et al*, 1995). The primitiveness of such cells has been tested with various *in vivo* studies. Earlier it was shown that ES cell derived livers of 14.5 day chimaeric foetuses, could reconstitute lethally irradiated hosts (Forrester *et al*, 1991). Furthermore haematopoietic cells from these hosts could be used to reconstitute secondary recipients.

Following this, it was demonstrated that some populations of immortalised haematopoietic cells of ES cell origin, could give rise to multiple lineages in lethally irradiated recipients (Chen *et al*, 1992). These experiments indirectly illustrate that ES cells can give rise to haematopoetic cells with reconstituting ability. However in order to test whether ES cells are able to give rise to self-maintaining haematopoietic stem cells, *in vivo* reconstitution studies with genetically unaltered ES cells must be carried out.

Such an experiment performed by Müller and Dzierzak (1993) showed that only the more mature CFU-S cell type with limited recontitution ability could be derived from ES cells. However recently long-term repopulation of lethally irradiated recipients with differentiated ES cells have been reported (Palacios *et al*, 1995; Hole *et al*, 1996). In the former study ES cells were co-cultured with a stromal cell line and cytokines such as IL-3 and IL-6 added. Following FACS sorting $Thy1^{+}Lin^{-}$ cell populations were injected into lethally irradiated hosts and donor cells found to contribute to all haematopoietic

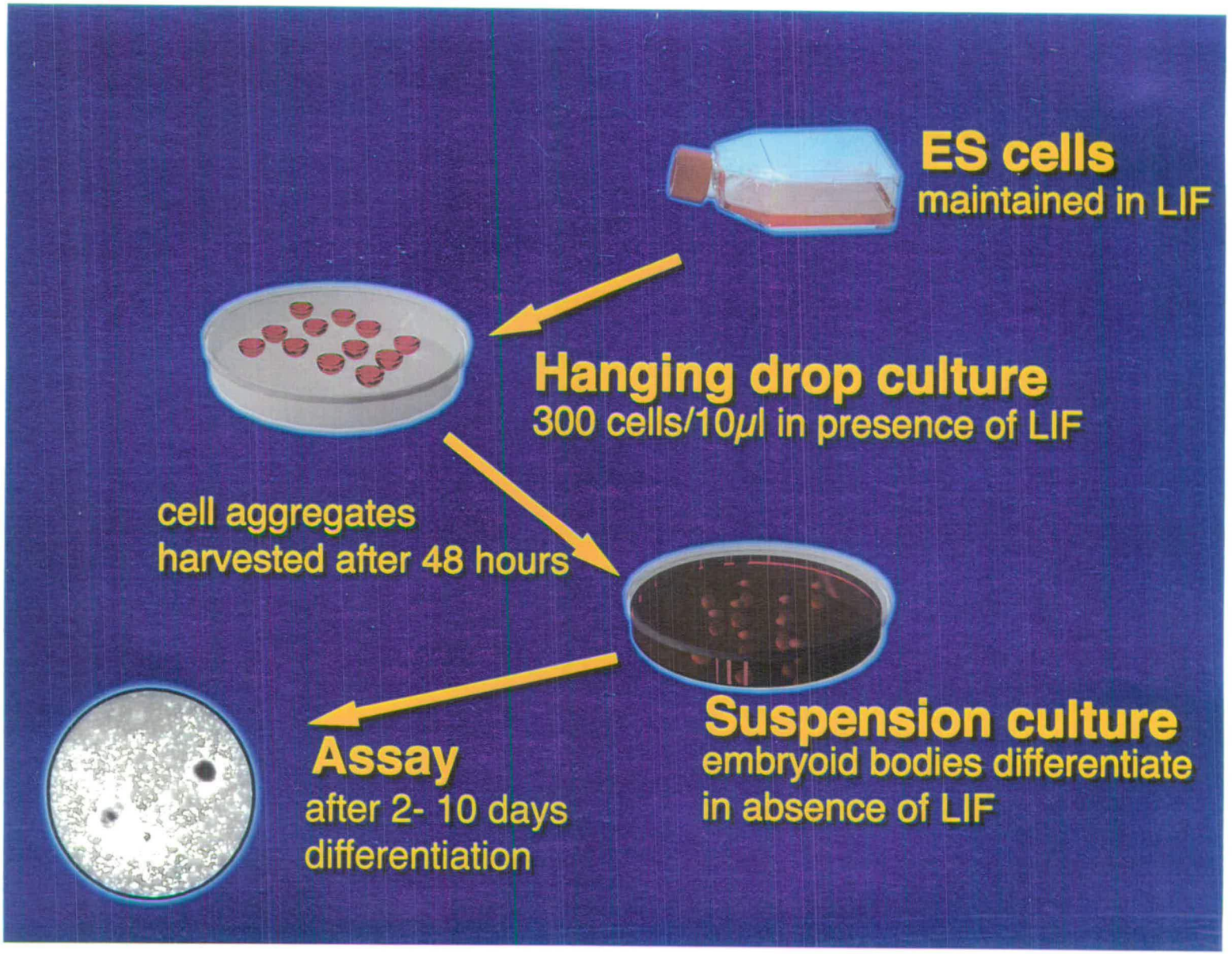


Figure 1.3. Outline of the culture system used.

lineages. In the latter study however embryoid bodies cultured in the absence of additional cytokines or stromal cell lines have been shown to reconstitute the haematopoietic system of lethally irradiated recipients.

With reports of the ability of ES cells to reconstitute haematopoietically compromised hosts, it is becoming increasingly apparent that the ES cell culture systems provides a unique model for studying both primitive and definitive haematopoiesis. Despite its many advantages, certain limitations are still imposed by the ES cell culture system, foremost of which is that it remains an *in vitro* model and as such may not be fully representative of *in vivo* haematopoiesis. Furthermore ES cell commitment and differentiation is temporally specific and results in the production of multiple cell types and not just haematopoietic lineages. Due to these reasons the study of the early developmental events which control ES cell haematopoietic commitment remain difficult to elucidate.

1.3.4 Aims and objectives of the current study.

As mentioned earlier, the detailed molecular events which control haematopoietic commitment and differentiation are poorly understood, since key genes which direct the process have yet to be identified. The main aim of this project has been to distinguish and isolate such regulatory elements. However in order to identify a relatively small population of genes involved in a specific process, requires a panning process whereby the larger unrelated pool can be eliminated. One means of achieving this is by altering gene expression patterns of the system under study to either enhance or down-regulate the expression of the genes of interest, which can in turn be achieved by altering the growth conditions of cells.

The culture system under study, outlined in Figure 1.3, utilises the CFU-A assay (colony forming unit-assay) to assess and quantify the haematopoietic differentiation of embryoid bodies (Figure 1.4). It has thus been found that approximately 30-50% of 5-7 day old embryoid bodies can give rise to CFU-A colonies. Embryoid bodies which have differentiated for less than 4 or more than 8 days, give rise to markedly fewer CFU-A colonies. This time course of differentiation has been found to be highly reproducible. Although few CFU-A colonies are obtained with 4 day embryoid bodies, the reconstituting ability reported by Hole *et al* (1996), appears to be restricted to this time point.

This project therefore seeks to modulate embryoid body haematopoietic commitment by the addition of exogenous factors to the culture environment of these cystic structures, thereby up- or down-regulating haematopoietic gene expression. This strategy could thereafter be used to devise a method of identifying genes which are either directly involved in ES cell haematopoietic commitment and differentiation, or which could act as surrogate markers for potential haematopoietic progenitors.

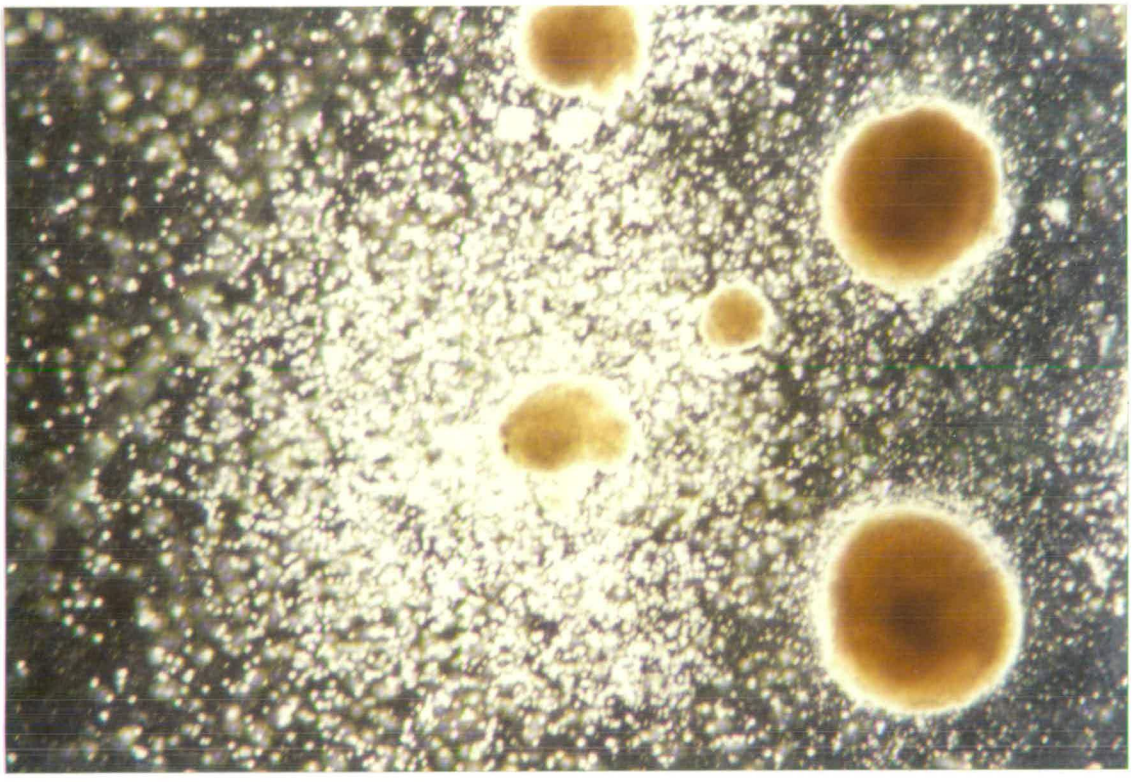


Figure 1.4. CFU-A assay of embryoid bodies. Embryoid bodies were assayed after 6 days differentiation in suspension culture, and assays scored after approximately 11 days incubation. A typical CFU-A colony is illustrated in the centre of the figure with macrophage and monocytes emanating from the central embryoid body. Two non-haematopoietic embryoid bodies are represented to the right of the colony.

2. Materials and Methods.

All chemicals were of analytical grade and were purchased from the suppliers indicated, the addresses for which are given in Apppendix II. All percentages are weight/volume unless specified otherwise.

2.1 Tissue culture techniques.

2.1.1 Routine maintenance of cells.

The embryonic stem (ES) cell line, EFC-1, derived from male embryos of 129J/Ola strain of mice were used throughout the course of the study (Nicholls *et al*, 1990). These were routinely karyotyped and checked for mycoplasma infection. All procedures were carried out essentially as described (Smith, 1991; Hole and Smith, 1994). Cells were thawed rapidly in a 37°C water bath and transferred to a 15ml centrifuge tube containing 9.5ml of media [1 x G-MEM (Gibco BRL) media supplemented with 10% foetal calf serum (GlobePharm), 1% non-essential amino acids (Gibco BRL), 2% L-glutamine/sodium pyruvate (Gibco BRL) (L-glutamine (200mM) and sodium pyruvate (100mM) were prepared as stocks in a 1:1 ratio), 1×10^{-4} M 2-mercaptoethanol (Sigma), 0.2% sodium bicarbonate (Gibco BRL)]. The suspension was centrifuged at 140g for 5 minutes, the media aspirated and 10ml of fresh media added. The cells were then transferred to a $25cm^2$ plastic flasks coated with 0.1% gelatine (Sigma), 100 U/ml of LIF added and incubated at 37°C in a humidified atmosphere of 5% CO_2. LIF was obtained from the supernatant of Cos-7 cells transfected with an expression vector containing the LIF gene. The volume of supernatant which gave a detectable inhibition of ES differentiation was defined as 1 unit. After approximately 6 hours incubation the media on the newly thawed cells was aspirated and fresh media added.

Cells were thereafter routinely passaged once every 48 hours being washed with 4-5ml of PBS (Oxoid) and then released into a single cell suspension by trypsinisation at 37°C with 2ml of TVP [1% chick serum (Gibco BRL), 1% trypsin (Gibco BRL), 8.4×10^{-4} M EDTA (MERCK-BDH) in PBS] for approximately five minutes. Following physical agitation the cells were transferred to a 15ml centrifuge tube, 8ml of media added and the suspension spun at 100g for 5

minutes. The supernatant was consequently removed and the pellet re-suspended in 10ml of fresh media. A sample of the suspension was then used to estimate cell numbers with a haemacytometer. This suspension was either used for passaging, passeges being seeded at approximately 1 x 10^6 cells per 10ml of media, or generating hanging drop suspensions. Cells of more than passage 30 were not used for experiments.

For the purposes of freezing, cells was trypsinised as described, resuspended in 7ml of media. The suspension was then centrifuged at 100g for 5 minutes, the supernatant aspirated and cells resuspended in 1.0ml of 10% DMSO (v/v) in media. 0.5ml of the suspension was then pipitted into a cryo-tube and placed in a -70°C freezer overnight and thereafter transferred to liquid nitrogen (BOC).

2.1.2 'Hanging Drop' method for the generation of ES cell aggregates

'Hanging drop' cultures for the formation of embryoid bodies were performed essentially as described by Rohwedel *et al*, (1994). Cell suspensions of approximately 3 x 10^4 cells per ml of media in the presence of 100U/ml of LIF were prepared and 10µl aliquots dispensed on the upturned lid of a bacteriological plate. The lid was subsequently replaced on the base of the plate, containing 8ml of sterile water to prevent dehydration of the drops. Plates were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 hours after which the undifferentiated ES cell aggregates were harvested into universal containers, centrifuged at 65g for 3 minutes, the LIF containing supernatant removed by aspiration and the aggregates resuspended in an equal volume of differentiation media [1 x G-MEM media supplemented with 10% foetal calf serum (Advanced Protein Products), 1% non-essential amino acids, 2% L-glutamine/sodium pyruvate, 1 x 10^{-4} M 2-mercaptoethanol, 0.2% sodium bicarbonate].

2.1.3 Culture of embryoid bodies.

Embryo bodies were cultured in differentiation media in non-tissue culture grade petri dishes, 50µg/ml of streptomycin/50 units penicillin (Gibco BRL) being included in the media during the first 48 hours of differentiation, and incubated at 37°C in a humidified atmosphere of 5% CO_2. Embryoid bodies were routinely harvested once every 48 hours by transfer into universal

containers and allowed to settle. The media was then aspirated and embryoid bodies resuspended in fresh differentiation media. The volume of media used was dependent on the numbers of embryoid bodies cultured, 1500-2000 embryoid bodies being cultured in 20ml of media in $10cm^2$ dishes and numbers of between 375-500 cultured in 10ml of media in $5cm^2$ dishes.

Chemical inducers were included in the cultures as required. Retinoic acid (Sigma) was dissolved in 100% ethanol (Merck-BDH) at a stock concentration of 10^{-2} M and stored in the dark at -20°C; α-tocopherol (Sigma) was dissolved in 100% ethanol at a stock cencentration of 1M and stored as retinoic acid. Iron (II) sulphate (Sigma) was made fresh in differentiation media at a stock concentration of 10^{-2}M and filtered through a 0.2μm syringe filter before addition to cultures. After the required period of exposure, embryoid bodies were harvested as described and washed once in 10-15ml of PBS before resuspension in fresh differentiation media.

2.1.4 CFU-A assay for the detection of primitive haematopoietic stem cells .

Assays were performed essentially as described (Pragnell *et al*, 1988; Lorimore *et al*, 1990). The assay is composed of 2 layers, a bottom feeder layer containing conditioned media which are a source of cytokines, and a top agar layer which is used to dispense embryoid bodies on the feeder layer and also maintains the CFU-A colonies intact. The bottom feeder layer consisting of 0.6% Noble agar (Difco), 50% 2 x stock media [50% (v/v) horse serum, 42% (v/v) α-MEM; 2% L-glutamine; 0.45% sodium bicarbonate], 1/10th of the total volume of conditioned media from the rat fibroblast cell line L929 (a source of CSF-1 (M-CSF) (Stanley and Heard, 1977)), and 1/20 of the total volume of conditioned media from the malignant histocytosis sarcoma virus-transformed cell line AF1-19T (a source of GM-CSF (Franz *et al*, 1985)), was prepared and 1ml layers dispensed into 3cm tissue culture grade dishes and allowed to set. Each embryoid body sample was assayed in triplicate.

Embryoid bodies were harvested into universal containers, allowed to settle and their media aspirated. The number of embryoid bodies per sample was estimated by placing three 10μl aliquots onto a microscope slide and calculating the mean number per 10μl. An aliquot of embryoid bodies containing approximately 150 embryoid bodies was then removed and placed

into a fresh universal container. These embryoid bodies were then mixed with 3ml of top agar [0.3% agar; 50% 2 x stock media] and dispensed onto the bottom agar layer, 1ml per plate, (approximately 50 embryoid bodies per plate). The plates were then incubated at 37°C in a humidified atmosphere with 5% O_2 and 10% CO_2 for 11 days. CFU-A colonies were quantified visually using an inverted light microscope.

CFU-A colonies are typically defined as those with a diameter of 2mm or more. However, since more primitive haematopoietic progenitors can potentially give rise to smaller CFU-A colonies (Pragnell *et al*, 1988), in order to avoid the exclusion of such a population of cells from quantification, CFU-A colinies were defined on the basis of the number of haematopoietic cells (typically macrophages and monocyates) surrounding an embryoid body. Therefore, embryoid bodies surrounded by 50 or more haematopoietic cells were defined as CFU-A colony forming. The same definition was applied for the quantification of CFU-A colonies from disrupted embryoid bodies. That is clusters of 50 or more cells were defined as CFU-A colonies.

2.1.5 Disruption of embryoid bodies.

Embryo bodies were harvested as described in section 1.3, their supernatant removed, washed once with PBS and then resuspended in 0.5ml of PBS per 375-500 embryoid bodies. An equal volume of Dispase (Boehringer) was added and the suspension incubated at 37°C for 1 hour with gentle agitation. The cell suspension was passed through a 23G needle several times to give a single cell suspension and then transferred to a 15ml centrifuge tube and centrifuged at 100g for 5 minutes. The supernatant was removed and the pellet re-suspended in 1ml of PBS and the cell number estimated. For CFU-A assays 1.8 x 10^5 cells were assayed in triplicate, 6 x 10^4 cells per plate, as described in section 2.1.4.

2.2 Animal dissections.

2.2.1 Dissection of mouse spleen and thymus, and single cell suspension preparartion.

Spleen cells were obtained from female 129/Ola mice by dissection. Incisions were made through the fur and peritoneum, the spleen removed and placed into a petri dish and covered with 1-2ml of PBS. Two 23G needles on 1ml syringes were bent to a 90° angle. The spleen was held in place with one needle while the second needle was used to 'nick' one end of the spleen membrane and then gently stroked across the organ to release the cells. Once the majority of the cells had been removed the spleen membrane was discarded and the volume was made up to 10ml with PBS. The spleen preparation was then passed through a 23G needle to make a single cell suspension, transferred to a 15ml centrifuge tube and the cell number estimated with a haemacytometer. Single cell suspensions from the thymus were obtained using the same technique. Spleen cells required for RNA isolation were extracted using a dissection kit that was sterilised at 200°C for 4 hours prior to use, 0.1% DEPC treated PBS was used and the cell suspension was maintained on ice to minimise RNA degradation.

2.2.2 Bone marrow single cell suspensions from mouse femur.

A female 129/Ola mouse was skinned to expose the leg and muscle tissue removed from the femur. The femur was then removed by incisions at the knee and hip joints and the epiphyses removed. Using a 1ml syringe and 23G needle 1ml of ice cold 0.1% DEPC treated PBS was flushed through the marrow and into a chilled 15ml centrifuge tube. The bone marrow cell preparation was then passed through a 23G needle to make a single cell suspension, the volume made up to 10ml with PBS and the cell numbers estimated with a haemacytometer.

2.2.3 Dissection of post coital (p.c.) mouse embryos for RNA extraction.

Timed matings were set up between 3 female and 1 male CBA/Ca mice and 6.5 days after vaginal plugs were observed one female mouse was killed. The uterus was cut away from the pubic bone and the uterine horns separated but left attached at the ovaries. The end of one

uterine horn was held in place with a pair of forceps and and the membrane cut along the length to expose the decidual masses which were then removed and placed in a petri dish containing 1-2ml of 0.1% DEPC treated PBS. With the use of a microscope the decidual masses were held in place with a pair of forceps and cut off-centre along the length. The embryo proper was teased out by gentle removal of the surrounding maternal and placental tissue. Once free, the embryo was removed with a sterilised Pasteur pipette and placed in a 1.5ml eppendorf tube and used for RNA extraction.

For the removal of day 13 p.c. embryos the uterus was once again cut away from the pubic bone and the uterine horns separated but left attached at the ovaries, as above. One uterine horn was held in place with a pair of forceps and the membrane cut along its length to expose the embryos. The embryos were then removed and placed in a petri dish containing 3-4ml 0.1% DEPC treated PBS. The allantois was peeled away and removed along with the placenta and yolk sac, and the foetal liver removed with a pair of forceps and placed into a 1.5ml eppendorf containing 0.5ml DEPC treated PBS. The foetal liver was then homogenised by passage through 18G and 23G needles respectively and then used for RNA extraction.

2.3 Molecular biology techniques.

All procedures were carried out essentially as described by Sambrook *et al* (1989) except where indicated. All glassware used for RNA work was sterilised at 200°C for 4 hours prior to use and all solutions were treated with 0.1% DEPC for 2 hours, or overnight at room temperature, before autoclaving.

2.3.1 Preparation of genomic DNA.

Blood samples collected from mice were centrifuged at 9500g for 5 minutes, the sera removed and 2 volumes of lysis buffer [25mM Tris (Fisons); 75mM sodium chloride (Fisons)), 1/5 of the total volume of blood of 10% SDS (Fisons)] and 1/20 of the volume of proteinase K (10mg/ml) (Boehringer) were added and the cells incubated overnight at 37°C. An equal volume of washed phenol (Fisons) was then added and the 2 phases thoroughly mixed for five minutes. The suspension was centrifuged for 2 minutes at 9500g, the aqueous layer transfered to a fresh

1.5ml eppendorf and the procedure repeated. An equal volume of chloroform (Fisons): isoamyl alcohol (Aldrich Chemical Co. Ltd.) (24:1) was then added and the 2 phases mixed thoroughly for 5 minutes. The suspension was centrifuged for 5 minutes at 9500g and the aqueous layer transfered into a fresh 1.5ml eppendorf tube. 1/10 of the total volume of 3 M sodium acetate (Fisons) (pH 7.0) and 2 volumes of 100% ethanol were then added and the DNA allowed to precipitate at -70°C for 20 minutes after which it was pelleted by centrifugation at 9500g for 20 minutes. The pellet was washed once with 70% ethanol, centrifuged as above and finally dissolved in an appropriate volume e.g. 200µl of TE buffer (10mM Tris.Cl pH 7.5; 1mM EDTA pH 8.0).

2.3.2 PCR amplification of ZFY and Myogenin markers.

The proportion of peripheral blood cells derived from male ES cells was determined by polymerase chain reaction (PCR) amplification of the male genomic marker ZFY, essentially as described (Koopman *et al*, 1991) with the exception that separate reactions were prepared for ZFY and Myogenin (control reaction). The reaction mixtures consisted of 10µl of genomic DNA, 0.3mM of each of dATP, dGTP, dCTP and dTTP (Promega), 1 x PCR buffer [50mM Tris-HCL pH 9.0, 15mM ammonium sulphate (Fisons), 7mM magnesium chloride (Fisons), 0.05% Nonidet P-40 (Merck-BDH)], 1µg oligonucleotide mix (Cruachem) (consisting of 1:1 ratio of 3' and 5' primers; Table 2.1), and 2.5 units of BIOPRO™ polymerase (Bioline) were added in a total volume of 50µl. The reactions were briefly centrifuged in a microcentrifuge, overlaid with 50µl of mineral oil (Sigma) and PCR amplification carried out. The template was denatured at 95°C for 5 seconds followed by annealing of primers for 30 seconds at the appropriate temperature (Table 2.1) and extension at 72°C for 30 seconds. This procedure was caried out for 25 cycles. Upon completion, 2µl of 6 x DNA gel loading buffer [0.25% bromophenol blue (Fisons); 0.25% xylene cyanol FF (Fisons); 30% glycerol in water (Fisons)] was added to 10µl of each PCR product and products electrophoresed on 2% agarose (Boehringer), 0.5 x TBE (0.045M Tris-borate; 0.001M EDTA), 0.5 µg/ml ethidium bromide (Sigma) gels, at 5 v/cm of gel and thereafter photographed. Gels were then denatured in 100-200ml of 1.5M sodium chlride/0.5M sodium hydroxide solution and Southern blotted.

2.3.3 Purification and [α-^{32} P] dCTP labelling of probe DNA.

Probe DNA was either obtained from PCR amplification of the relevant fragment from mouse genomic DNA or cDNA, or excised from plasmid vectors by restriction endonuclease digestion. Restriction endonuclease reactions typically consisted of approximately 5μg of plasmid DNA, 5 units restriction enzyme(s) (Boehringer), 1 x of appropriate reaction buffer, in a total volume of 20-50μl and digested at the required temperature (typically 37°C) for at least 1 hour. PCR products and/or restriction endonuclease reaction products were resolved on 1-2% agarose, 0.5% TBE, 0.5μg/ml ethidium bromide gels. The appropriate band was then cut from the gel and placed in a 0.5ml eppendorf tube which had been pierced once with a 26G needle and plugged with siliconised glass wool. DNA was collected into a 1.5ml eppendorf by centrifugation for 10 minutes at 2000g in a microcentrifuge. The DNA eluate was extracted twice with phenol/chloroform and precipitated with 1/10 volume 3M sodium acetate, pH 7.0, and 2 volumes of ethanol.

Once purified the probe DNA was labelled with radioactive [α-^{32} P] dCTP (Amersham), using the Promega nick translation system according to the manufacturer's instructions. The labelling reaction consisted of 180μM dNTP mixture (60μM of each dNTP excluding dCTP), 1 x Nick translation buffer (50mM Tris-HCl, pH 7.2; 10mM magnesium sulphate; 0.1mM DTT), 1μg probe DNA, 50μCi [α-^{32}P] dCTP, 5μl Promega Nick Translation Enzyme Mix [1 unit/μl of DNA polymerase I, 0.2 ng/μl DNase I in a 50% glycerol in a solution containing 50mM Tris-HCl pH 7.2, 10mM magnesium sulphate, 0.1mM DTT and 0.5mg/ml nuclease free BSA], in a total volume of 50μl. The reaction was incubated at 15°C for 1 hour and subsequently 5μl of stop solution (0.25M EDTA pH 8.0, 0.5M sodium phosphate pH 6.8) added and the total volume made up to 100μl with double distilled water. Unincorporated dNTPs were removed by centrifugation of the product through a 1ml Sephadex G-50 (Pharmacia) column. The columns were prepared by filling a 1ml syringe with Sephadex G-50 and centrifuging for 5 minutes at 350g. This was repeated once more and the column washed once with 0.5ml of 0.1% SDS and centrifuged at 350g for 5 minutes. The radioactive labelled probe was then centrifuged through the column at 350g for 5 minutes and the eluate collected into a 1.5ml eppendorf . The double stranded DNA probe was denatured by boiling in a water bath for 5 minutes and subsequetly chilled rapidly on ice.

2.3.4 Extraction of total RNA and agarose/formaldehyde gel electrophoresis.

Total RNA was extracted from embryoid bodies and/or tissue samples using RNAzol B solution (Biotecs) according to the manufacturer's instructions. Briefly the embryoid body or homogenised tissue sample was washed with DEPC treated PBS at least 3 times and 0.2ml of RNAzol B solution per 10^6 cells was added, each embryo body being estimated to contain an average of 5000 cells. The lysate was then passed through a 23G needle several times, followed by addition of 1/10 volume of chloroform (Fisons), mixed thoroughly by agitation and incubated at 4°C for 5 minutes. The sample was then centrifuged at 4°C at 12000g for 15 minutes and the upper aqueous phase transferred to a fresh tube. An equal volume of propan-2-ol (Merck-BDH) was added to precipitate the RNA and the sample incubated at 4°C for at least 25 minutes. The RNA was subsequently pelleted by centrifugation at 7500g for 10 minutes at 4°C, washed once in 70% ethanol, re-centrifuged and dissolved in an appropriate volume of DEPC treated 1mM EDTA, pH 7.0. The absorbance of the RNA samples were measured at 260nm and 280nm to determine concentration and purity, and samples stored at -20°C.

Electrophoresis of total RNA extracts on denaturing agarose formaldehyde gels were carried out as follows. 1% agarose gel were prepared by dissolving the appropriate amount of agarose in DEPC treated water, cooling to 60°C, and adding 1/5 of the total volume of 5 x formaldehyde gel running buffer [0.1M MOPS pH 7.0, 40mM sodium acetate (Merck-BDH), 5mM EDTA] and formaldehyde (Merck-BDH) to give a final concentration of 2.2M. The RNA samples (3-10µg) were then prepared by adding 1/10 of the total volume of 5 x formaldehyde gel running buffer, 50% formamide (Merck-BDH) and formaldehyde to give a final concentration of 2.2M. The samples were then incubated at 65°C for 15 minutes, chilled on ice and 1/10 of the total volume of RNA gel loading buffer [0.25% bromophenol blue; 0.25% xylene cyanol FF (Fisons); 1mM EDTA pH8.0; 50% glycerol in DEPC treated water] and 1/100 of the total volume of 10mg/ml ethidium bromide added and mixed thoroughly. The formaldehyde gel was pre-run in 1 x formaldehyde gel running buffer for 5 minutes at 5 volts/cm of gel, then the samples loaded and electrophoresed. The gels were subsequently photographed, washed in copious amounts of DEPC treated water with 3-4 changes to remove the formaldehyde and blotted on to nitrocellulose membranes as described in section 2.3.5.

2.3.5 Southern and Northern transfer and hybridisation with probe DNA.

Capillary transfer of DNA and RNA onto a solid support was performed essentially as described by Southern (1975) and Alwine (1977) respectively. The capillary transfer apparatus was prepared by pouring 20 x SSC (3M sodium chloride; 0.3M sodium citrate) in to large tray and placing a platform support over the tray. A folded sheet of Whatman 3MM paper soaked in 20 x SSC was then placed on the support with the overhanging edges imersed in 20 x SSC, avoiding the entrapment of air bubbles. The gel was then placed upside down on the 3MM paper, once again avoiding air bubbles and a of piece of nitrocellulose filter (Boehringer) which had been cut to the size of the gel and pre-soaked in 2 x SSC carefully placed over the gel. To assist the transfer two pieces of 3MM Whatman paper were placed over the filter and covered with a stack of paper towels and an even weight of approximately 500 grams balanced on the towels. In order to avoid short circuiting of the transfer the gel was surrounded with clingfilm and transfer allowed to take place over night. Once complete, the apparatus was dismantled, the filter removed and placed between 2 pieces of Whatmann paper and baked at 120°C for 40 minutes. Northern transfer of RNA was carried in the same way with the exception that 0.1% DEPC treated SSC was used with baked glass trays and platforms. Denaturation of RNA was not necessary since RNA was electrophoresed on denaturing gels, (see below).

Filters were first pre-hybridised in 10-25ml of Church/Gilbert buffer (0.5M di-sodium hydrogen orthophosphate (Fisons); 7% SDS; 1mM EDTA; pH 7.2 with orthophosphoric acid) containing 1/100 volume denatured salmon sperm DNA (10mg/ml) (Boehringer) for up to 1 hour at 65°C with rotation, after which the denatured radioactive DNA probe was added and hybridisation allowed to take place over night under the same conditions. Following hybridisation, unbound and non-specifically bound probe was removed by washing with 30-50ml of 5% SDS, 1mM EDTA, 0.04M di-sodium hydrogen orthophosphate and 1% SDS, 1mM EDTA, 0.04M di-sodium hydrogen orthophosphate in sequence, for upto 1 hour in each wash at 65°C with rotation. The filter was subsequently wrapped in clingfilm and exposed to a phosphorscreen over night. The screen was then scanned using a Molecular Dynamics laser scanner and analysis performed using Molecular Dynamics software, run on a Macintosh computer.

GENE	5′ PRIME SEQUENCE	3′PRIME SEQUENCE	PRODUCT SIZE (bp)	ANNEALING TEMPERATURE	MAGNESIUM CHLORIDE	REF
ß - ACTIN	5′-GTGACGAGGCCCAGAGCAAGAG-3	'5′-AGGGGCCGGACTCATCGTACTC-3′	934	63 °C	2 mM	Schmitt *et al* (1991)
FOETAL -ß GLOBIN	5′-AACCCTCATCAATGGCCTGTGG-3′	5′-TCAGTGGTACTTGTGGGACAGC-3′	415	63 °C	2mM	Schmitt *et al* (1991)
BRACHYURY	5′-TCCAGGTGCTATATATTGCC-3′	5′-TGCTGCCTGTGAGTCATAAC-3′	947	50 °C	4mM	Keller *et al* (1993)
CD - 45	5′-CCTGAGTCTGCATCTAAACCCC-3′	5′-TGCTTGGCCAGTATTCTGCGCA-3′	1067	63 °C	4mM	Schmitt *et al* (1991)
c-kit receptor	5′CAACAGCAATGGCCTCACGAGT3′	5′GTGGTACACCTTTGCTCTGCTC3′	1069	68 °C	2mM	Schmitt *et al* (1991)
GATA - 1	5′-ATGCCTGTAATCCCAGCACT-3′	5′-TCATGGTGGTAGCTGGTAGC-3′	581	55 °C	1mM	Keller *et al* (1993)
HAEMATOPOIETIC CELL PHOSPHATASE (HCP)	5′-CAGGACTGCAGGTTGGCTTTAG-3′	5′-ATGTCAGCTGGGTTTACCCGAG-3′	800	58 °C	2mM	
IL-3	5′-TTCTTGCCAGCTCTACCACCAG-3′	5′-ACTTTAGGTGCTCTGCCTGCTG-3′	522	63 °C	2mM	
Ly - 6A/E/Sca1	5′-CCCCTACCCTGATGGAGTCTGT-3′	5′-GGATTAGAGCACCTACCTACCC-3′	450	58 °C	2mM	
MIP-1α			299	65 °C	2mM	
MYOGENIN	5′-TCACGGTGGAGGATATGTCT-3′	5′-GAGTCAGCTAAATTCCCTCG-3′	350	60 °C	7mM	Koopman et al (1991)
ZFY	5′-CCTATTGCATGGACTGCAGCTTATG-3′	5′-GACTAGACATGTCTTAACATCTGTCC-3′	180	65 °C	7mM	Koopman *et al* (1991)

Table 2.1. Deoxyribonucleotide primers and reaction conditions used for the PCR reactions.

2.3.6 Reverse transcription of RNA and PCR amplification of products.

The Promega AMV reverse transcription system was used to synthesise single stranded cDNA from total RNA extracts, according to the manufacturer's instructions. In brief, 1μg of total RNA extract was reverse transcribed in a reaction containing reverse transcriptase buffer [10mM Tris-HCl (pH 8.0), 50mM potassium chloride, 0.1% Triton X-100], 1mM of each dNTP, 1u/μl of rRNasin ribonuclease inhibitor, 15u/μg of AMV reverse transcriptase, 0.5μg/μg RNA of random primers in a total volume of 20μl. The reaction mixture incubated at 42°C for 15 minutes.

PCR amplification of the cDNA product was carried out in a total volume of 50μl consisting of 1μl of the reverse transcription product, 1 x ammonium buffer [16mM ammonium sulphate, 67mM Tris-HCl pH 8.8, 0.01% Tween-20], 2-5mM magnesium chloride (depending on the specific reaction, see Table 2.1), 1μg oligonucleotide mixture (consisting of 1:1 ratio of 3' and 5' primers; Table 2.1), 2.5 units BIOPRO™ polymerase and 1mM dNTP mixture (consisting of 1:1:1:1 ratio of dATP, dCTP, dGTP and dTTP). The reactions were overlaid with 50μl mineral oil and amplification carried out. The template was denatured at 95°C for 20 seconds, followed by annealing of primers for 1 minute at the appropriate temperature (Table 2.1), and extension of primers at 72°C for 1 minute. The procedure was repeated for 30 cycles. Upon completion 1/6 of the total volume of DNA gel loading buffer was then added to 10μl of RT-PCR product and ran on a 1-2% agarose, 0.5% TBE, 0.5μg/ml ethidium bromide gels. If products were not readily visible with ethidium bromide staining, they were blotted and hybridised with the relevant probe.

2.4 Protein isolation and analysis.

2.4.1 L-[^{35}S] methionine labelling of embryoid body proteins.

Approximately 50-100 embryoid bodies from each sample were washed twice in PBS and resuspended in 0.9ml of CO_2 -gassed methionine free-media [1 x D-MEM (Gibco) L-methionine and L-glutamine free; 10% dialysed amino-acid free FCS; 1% glutamine; kept at 5% CO_2] and transferred to a tissue culture grade 24 well plate. 0.1ml of media containing 0.25 mCi of L-[^{35}

S] methionine was then added to each well and incubated for 1 hour at 37°C in a humidified atmosphere of 5% CO_2. The embryoid bodies were then transferred to 1.5ml eppendorf tubes, washed once in cold PBS and 100µl of lysis buffer added [9.9M urea; 4% Nonidet P-40; 2.2% ampholytes pH range 3-10 (Sigma); 100mM DTT)].

2.4.2 Estimation of protein concentration .

Protein concentrations in embryoid body lysates was determined using the Lowry method (1951) modified by the addition of sodium deoxycholate. 5µl of each lysate was made 0.25 M with respect to sodium hydroxide and 2% with respect to sodium deoxycholate (Sigma) in a total volume of 0.5ml. 2.5ml of copper alkali reagent [0.05% sodium hydroxide; 2% sodium carbonate (Fisons) 0.02% sodium potassium tartrate (Fisons) 0.01% copper sulphate (Sigma)] was then added to the protein solution, mixed and incubated at room temperature for 10 minutes. 0.25 ml of 1.0M Folin-Ciocalteu's phenol reagent (Sigma) was then added, mixed thoroughly and the colour allowed to develop in the dark for 90 minutes. The absorbance of the samples was measured at 660nm against a reagent blank. A standard curve was calculated using dilutions of a known BSA solution in the same assay and the protein concentration of the embryoid body samples calculated from this curve.

2.4.3 Two dimensional SDS - polyacrylamide gel electrophoresis (2D-PAGE) and staining of gels.

2D-PAGE was carried out essentially as described by O'Farrel (1975). Isoelectric gel mixture consisting of 9M urea, 4% acrylamide (Fisons), 0.24% N,N'-methylbisacrylamide (Fisons), 300-450µl ampholines pH range 3-10, 2% Nonidet P-40 (added after degassing the mixture for 10 minutes), was prepared in a total volume of 15ml and polymerisation activated by the addition of 70µl 10% ammonium persulphate (Fisons) and 10µl of TEMED. The IEF gels were then cast to a height of approximately 12cm, in capillary tubes of internal dimensions of 0.15 x 17cm, by carefully placing the tubes in the gel mixture and overlayering with water. 1 hour was allowed for polymerisation after which the gels were prefocussed at 200 volts for 1-2 hours using 0.085% orthophosphoric acid as the anode electrode buffer and 0.085% sodium hydroxide as the cathode electrode buffer. 0.5mg of each protein sample was then loaded onto

a gel with a Hamilton syringe under the cathode electrode buffer and isoelectric focusing carried out for 10000-12000 volt-hours. Once complete the gels were removed by inserting a yellow eppendorf pipette tip attached to a water-filled 1ml syringe into the cathode end of the gel and carefully removing the gel. The gels were equilibriated in equilibriation buffer [0.125M Tris-base, pH 6.8 (Sigma); 2% SDS; 10% glycerol; 0.2-0.8% 2-mercaptoethanol added just before use] for 15 minutes. If necessary they were frozen at -20°C before equilibration and thawed in equilibration buffer just before electrophoresis across the second dimension.

Resolving gels for SDS-PAGE electrophoresis consisting of 10% acrylamide, 0.27% N,N'-methylbisacrylamide, 0.38 M Tris (pH 8.8), 0.1% SDS, were prepared in a total volume of 50ml and the polymerisation reaction was initiated by the addition of 0.5ml 10% ammonium persulphate and 20µl of TEMED. The gels were cast between sandwiches of glass plates (16 x 18cm) separated by 0.15cm spacers and overlaid with water saturated butanol. After polymerisation the butanol and un-polymerised acrylamide were washed off with distilled water and the tube gel laid horizontally across the resolving gel, alongside molecular weight markers (Sigma) [mixed with an equal volume of 1% agarose and formed into a tube with the aid of a Pasteur pipette] and electrophoresed at 5 volts/cm of gel in Tris-glycine buffer [25mM Tris; 250mM glycine (Fisons); 0.1% SDS].

After electrophoresis the gels were either Western blotted or stained overnight in Coomasie blue stain with shaking [0.5% Coomasie blue (Fisons); 10% glacial acetic acid; 10% ethanol] destained in 10% glacial acetic acid/10% ethanol with shaking until the background was clear, and dried under vacuum onto Whatmann 3MM paper. L-[^{35}S] methionine labelled gels were exposed to Kodak film and the autoradiographs scanned on a Molecular Dynamics laser densitometer. The images were then analysed using PDQuest™ software (Protein Databases Incorporated) run on a Sun Workstation.

2.4.4 Western transfer and immunological detection of proteins.

Western blotting was carried out essentially as described by Towbin (1979). The transfer apparatus was prepared by placing 3 sheets of 3MM paper cut to the size of the gels and soaked in transfer buffer [39mM glycine; 48mM Tris base; 0.037% SDS; 20% methanol] onto the anode side of the transfer cassette. The PVDF (Boehringer) membrane [pre-soaked in

methanol and washed in water] was then placed onto the 3MM paper, the 2-dimensional PAGE gel removed from the glass plates and placed over the membrane, avoiding the entrapment of air bubbles. A further 3 sheets of 3MM paper soaked in transfer buffer were then placed over the gel and the cathode side of the transfer cassette clipped into place adjusted into place. Transfer was carried out over a period of at least 2 hours at 0.65 milliamps/cm^2 of gel after which the apparatus was disassembled and the membrane briefly stained with Ponceau S (Sigma) to ensure that transfer had taken place and washed in several changes of distilled water. The lane containing markers was cut from the membrane and stained with Amido black [0.5% amido black, 10% galcial acetic acid; 10% ethanol] and destained in 10% glacial acetic acid/10% ethanol until the bands were visible.

The remainder of the membrane was placed in 0.1ml/cm^2 of membrane of blocking buffer [0.05% Tween-20 (Sigma) in PBS; 5% non-fat dried milk] and incubated at room temperature with shaking, for 2 hours. The membrane was then washed 4 times in washing buffer (0.05% Tween-20) for five minutes each time. The primary antibody (1:500 dilution) was added in fresh blocking buffer (0.1ml/cm^2 membrane) and the filter incubated as before. Unbound and non-specifically bound antibody was removed by 4 times in washing buffer. After the last wash the membrane was washed in Tris-saline [150mM sodium chloride; 50mM Tri-HCl (pH7.5)] for 5 minutes. HRP-conjugated secondary antibody (Law Hospital) (1:200 in Tris-saline/5% non-fat dried milk) was added to the filter and incubated for 2 hours with shaking. Unbound secondary antibody was removed by washing the filter in 4 changes of Tris-saline for 5 minutes each time. The chromogenic reagent [0.3% 4-chloronaphthol in methanol] was diluted 1:5 in Tris-saline and added to the membrane and the colour allowed to develop. Once at the desired intensity the reaction was stopped by washing in water, the filter allowed to dry and scanned with an Epson scanner and analysed with Adobe Photoshop™ software run on a Macintosh computer.

3. Effects of Exogenous Factors on the Haematopoietic Differentiation of Embryoid Bodies.

3.1 Introduction.

Work already carried out with the culture system under study, suggests that primitive HSCs can be derived from cultured embryoid bodies, in sufficient quantities to be detected *in vitro* by the CFU-A assay and *in vivo* by reconstitution experiments. It would however be useful to increase HSC yields from embryoid bodies, for the purposes of providing a larger pool of stem cells for long term repopulation. The ability to modulate haematopoiesis would also be beneficial for differential gene expression studies, facilitating the identification of genes involved in haematopoietic development.

One means of modifying the level of embryoid body haematopoiesis would be by the addition of cytokines to the culture system. As discussed previously, the addition of cytokines to bone marrow and/or spleen haematopoietic precursors in culture can enhance cell colony formation (Tsuji *et al*, 1991), and have also been shown to have an effect on embryoid body haematopoiesis during later stages of differentiation (Keller *et al*, 1993). However most cytokines exert their effect in combination, and deciphering the assortment of cytokines that may yield the required results can, not only be time consuming but also expensive. The presence of foetal calf serum in the culture media with a many unidentified growth factors may also mask and/or complicate the effects of any added cytokines. For the purposes of this study therefore more accessible chemical inducers, mainly DMSO and RA, were used to regulate the haematopoietic differentiation of embryoid bodies.

Dimethyl sulphoxide is a low molecular weight polar planar molecule which is commonly used in tissue culture systems as a cryoprotectant for freezing cells. It is able to induce cardiac and skeletal muscle differentiation from EC and ES cell lines (McBurney *et al*, 1982; Pari *et al*, 1991; Dinsmore *et al*, 1996), and has also been shown to influence haematopoietic cell differentiation. DMSO was the first chemical agent shown to induce terminal differentiation of the transformed murine erythroid precursor cell line, MEL (Friend *et al*, 1971), and the human promyelocytic leukaemia cell line, HL-60 (Collins *et al*, 1979). DMSO can also induce the differentiation of other malignancy-derived cell lines such as neuroblastomas (Kimihi*et al*, 1976). Studies regarding the effects of DMSO on ES cell haematopoietic differentiation have not been reported to date.

Since DMSO is not known to occur naturally in mammalian cells and is required at relatively high concentrations (usually between 1-2%) in order to elicit an effect, it is generally believed to act by a non-specific signalling mechanism(s). DMSO is a lipid soluble molecule and could therefore enter cells directly without further requirement for cell surface proteins/receptors. However DMSO has been found to inhibit the binding of GM-CSF to its receptor in HL-60 cells and is thus thought to inhibit proliferation and trigger cell differentiation (Brennan *et al*, 1991; Schwartz *et al*, 1993). Biochemical studies in MEL and EC cell lines have shown that DMSO releases intracellular stores of the secondary messenger calcium, by a process which by-passes phosphatidyl inositol breakdown (Morley and Whitfield, 1993). Similar studies with the rat neuroblastoma cell line N1E-115 however, suggest that DMSO activates the production of lipid second messengers including diacylglycerol which is a breakdown product of phosphatidyl inositol (Clejan *et al*, 1996). Despite such studies the manner in which DMSO exerts its effects remains largely elusive.

Retinoic acid (RA) is a metabolite of retinol (vitamin A), and its involvment in embryogenesis and its roles as a teratogen and morphogen are now well documented (reviewed by Morriss-Kay, 1993). RA occurs naturally in living systems and the mechanism of its action via its nuclear receptors is now well understood (reviewed by Chambon, 1993). Both EC and ES cell lines can be induced to differentiate towards neuronal cell lineages in response to certain concentrations of RA. Studies of retinoic acid receptors (both RARs and RXRs) and retinoic acid-responsive genes in murine and human EC cell lines, have been carried out which strongly suggest that retinoic acid directly induces the terminal differentiation of these cells via its receptors (Minucci *et al*, 1994; Moasser *et al*, 1995; Roy *et al*, 1995).

The involvement of various stereo-isomers of RA, such as all-*trans* retinoic acid (atRA) and 13-*cis* retinoic acid (13cRA), and their receptors in haematopoiesis have been widely reported. Retinoic acid receptors have been found to be expressd in haematopoietic cells of both leukaemic and non-leukaemic origin (de Thé *et al*, 1990). Like DMSO, retinoic acid is able to induce the *in vitro* differentiation of HL-60 cells (Brietmann *et al*, 1988), and is now widely used clinically for the treatment of acute promyelocytic leukaemia (Huang *et al*, 1988). Furthermore gene transfection assays in RA resistant HL-60 cells have shown that retinoic acid exerts its effect directly via the RARα receptor (Collins *et al*, 1990). 13-*cis* retinoic acid has also been used in the induction of promyelocyte differentiation *in vitro* and *in vivo*, but has been found to be at least ten fold less effective than atRA (Chomienne *et al*, 1990).

The effects of RA can vary depending on the concentration used and the type of retinoid administered (Edwards and McBurney, 1983a). The ability of different retinoids to exert differential effects is mediated through their receptors which display ligand specificity. The RAR receptors for instance have been found to bind both all-*trans* and 9-*cis* retinoic acid with high affinity, whereas the RXR receptors only bind 9-*cis* retinoic acid with high affinity (Levin *et al*, 1992).

Various studies of the effects of retinoic acid on the haematopoiesis of murine and human haematopoietic progenitors have been carried out. These *in vitro* studies on normal haematopoietic cells remain inconclusive, with some investigators reporting an enhancement of haematopoiesis after RA administration to cultured cells (Douer and Koeffler, 1982; Sakashita *et al*, 1993), while others have reported a marked decrease (van Bockstaele *et al*, 1993; Smeland *et al*, 1994). Detailed studies of the haematopoietic differentiation of ES cells after RA treatment have to date not been published.

One means by which to modulate haematopoietic commitment, is by influencing mesoderm differentiation. A number of specific factors are known to be directly involved at this stage of differentiation such as BMP-4, activin-1 and bFGF. The putative roles of BMP-4 and bFGF with respect to haematopoietic development have already been outlined in section 1.2.1. Furthermore bFGF has been reported to directly enhance the differentiation of human bone marrow haematopoietic progenitors *in vitro* (Wilson *et al*, 1991). Such factors could therefore be theoretically used in ES cell cultures to induce mesoderm lineages and hence to indirectly influence haematopoiesis.

The influence of DMSO, atRA, 13cRA, bFGF and activin-1 on the haematopoietic differentiation of embryoid bodies was consequently investigated. In order to study the effect of these factors on the earliest stages of ES cell commitment to haematopoietic stem cells, undifferentiated ES cell aggregates were incubated with one of the above named factors in the absence of LIF, immedately after being harvested from 'hanging-drop' cultures. Embryoid body formation in the presence of the added factor was allowed to occur for the first 48 hours of differentiation. Thereafter embryoid bodies were removed from the morphogen/chemical supplemented media and allowed to differentiate in the absence of inducer. Subsequently the presence of haematopoietic progenitors in the embryoid bodies was detected by the CFU-A assay. Dose-response and time-course studies were performed and the influence of the said factors on haematopoietic differentation of embryoid bodies assessed.

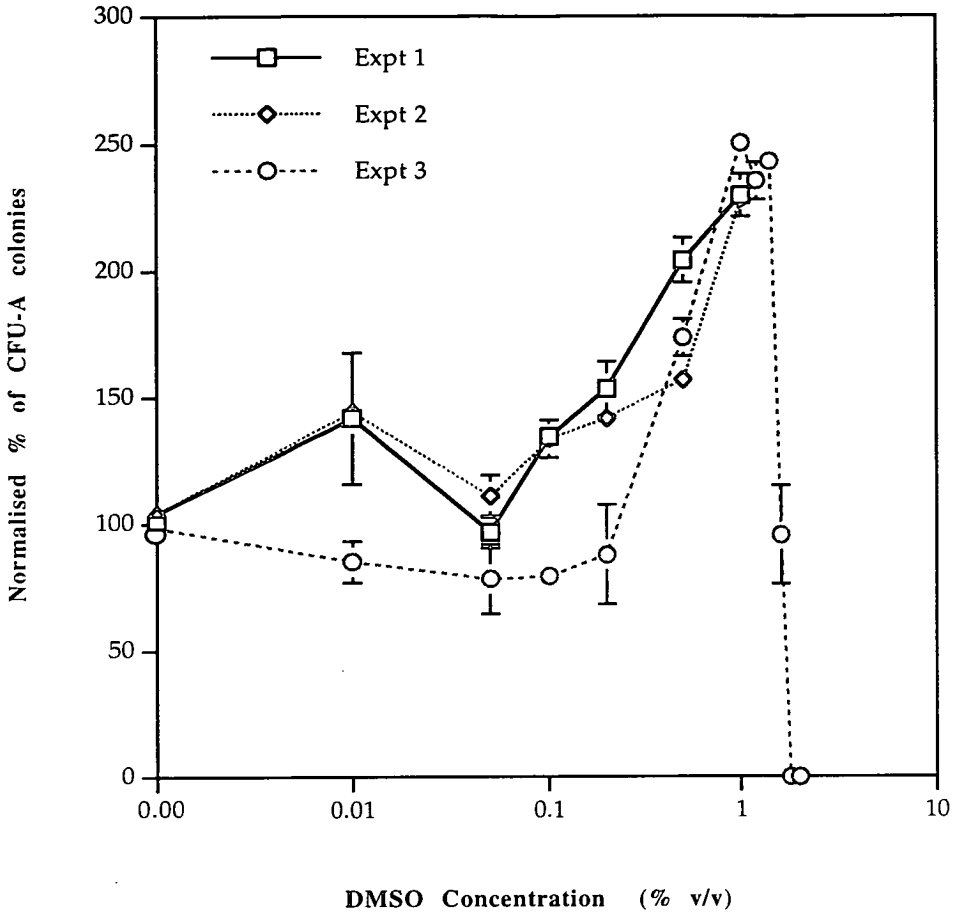


Figure 3.1A. DMSO dose response curve. Embryoid bodies were treated with varying concentrations of DMSO for the first 48 hours of differentiation and assayed after a total of 6 days in culture. Each point represents the mean of three plates assayed ± standard deviation. The normalised percentage of embryoid bodies giving rise to CFU-A colonies is shown on the y-axis. DMSO concentration is expressed as percentage (v/v), on a logarithmic scale on the x-axis .

3.2(i) Results of studies with DMSO

DMSO concentration is expressed as a percentage of volume/volume.

3.2(i).1 DMSO dose-response experiments.

The effects of DMSO on the haematopoietic differentiation of embryoid bodies was initially analysed by dose response studies and the effects of the compound assessed by the CFU-A assay. Approximately 300-500 undifferentiated embryonic stem cell aggregates were incubated either in the absence of DMSO or with increasing concentrations of DMSO for the first 48 hours of differentiation, in the absence of LIF. Antibiotics were also included in the suspension cultures at this point, since the differentiating aggregates are particularly prone to infections during this period. After exposure to the chemical inducer, embryoid bodies were washed with PBS and resuspended in unsupplemented differentiation media in the absence of antibiotics. Since previous studies have shown that the maximum percentage of haematopoietic colonies are obtained with 6 day old embryoid bodies, they were allowed to differentiate for a further 4 days, being replenished with fresh media once every 48 hours.

Since the number of CFU-A colonies decrease when large numbers of embryoid bodies are plated, due to glucose depletion, no more than 50 embryoid bodies were included in each plate. Each sample was assayed in triplicate. The numbers of CFU-A colonies were scored after 8-11 days incubation and the percentage of embryoid bodies giving rise to CFU-A colonies calculated. Examples of three independent DMSO dose response experiments are illustrated in Figure 3.1, where each point represents the mean percentage of CFU-A colonies from triplicate dishes.

The results show a dose dependent response with higher percentages of embryoid bodies giving rise to CFU-A colonies as the concentration of DMSO is increased. Concentrations of between 0.01-0.2% appear to have little or no effect on the number CFU-A colonies, when compared with the untreated control samples. An apparent increase in haematopoiesis is observed with 0.5% DMSO treatment. Incubation of embryoid bodies with 1.0% DMSO however results in a more than 2 fold increase in the percentage of CFU-A colonies. Further experiments have shown that this increase in haematopoiesis is reproducible and can be achieved by concentrations of up to 1.4% DMSO, however any further increase in DMSO results in a rapid decline in the numbers of CFU-A colonies. In order to minimise

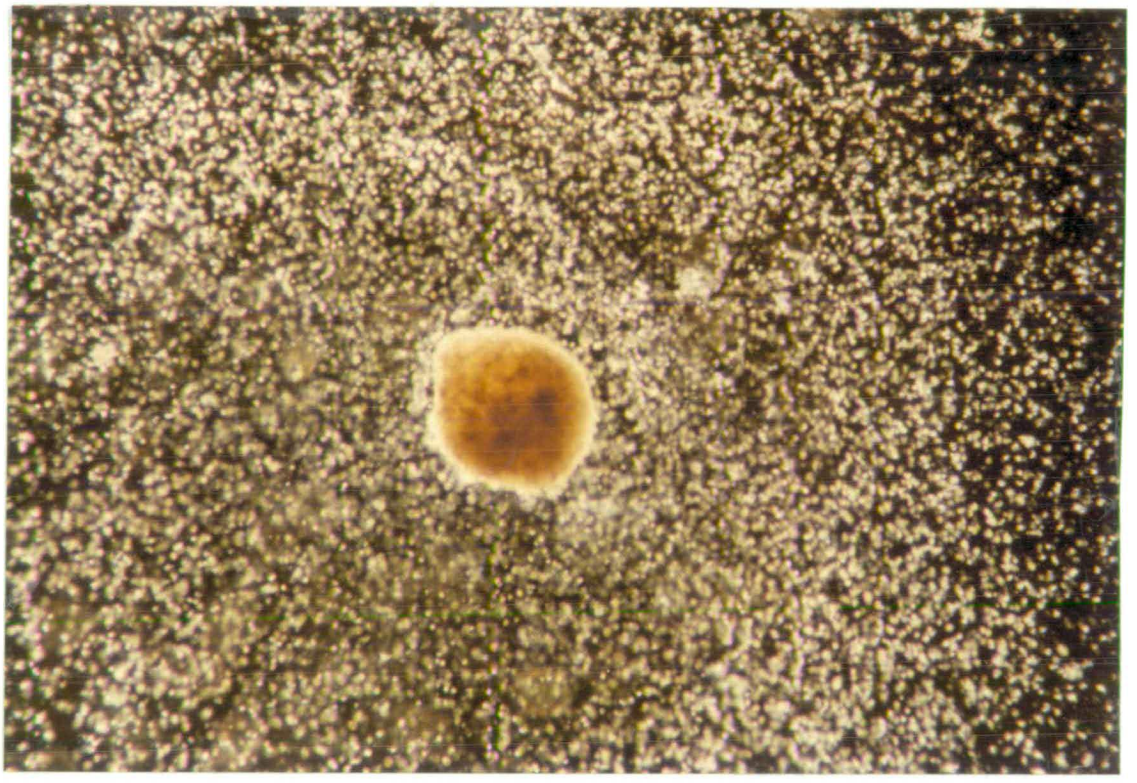


Figure 3.1B. CFU-A assay of DMSO treated embryoid bodies. Embryoid bodies were treated with 1% DMSO for the first 48 hours of differentiation and assayed after a total of 6 days, in suspension culture. Macrophages and monocytes are seen to emanate from the central embryoid body.

any toxic effect the compound may have, subsequent experiments were carried out with 1.0% DMSO.

In order to determine whether there was a qualitative difference in the CFU-A colonies obtained after DMSO treatment, the size of colonies produced by untreated and 1.0% DMSO treated embryoid bodies were compared (Table 3.1). The diameters of CFU-A colonies from DMSO treated and untreated samples in three independent experiments, were measured with a graticule to the nearest millimetre and the means compared. Assuming that the diameter of CFU-A colonies follows a normal distribution a t-test at the 5% level of significance was carried out on these values. The results show that there is no statistically significant increase in the mean diameter of CFU-A colonies after DMSO treatment.

	Mean diameter of CFU-A colonies (mm)	
	Untreated	1% DMSO Treated
Experiment 1; n=12	2.6 ± 1.0	2.8 ± 1.2
Experiment 2; n=12	3.3 ± 1.2	4.2 ± 0.8
Experiment 3; n=6	4.0 ± 1.1	5.2 ± 0.8

Table 3.1. CFU-A colony sizes in untreated and DMSO treated embryoid bodies. Colonies were measured, to the nearest millimetre, from triplicate assays of each sample. Results are from 3 independent experiments. Results are shown as mean diameter of colonies ± standard deviation.

Embryoid bodies also give rise to skeletal and cardiac muscle in culture, these being visually distinguishable as they contract and relax in a rhythmic fashion. Approximately 5-10% of untreated embryoid bodies are seen to 'beat' in this manner in CFU-A assays. In DMSO treated samples the percentage of beating embryoid bodies that lack haematopoietic colonies decreases to less than 5%. In contrast however, up to 50% of the haematopoietic embryoid bodies in these samples are also seen to beat.

3.2(i).2 DMSO time-course experiments.

Previous time course studies with untreated embryoid bodies have shown that the maximum percentage of CFU-A colonies are observed at 6 days of differentiation. Untreated embryoid bodies and those treated with 1.0% DMSO for the first 48 hours of

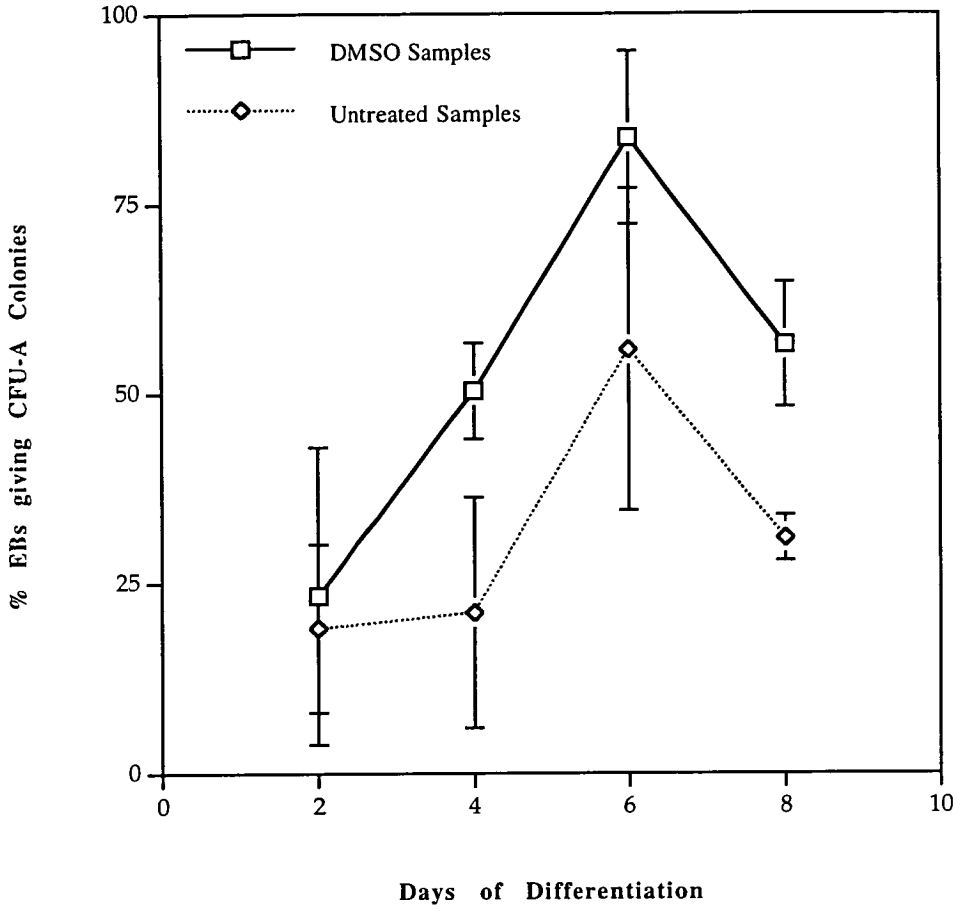


Figure 3.2. Time course of embryoid body CFU-A differentiation. Untreated and 1% DMSO treated embryoid bodies were assayed after 2, 4, 6 or 8 days in suspension culture. The graphs are means of two independent experiments, a set of untreated and DMSO treated samples being cultured and assayed at the same time. The normalised percentage of embryoid bodies giving rise to CFU-A colonies is shown on the y-axis, and the total number of days of differentiation, shown on the x-axis. Error bars represent the standard error.

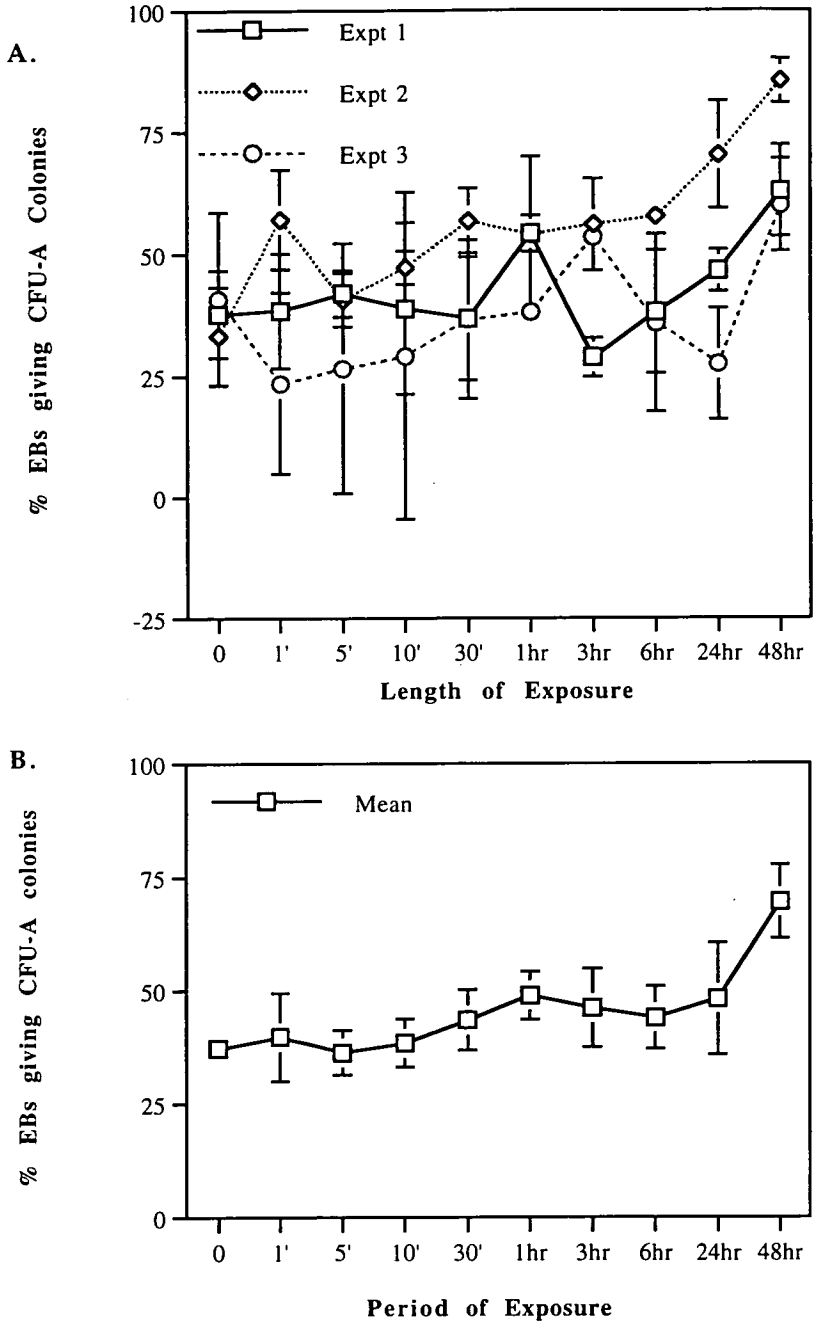


Figure 3.3A. Effect of length of exposure to 1% DMSO on embryoid body differentiation. Embryoid bodies were exposed to 1.0% DMSO for varying lengths of time (x-axis) and assayed after a total of 6 days differentiation. Each point represents the mean of 3 plates assayed ± standard deviation. Percentage of embryoid bodies (EBs) giving rise to CFU-A colonies is shown on the y-axis.

Figure 3.3B. Mean percentage of embryoid bodies giving rise to CFU-A colonies ± standard error, after varying lenths of exposure to 1% DMSO, as calculated from Figure 3.3A

differentiation were assayed after 2, 4, 6 and 8 days of differentiation in order to assess whether the treatment affects this time course of CFU-A differentiation.

Time course experiments in which a set of DMSO treated and untreated embryoid bodies were cultured and assayed at the same time, have shown that DMSO treatment does not appear to alter the time course of embryoid body haematopoietic differentiation. DMSO treated embryoid bodies do however show an elevated level of haematopoiesis compared with their untreated counterparts, as shown by Figure 3.2. Furthermore when compared statistically, the DMSO treated population of embryoid bodies give rise to a significantly higher number of CFU-A colonies than the untreated population, at the 5% level of significance.

3.2(i).3 DMSO timed-exposure experiments.

By exposing embryoid bodies to chemicals during the first 48 hours of differentiation, it is assumed that the very early stages of embryonic stem cell haematopoietic commitment is influenced. In order to determine the period of exposure in this time-frame which produces the greatest increase in the percentage of CFU-A colonies from embryoid bodies, timed DMSO exposure experiments were carried out. Embryoid bodies were exposed to 1% DMSO for varying lengths of time and assayed after a total of 6 days differentiation.

Three independent experiments are illustrated in Figure 3.3A, the mean of which is represented by Figure 3.3B. Considered together the graphs suggest that the increase in the percentage of CFU-A colonies after DMSO treatment, is cumulative and dependent on the length of time of treatment. A slight increase in the percentage of CFU-A colonies is observed after approximatey 1-24 hours of exposure, however this increase is not statistically significantly higher than the control population, at the 5% level of significance. The largest increase is observed with embryoid bodies that have been treated with 1.0% DMSO for 48 hours. This increase was found to be significantly higher than the untreated population when tested in a t-test at the 5% level of significance.

3.2(i).4 Experiments with α-tocopherol.

DMSO is an antioxidant and can therefore acts as a free-radical scavenger. Free radicals which are generated during normal cellular metabolism, are known to cause oxidative

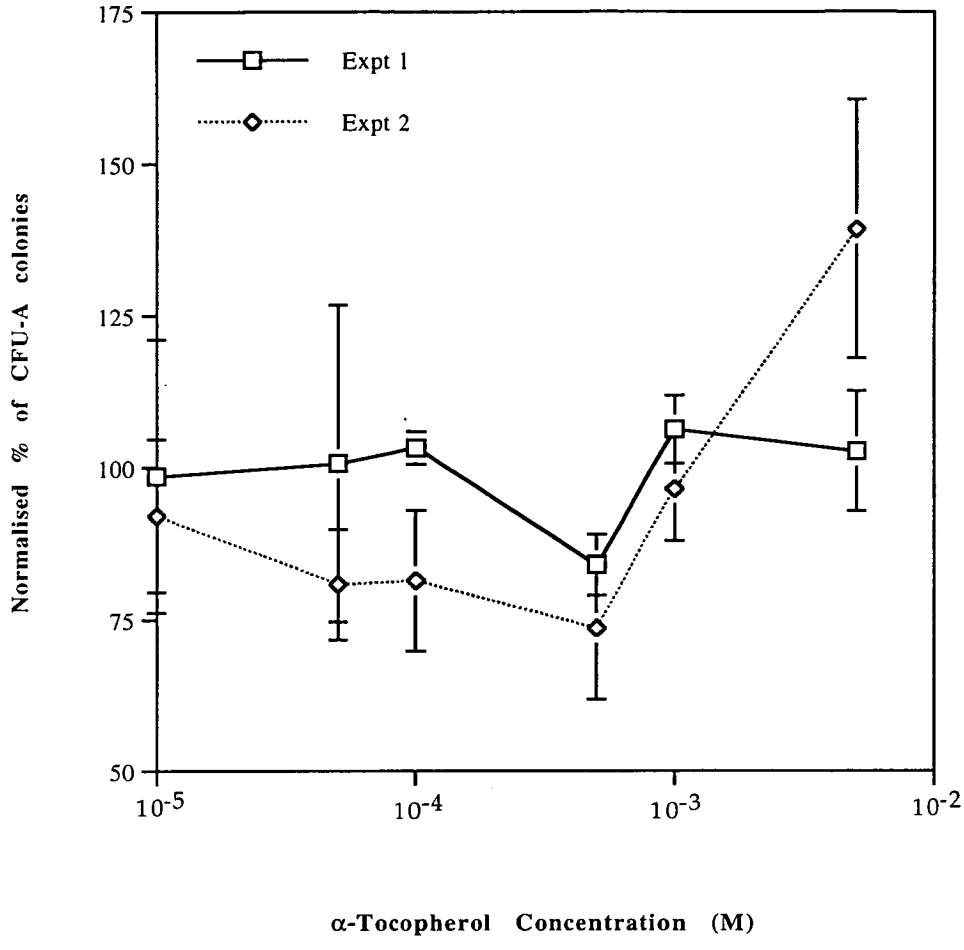


Figure 3.4. α–Tocopherol dose response curve. Embryoid bodies were exposed to varying concentrations of α-tocopherol for the first 48 hours of differentiation, and assayed after a total of 6 days differentaition. The normalised percentage of embryoid bodies giving rise to CFU-A colonies is shown on the y-axis. α-Tocopherol concentration is expressed as volume per volume on the x-axis. Each point represents the mean of three plates assayed ± standard deviation.

damage and hence cell death. The ability of DMSO to increase the percentage of haematopoietic embryoid bodies could in part be attributable to its antioxidant properties. DMSO could in theory, prevent the cumulative build-up of free-radicals in cell cultures thus reducing cell death, resulting in increased numbers of CFU-A colonies. In order to test this hypothesis, the effects of α-tocopherol (vitamin E) a known antioxidant, on the haematopoietic differentiation of embryoid bodies was also tested and compared with the effects of DMSO.

Embryoid bodies were treated with varying concentrations of α-tocopherol for the first 48 hours of differentiation and assayed after a total of 6 days in culture. The results of two independent experiments are shown in Figure 3.4. These clearly do not mirror the observations made with DMSO; in fact the drug had little or no effect on the percentage of CFU-A colonies even at concentrations as high as 5mM. Higher concentrations had severe toxic effects causing the disruption of embryoid bodies.

α-tocopherol is not water soluble and therefore either DMSO or ethanol are usually used as carriers for the drug. In these experiments the drug was dissolved in ethanol, and to determine whether the use of ethanol affected the haematopoietic differentiation of embryoid bodies, embryoid bodies were treated with 0.5% ethanol for the first 48 hours of differentiation and assayed; 0.5% being the highest concentration of ethanol embryoid bodies were exposed to when cultured with 5mM α-tocopherol. Treatment with this concentration of ethanol had no effect on the percentage of CFU-A colonies compared with that of untreated control samples (data not shown).

3.2(ii) Results of studies with atRA.

3.2(ii).1 atRA dose-response experiments.

As with DMSO, the initial experiments to be carried out with atRA were dose response studies, to assess the the effect of the drug on the early stages of embryoid body haematopoietic differentiation, and to determine the concentration at which these effects, if any, would be exerted. Embryoid bodies were treated with varying concentration of atRA during the first 48 hours of differentiation, in the presence of antibiotics and the absence of LIF. After treatment the samples were washed in PBS and allowed to differentiate in the absence of antibiotics and morphogen and the media routinely renewed once every 48 hours.

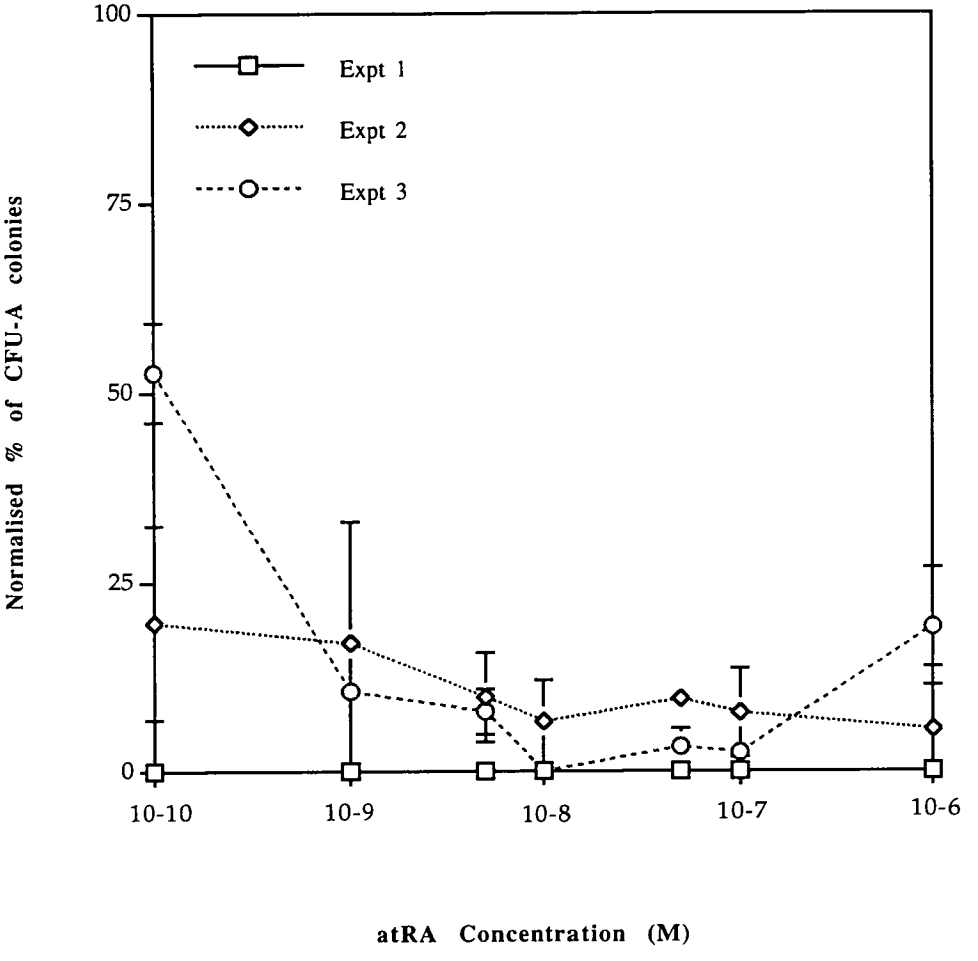


Figure 3.5A. atRA dose response curve. Embryoid bodies were exposed to various concentrations of atRA (dissolved in 95% ethanol) for the first 48 hours of differentiation and assayed after a total of 6 days in suspension culture. Each point represents the mean of three plates assayed ± standard deviation. The normalised percentage of embryoid bodies giving rise to CFU-A colonies is shown on the y-axis. The concentration of atRA used is shown on the x-axis on a logarithmic scale.

After a total of 6 days differentiation each sample was assayed in triplicate and incubated for 8-11 days subsequent to which the number of CFU-A colonies were scored and the percentage of embryoid bodies giving rise to CFU-A colonies calculated.

Examples of three such experiments are illustrated in Figure 3.5A, where each point represents the mean percentage of CFU-A colonies for triplictate plates. As can be seen, the treatment of embryoid bodies with gradually increasing concentrations of atRA reduces the percentage of CFU-A colonies even with concentrations as low as 10^{-10} M. A ten fold increase in the dose to 10^{-9} M results in a less than 50% reduction in the number of colonies, with 10^{-8} M atRA reducing the number of CFU-A colonies to below 5% (Figure 3.5B). Any further increase in atRA reduces this percentage further to below 1%. The percentage of CFU-A colonies from embryoid bodies treated with 10^{-11} M and 10^{-12} M atRA, was neither increased nor decreased in comparison with the untreated control samples (data not shown). Further experiments have shown the observations made with atRA to be highly reproducible.

Retinoic acid is not water soluble and therefore either DMSO or ethanol are usually used as carriers for the drug. Ethanol was used as the carrier for atRA throughout the present study. To determine whether ethanol affected the haematopoietic differentiation of embryoid bodies, samples were treated with 0.01% (v/v) ethanol for the first 48 hours of differentiation and assayed; 0.01% being the highest concentration of ethanol embryoid bodies were exposed to when cultured with 10^{-6} M atRA. Treatment with this concentration of ethanol had no effect on the percentage of CFU-A colonies compared with that obtained for untreated control samples (data not shown).

The diameter of CFU-A colonies from atRA treated and untreated samples were measured as before, to the nearest millimetre, and the mean diameter of CFU-A colonies from triplicate plates compared. As can be seen from Table 3.2 there is a gradual decrease in the mean diameter of CFU-A colonies as the concentration of atRA increases. In a t-test, this decrease was found to be statistically significant ($P<0.05$) between the untreated and the $5x10^{-8}$ M atRA treated samples.

atRA (M)	0	10^{-10}	10^{-9}	$5x10^{-8}$
Mean diameter of CFU-A colonies (mm)	3.2 ± 0.4	2.8 ± 1.5	1.6 ± 1.6	1.2 ± 0.3

Table 3.2. The mean diameter of CFU-A colonies from untreated and atRA treated embryoid bodies. The diameter of colonies were measured, to the nearest millimetre, from triplicate assays of each sample. Diameters are expresses as the mean of 6 colonies ± standard deviation.

No significant increase or decrease was observed in the percentage of beating muscle embryoid bodies after atRA treatment at any of the concentrations used. In subsequent experiments the concentration of atRA was limited to 10^{-8} M. This concentration not only reduces the percentage of CFU-A colonies significantly and reproducibly, but also minimises any toxic effects the drug may have at higher concentrations.

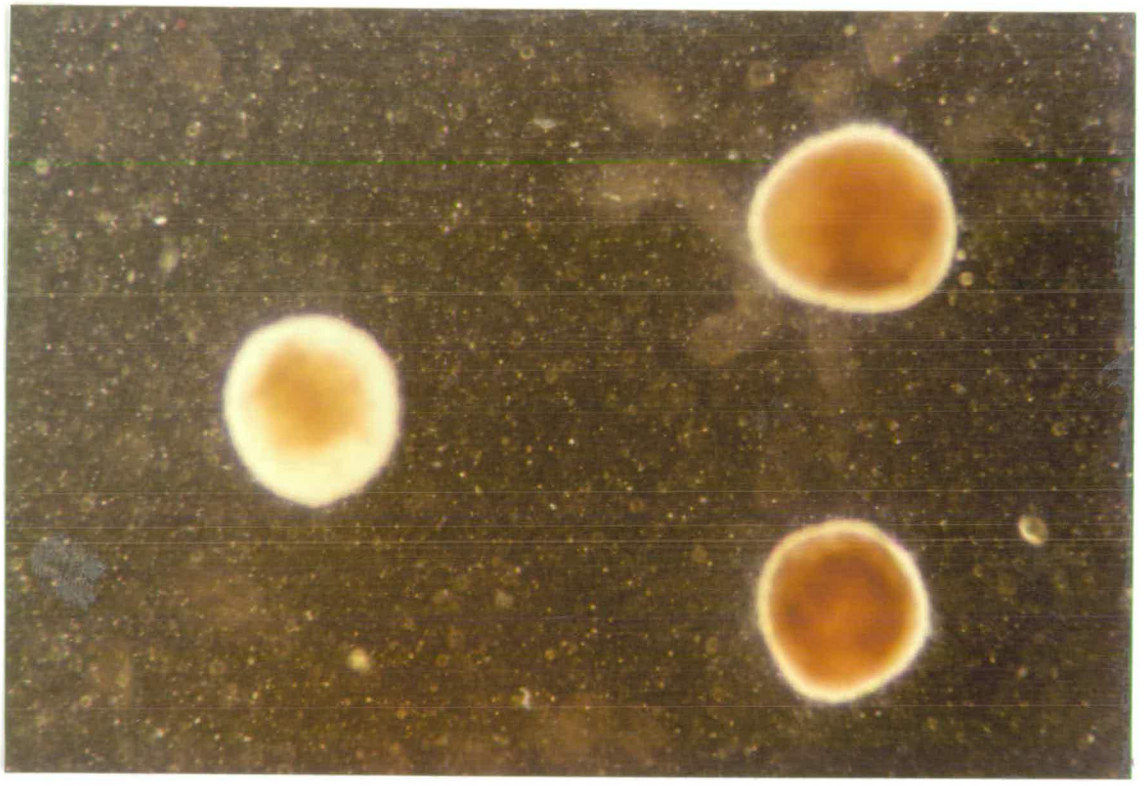


Figure 3.5B. CFU-A assay of atRA treated embryoid bodies. Embryoid bodies were treated with 10^{-8} M atRA for the first 48 hours of differentiation and assayed after a total of 6 days, in suspension culture. Subsequent analysis of assays showed a marked absence of CFU-A colonies (see Figure 1.4)

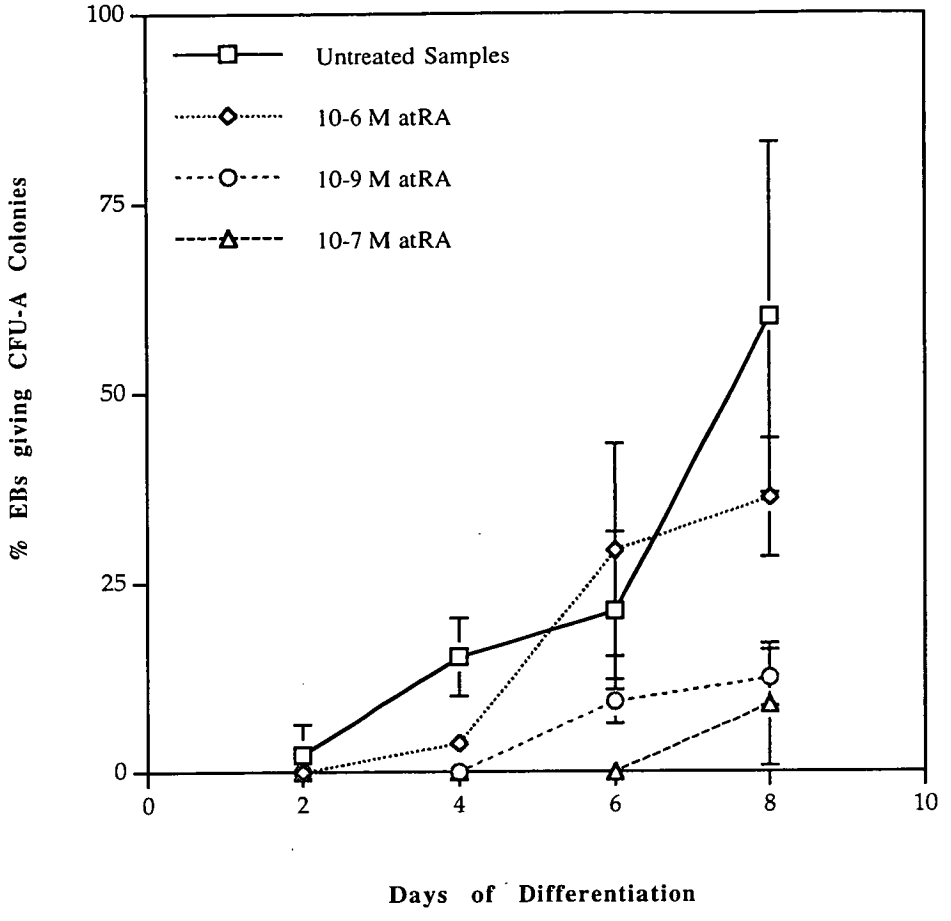


Figure 3.6. Time course of differentiation of atRA treated embryoid bodies (EBs). Embryoid bodies, untreated and atRA treated for the first 48 hours of differentiation, were assayed after 2, 4, 6 and 8 days in suspension culture (x-axis). Each point represents the mean of three plates assayed ± standard deviation. The percentage of embryoid bodies giving rise to CFU-A colonies is shown on the y-axis.

3.2(ii).2 atRA time-course experiment.

Time course experiments, similar to those carried out with DMSO, were also performed with atRA, at three different concentrations. The results are shown in Figure 3.6; each point represents the mean percentage of embryoid bodies giving rise to CFU-A colonies, calculated from three plates assayed for each sample. The atRA treated embryoid bodies follow the same time course of haematopoietic differentiation as the untreated samples, however as the concentration of atRA is increased so the percentage of CFU-A colonies decreases.

3.2(ii).3 atRA timed exposure experiments.

To determine the minimum length of time of atRA treatment required within the first 48 hours of differentiation before a decrease in the number of CFU-A colonies is observed, the length of time of treatment with 10^{-8} M atRA was varied and the samples assayed after a total of 6 days differentiation. Three such experiments are illustrated in Figure 3.7A the mean of which is shown by Figure 3.7B. The percentage of CFU-A colonies is not reduced significantly when embryoid bodies are treated for up to 6 hours. The largest decrease in the percentage of CFU-A colonies is seen after 16 hours treatment when less than 10% of embryoid bodies give rise to CFU-A colonies. This value does not change after 24 hours treatment. But after 48 hours in the presence of 10^{-8} M atRA the percentage of CFU-A colonies is reduced further to less than 5%.

In order to determine if atRA is able to influence the haematopoietic differentiation of embryoid bodies, whether positively or negatively, at any other time point during the 6 days of differentiation, embryoid bodies were treated with 10^{-8} M atRA for 24 hour periods and subsequently assayed after a total of 6 days differentiation in culture. Two such experiments are represented by Figure 3.8.

The results show that atRA reduces the number of CFU-A colonies if embryoid bodies are treated within the first 72 hours of differentiation. No colonies are obtained when

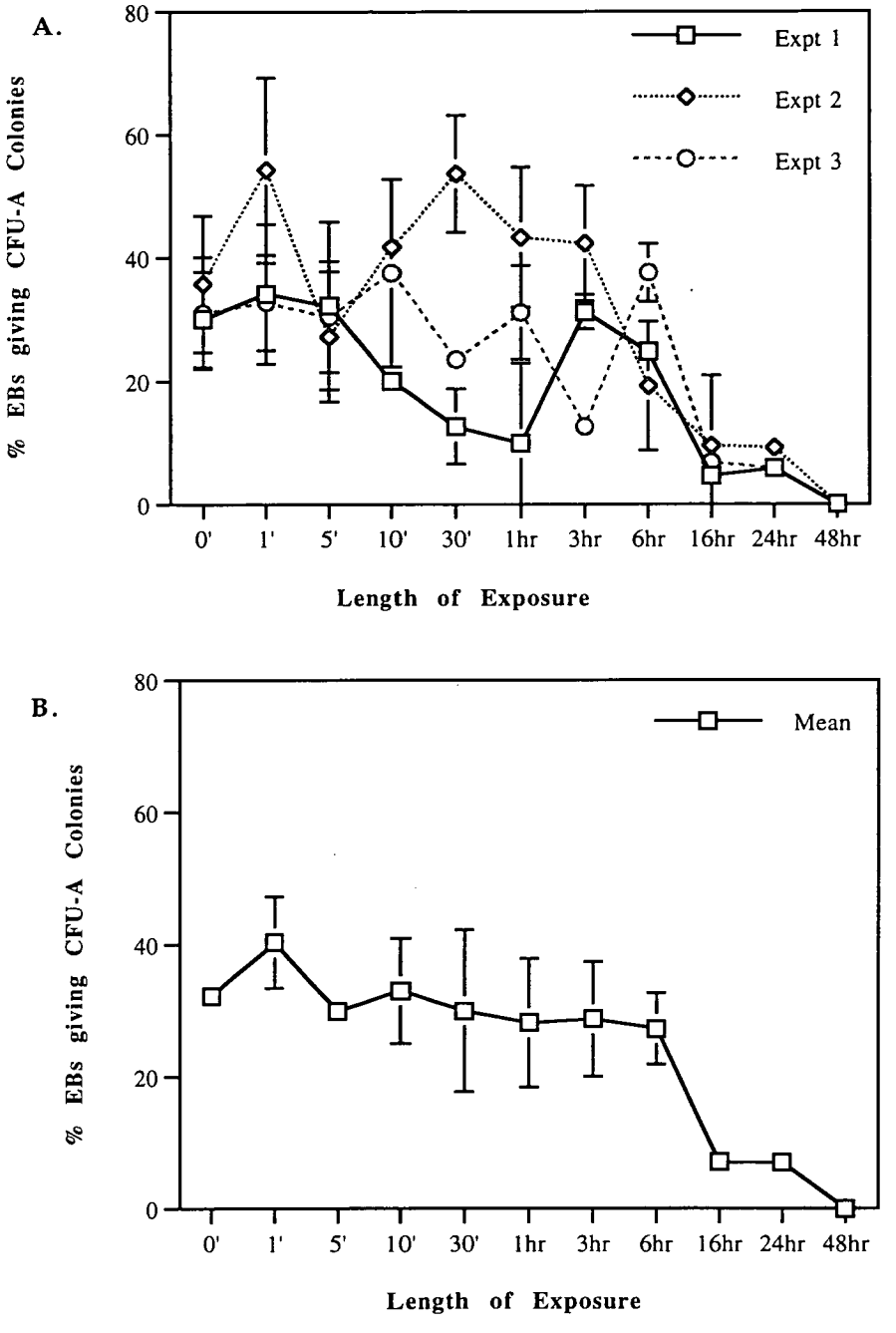


Figure 3.7A. Effect of length of exposure to atRA on embryoid body differentiation. Embryoid bodies (EBs) were exposed to 10-8 M atRA for different lengths of time immediately after being removed from LIF containing media and assayed after a total of 6 days differentiation. Each point represents the mean from three plates assayed ± standard deviation. The percentage of embryoid bodies producing CFU-A colonies is shown on the y-axis.

Figure 3.7B. The mean of graphs in Figure 3.7A. Error bars represent standard errors.

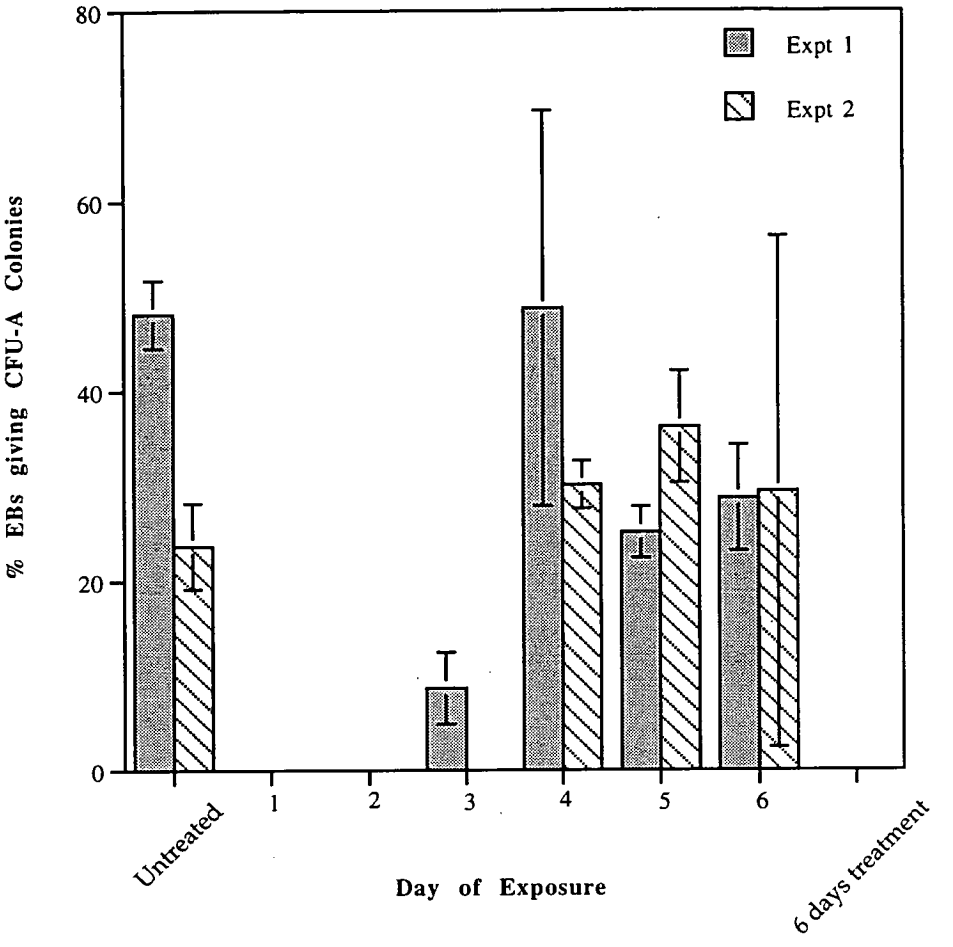
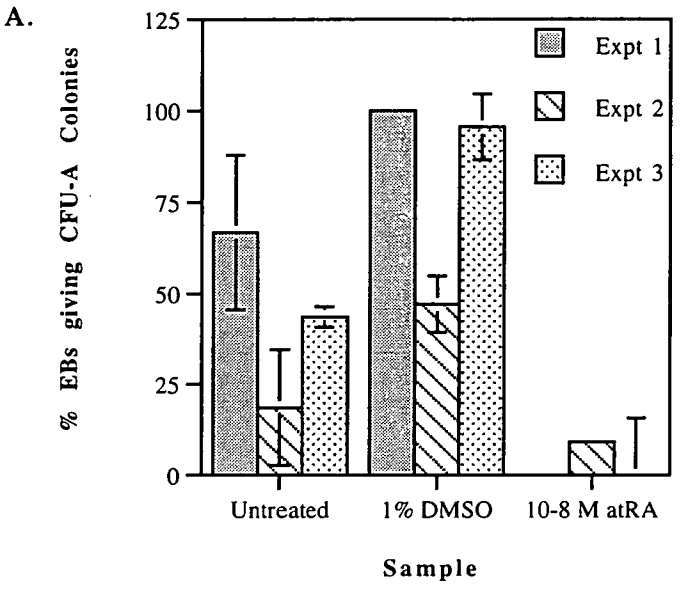


Figure 3.8. Embryoid bodies (EBs) were treated with 10-8 M atRA for 24 hour periods during 6 days differentiation. All samples were allowed to differentiate for a total of 6 days. Each bar represents the mean of 3 plates assayed ± standard deviation. The percentage of embryoid bodies producing CFU-A colonies is shown on the y-axis. The x-axis shows the day of treatment.



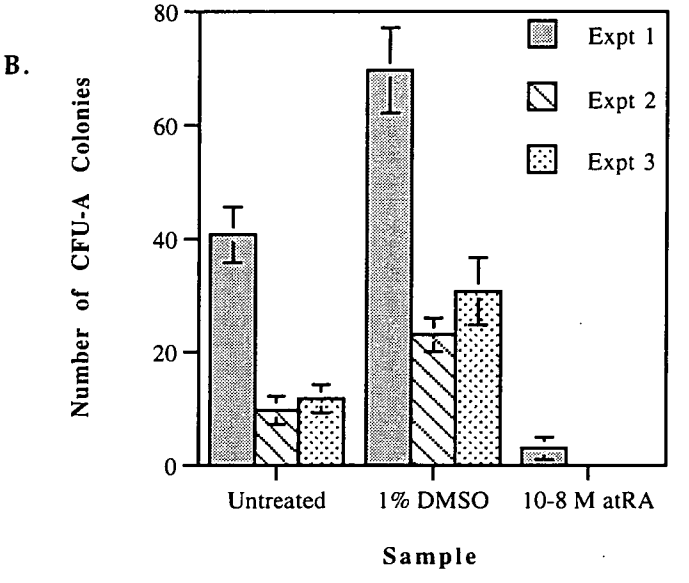


Figure 3.9A. Untreated embryoid bodies and those treated with 1% DMSO or 10-8 M atRA during the first 48 hours of differentiation, were assayed after a total of 6 days in suspension culture. The percentage of intact embryoid bodies (EBs) producing CFU-A colonies is shown on the y-axis, and the type of treatment on the x-axis. Each column represents the mean of 3 plates assayed ± standard deviation.

Figure 3.9B. The number of CFU-A colonies produced by single cells from disrupted embryoid bodies, after 6 days differentiation. Each point is the mean of 3 plates assayed ± standard deviation.

samples are treated during the first and second 24 hours of differentiation; the number of CFU-A colonies obtained after atRA treatment during the third day of differentiation increases marginally but remains below 10%. The percentage of CFU-A colonies obtained after treatment during the 4th, 5th and 6th days of embryoid body differentiation are similar to those observed for untreated samples. Samples treated with 10^{-8} M atRA for the duration of the 6 days, the atRA being renewed once every 48 hours, produced no CFU-A colonies as expected.

3.2(ii).4 CFU-A assays with disrupted embryoid bodies.

The haematopoietic lineages which give CFU-A colonies their distinctive morphological appearance, are the mobile macrophages and monocytes which migrate out into the surrounding agar, forming a halo around the embryoid bodies. If the surface properties of embryoid bodies are altered, thereby preventing the migration of macrophages/ monocytes, typical CFU-A colonies will not be observed.

To test whether the reduction of CFU-A colony numbers after atRA treatment is due to the inability of macrophages/monocytes to migrate away from the cystic structures, embryoid bodies were cultured with 10^{-8} M atRA for the first 48 hours of differentiation. After 6 days differentiation a sample of embryoid bodies was disrupted into a single cell suspension with dispase treatment, and 1.8×10^5 cells were assayed in triplicate as were the remainder of intact embryoid bodies. The number of CFU-A colonies formed by either single cells or intact embryoid bodies were scored after 8-11 days incubation. Untreated and 1.0% DMSO treated embryoid bodies were also cultured at the same time and treated in the same way.

The results of three independent experiments are shown by Figure 3.9; Figure 3.9A shows the number of colonies obtained from disrupted embryoid bodies, and Figure 3.9B, the percentage of the corresponding samples of intact embryoid bodies giving rise to CFU-A colonies. Each point represents the mean of triplicate plates. As can be seen the observations made with intact embryoid bodies are mirrored by the disrupted samples. Less than five CFU-A colonies were obtained from the three experiments with disrupted atRA treated embryoid bodies. The results from the three experiments were furthermore pooled and compared to the untreated samples using a t-test at the 5% level of significance. The number of CFU-A colonies obtained from intact and disrupted atRA treated embryoid bodies were found to be significantly lower than the control samples.

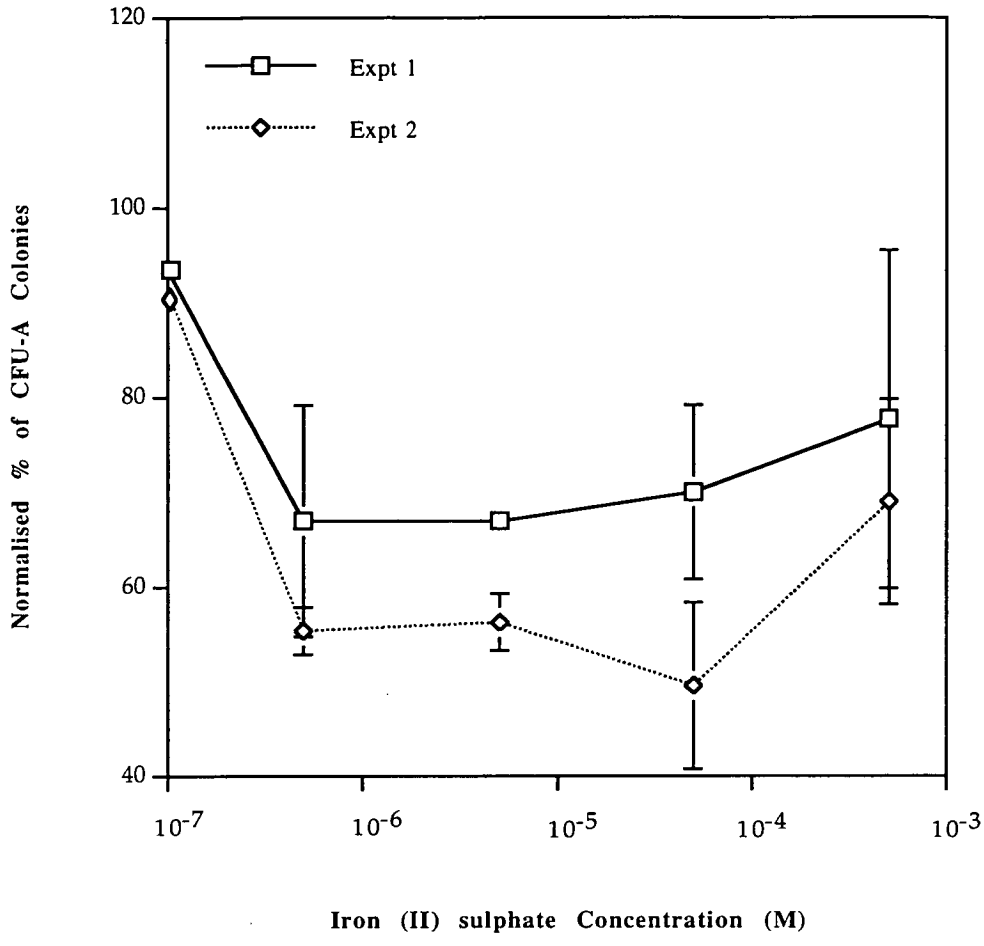


Figure 3.10. Iron (II) sulphate dose response curve. Embryoid bodies were treated with various concentrations of iron (II) sulphate for the first 48 hours of differentiation and assayed after a total of 6 days in suspension culture. Each point represents the mean of three plates assayed ± standard deviation. The normalised percentage of embryoid bodies producing CFU-A colonies is shown on the y-axis, and the concentration of iron (II) sulphate used on the x-axis .

Cells from disrupted 1.0% DMSO treated embryoid bodies gave rise to approximately twice as many CFU-A colonies as disrupted untreated embryoid bodies. However, in a t-test at the 5% level of significance, the number of CFU-A colonies obtained from disrupted DMSO treated embryoid bodies was not found to be significantly higher than the untreated sample, even though a similar t-test with the intact embryoid bodies showed the increase in the number of CFU-A colonies to be significantly higher than the untreated population.

3.2(ii).5 Experiments with iron (II) sulphate.

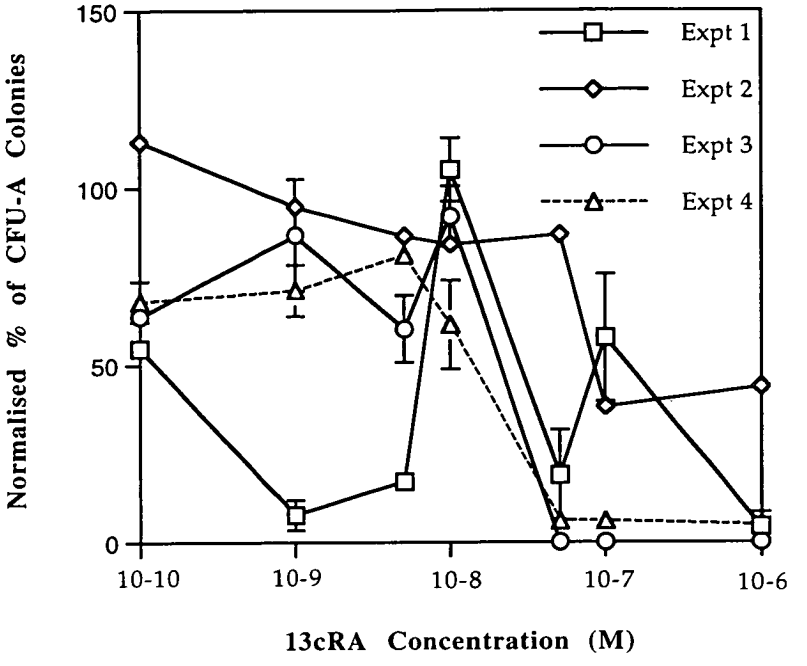
Retinoids have oxidative properties and are therefore capable of causing cellular and molecular damage thus inducing cell death. The ability of atRA to reduce the percentage of CFU-A colonies from embryoid bodies could in part be attributable to these properties whereby through oxidation it causes cell death which ultimately results in the dramatic reduction of CFU-A colonies. This hypothesis was tested by comparing the effects of atRA with those of iron (II) sulphate which is an oxidant that splits into its constituent ions when in solution. Fe^{2+} ions subsequently promote the production of superoxides, such as O_2^-.

Embryoid bodies were treated with various concentrations of iron (II) sulphate during the first 48 hours of differentiation and assayed after a total of 6 days in culture. The results of two independent experiments are shown in Figure 3.10. Although overall there may be a small reduction in the percentage of CFU-A colonies these effects are not as marked or potent as those observed after atRA treatment during the same phase of differentiation. In fact concentrations of iron (II) sulphate as high as $5x10^{-4}$ M failed to reproduce the observations made with 10^4 fold less concentrated atRA solutions.

3.2(ii).6 Experiments with 13-*cis* retinoic acid (13cRA).

Since different retinoids can exert different effects, experiments with 13cRA, were also carried out in order to determine whether another retinoid could elicit the same effects as atRA. Similar dose-response experiments to those described previously were carried out. Embryoid bodies were exposed to different concentrations of 13cRA for the first 48 hours of differentiation and subsequently assayed after a total of 6 days differentiation. The results of 4 such experiments are shown in Figure 3.11A where each point represents the

A.



B.

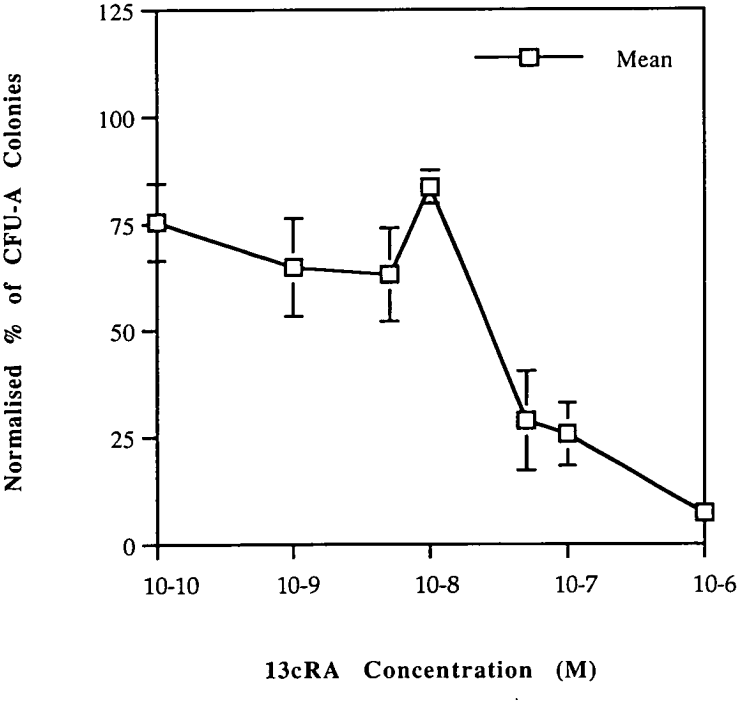


Figure 3.11A. 13cRA dose response curve. Embryoid bodies were treated with various concentrations of 13cRA during the first 48 hours of differentiation and assayed after a total of 6 days in culture. Each point represents the mean of 3 plates assayed ± standard deviation.

Figure 3.11B. The mean of graphs in Figure 3.11A ± standard error.

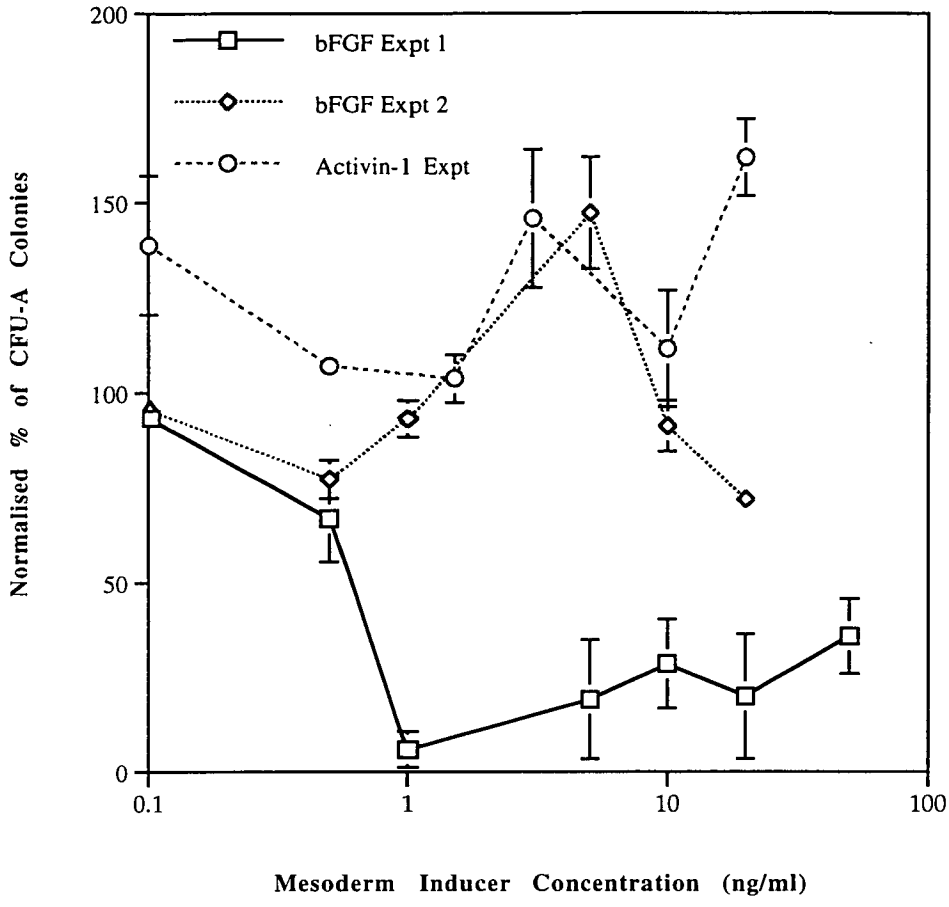


Figure 3.12. Dose response curves for bFGF and activin-1. Embryoid bodies were treated with various concentrations of the mesoderm inducers bFGF and activin-1 during the first 48 hours of differentiation and assayed after a total of 6 days in culture. Each point represents the mean of 3 plates assayed ± standard deviation. The normalised percentage of embryoid bodies giving rise to CFU-A colonies is shown on the y-axis, and the concentration of mesoderm inducer on the x-axis.

mean percentage of CFU-A colonies from three plates assayed. The mean for these experiments is shown in Figure 3.11B.

With reference to Figure 3.11B, it can be seen there is a gradual decline in the percentage of CFU-A colonies as the concentration of 13cRA is increased, the percentage being reduced to less than 5% with 10^{-6} M 13cRA. This level of reduction in CFU-A colony numbers however was reached with 10^{-8} M atRA, 100 fold less than the dose necessary to produce the same effect with 13cRA. In Figure 3.11A there is a slight increase in the numbers of CFU-A colonies at 10^{-8} M 13cRA, an effect which is more pronounced in experiment 1. However since this observation was not reproduced by the other 3 experiments to the same extent, the increase at this concentration could be an experimental anomaly.

3.2(iii) Experiments with mesoderm inducing factors

To test whether mesoderm inducing factors would have an effect on the haematopoietic differentiation of embryoid bodies in this culture system, similar to the effects observed with either DMSO or retinoic acid, dose-response experiments were carried out with murine basic fibroblast growth factor (bFGF) and activin-1. Embryoid bodies were exposed to one of the two factors for the first 48 hours of differentiation and assayed after 6 days differentiation. The results for these experiments are shown in Figure 3.12, where each point represents the mean percentage of CFU-A colonies calculated from three plates.

The results for bFGF show that at the concentrations used, this factor may reduce the percentage of CFU-A colonies; however to determine whether this reduction is reproducible further experiments are required. Activin-1 treatment of embryoid bodies at the concentrations used did not appear to alter the number CFU-A colonies in comparison with untreated samples. However once again further experiments are required to confirm this. The numbers of contracting embryoid bodies also did not increase in comparison to untreated samples, as may have been expected.

3.3 Discussion.

3.3(i) Discussion of results of studies with DMSO

Approximately 30-50% of 6 day embryoid bodies are able to give rise to CFU-A colonies. After treatment with DMSO for the first 48 hours of differentiation the percentage of CFU-A colonies increases; this increase being dependent on the dose of DMSO used. The percentage of CFU-A colonies increases gradually as the concentration of DMSO is increased. The largest increase of more than 2 fold was obtained with 1.0%-1.4% DMSO (160-220 mM). Further increases in DMSO concentration caused a rapid decline in the percentage of CFU-A colonies. This 2 fold increase is furthermore reproducible and was also observed in assays of single cell suspensions derived from embryoid bodies disrupted after 6 days differentiation (Figure 3.9A). In this experiment twice as many CFU-A colonies were observed with single cells derived from DMSO treated embryoid bodies than from untreated samples.

In a t-test at the 5% level of significance, the mean diameter of CFU-A colonies produced by embryoid bodies after DMSO treatment was not found to be significantly larger than the mean diameter of colonies obtained with untreated samples. Treatment with DMSO does not appear to alter the time course of embryoid body CFU-A differentiation. Moreover morphological assessment of CFU-A colonies produced by DMSO treated embryoid bodies has shown that these colonies are not different from those produced by untreated samples (Figures 1.4 & 3.1B).

As mentioned in section 3.1, DMSO has been shown to induce the terminal differentiation of promyelocytic and other malignancy derived cell lines, *in vitro*. DMSO can also induce EC cells to differentiate toward cardiac and skeletal muscle lineages (McBurney *et al*, 1982). Few studies of the effects of DMSO on ES cells have been published. In a recent publication Dinsmore *et al* (1996), have found DMSO to induce skeletal muscle cells from ES cells. In all these instances the most potent differentiation inducing concentrations of DMSO have been found to be between 1.0%-1.4%, with higher concentration being toxic. These results are consistent with those shown in Figure 3.1.

A 2 fold increase in the percentage of CFU-A colonies was observed after treatment of embryoid bodies with DMSO during the first 48 hours of differentiation. It is assumed that by exposing embryoid bodies to DMSO during this phase of differentiation, the very early

stages of ES cell commitment are being influenced, an assumption which is supported by the increase in CFU-A colonies after only 24 hours treatment. Timed exposure experiments, however, suggest that this effect is cumulative with the percentage of colonies increasing as the length of time of treatment is increased. Similar results have been obtained by Edwards *et al* (1983) with EC cells. In the latter study the number of beating muscle EC cell aggregates increased gradually as the length of time of exposure to 1.0% DMSO was increased, with the largest number of beating aggregates being observed after treatment for the first 50 hours of differentiation.

DMSO was found to influence embryoid bodies early on during differentiation (Figure 3.2). Furthermore, the numbers of CFU-A colonies obtained after disruption of embryoid bodies to single cell suspensions was found to mirror the proportion of intact DMSO treated embryoid bodies producing CFU-A colonies (Figure 3.9A). This suggests that DMSO is acting to increase haematopoietic commitment, as opposed to expanding the population of committed progenitors. The question of whether DMSO can expand the population of committed progenitors could be addressed by experiments which involve the treatment of embryoid bodies for 24 hour periods during the standard 6 day differentiation period, as carried out with retinoic acid (section 3.2(ii).3).

DMSO has been found to induce EC and ES cells to differentiate towards skeletal and cardiac muscle tissue (McBurney *et al*, 1982; Edwards *et al*, 1983; Pari *et al*, 1992; Dinsmore *et al*, 1996), which like haematopoietic tissue is derived from mesoderm. DMSO could therefore, be acting essentially as a mesoderm inducer in differentiating EC and ES cell aggregates. The culture system used throughout the course of this study is optimised for haematopoietic differentiation, providing the right environment for haematopoietic differentiation of the possibly DMSO induced mesoderm. Although the culture conditions do not support haematopoietic differentiation exclusively, since approximately 50% of the embryoid bodies giving rise to CFU-A colonies also contracted, indicating the presence of either skeletal or cardiac muscle.

DMSO has anti-oxidative properties and can therefore act as a free-radical scavenger. Since free radicals can cause cellular and molecular damage and thereby cell death (reviewed by Parchment, 1993), it was hypothesised that the increase in CFU-A colonies after DMSO treatment could in part, be due to fewer free-radicals in the culture media. α-tocopherol is a known antioxidant the effects of which have been studied in other systems such as tumours and lymphocytes (Masotti *et al*, 1988; Poulin *et al*, 1996). Therefore the influence of α-tocopherol on the haematopoietic differentiation of embryoid bodies was

assessed, and compared with the observations made with DMSO. The results show that α-tocopherol has little or no effect on the percentage of CFU-A colonies (Figure 3.4). On the basis of this comparison the effect of DMSO as an anti-oxidant in this culture system is unlikely to be significant.

The mechanism of action of DMSO is not understood although various suggestions have been made, including the direct influence of the molecule on the expression of genes involved in differentiation (Tanaka *et al*, 1975). However there is increasing evidence to suggest that DMSO acts indirectly on gene expression, explaining the requirement for the high concentrations necessary to induce differentiation. Studies with various cell lines have shown that the drug stimulates the release or production of intracellular secondary messengers such as calcium and lipid secondary messengers (Morley and Whitfield, 1993; Clejan *et al*, 1996).

DMSO has also been shown to bind to the GM-CSF and insulin receptors, and suggested to thereby stimulate cell differentiation (Brennan *et al*, 1991; Schwartz *et al*, 1993). RT-PCR studies with extracts from the CCE line of ES cells have shown the transcript for the GM-CSF receptor to be present, demonstrating that ES cells can express this receptor amongst others (McClanahan *et al*, 1993). PCR detection for the β-chain of the GM-CSF receptor transcript in EFC-1 cells has been carried out, but it has not been found to be expressed in embryoid bodies of less than 6 days. EFC-1 cells have however been shown to express the gp130 common subunit required for dimerisation with various interleukin receptors (Hole *et al*, 1996). DMSO could therefore be acting via one or more of these receptors to induce haematopoietic differentiation. Competitive binding assays between DMSO and ligands for the receptors thought to be expressed in EFC-1 cells, could help to clarify this question.

It has also been suggested that DMSO effects the permeability of the cell membrane (Lyman *et al*, 1976), and has even been shown to enhance DNA transfection (Kawai and Nishizawa, 1984). Edwards *et al* (1983) have suggested that in experiments with cultured EC cells, DMSO acts as a carrier for retinoids that are present in the serum used in the media, implying that the results obtained are not directly due to DMSO. The group therefore investigated the effects of DMSO on EC cells in delipidised serum, but found no alteration in the results. DMSO could be acting as a carrier for retinoic acid and/or other serum factors present in the culture system throughout the present study. However the results obtained by Edwards *et al*, as discussed above, suggest that this is probably not the case, although the only way to ascertain this would be to perform the same experiments with delipidised serum. However, the disadvantage with delipidised serum is that many

other factors, the combinations of which may be necessary for ES cell/embryoid body differentiation and proliferation, could also be removed.

As with many other cell culture systems, DMSO is used as a cryoprotectant for the freezing of the cells used in this study. It is unlikely that the 10% DMSO solution used, could skew subsequent differentiation of ES cells, for a number of reasons. When suspended in the freezing-solution, the cells are immediately frozen and when thawed the solution is diluted and aspirated promptly and the cells washed several times with media before culture. Such practices should ensure that DMSO does not have an effect on the cells, particularly since exposure times in excess of 24 hours are required before an effect is observed (Figure 3.3). However, the only way to determine the effects of DMSO in this procedure on ES cells, would be to use an alternative cryoprotectant.

3.3(ii) Discussion of results of studies with retinoic acid

Retinoic acid affects the haematopoietic differentiation of embryoid bodies, as detected by the CFU-A assay, in a dose dependent fashion. After treatment with atRA during the first 48 hours of differentiation, the percentage of CFU-A colonies obtained from 6 day embryoid bodies is found to decrease with increasing concentrations of atRA. Concentrations of less than 10^{-10} M have been found not to affect the percentage of CFU-A colonies compared to untreated control samples. The first decrease in the percentage of colonies is observed with 10^{-10} M atRA, with any further increase causing a sharp decrease in the percentage of CFU-A colonies. Treatment with 10^{-8} M atRA reduces the percentage of CFU-A colonies to below 5%, while higher concentrations reduce the percentage further to less than 1%.

Morphological analysis of the limited CFU-A colonies obtained from embryoid bodies treated with lower doses of atRA (10^{-10} M, 10^{-9} M) have shown that these colonies do not differ from those derived from untreated control samples. Furthermore, time course experiments show that like DMSO, atRA is not shifting embryoid body CFU-A differentiation to either an earlier or later time point (Figure 3.6).

In a t-test at the 5% level of significance, the mean diameter of CFU-A colonies produced by intact atRA treated embryoid bodies were also found to be significantly smaller than those produced by the untreated samples. Assays of single cells derived from disrupted atRA treated embryoid bodies also show a marked decrease in the number of CFU-A

colonies compared with untreated samples. This could suggests that atRA is acting to reduce the number of haematopoietic progenitors capable of forming CFU-A colonies, and that the absence of colonies after atRA treatment is not due to the inability of macrophages/monocytes to migrate away from intact embryoid bodies. Alternatively treatment of embryoid bodies with atRA could be affecting the self-maintenance capacity of CFU-A cells, resulting in the smaller colony sizes.

Retinoic acid is known to induce the differentiation of EC cells (Hogan *et al*; 1981; Edwards and McBurney, 1983a) and reports of its ability to induce ES cell differentition have also been published recently. Few reports regarding its influence on the haematopoietic differentiation of either of these cell lines have however been published. Reporter gene studies in the human EC cell line NT2, have shown atRA concentrations of 10^{-5}M and $5x10^{-7}$ M can activate the MHC class I promoter, and immunoprecipitation studies have shown the promoter to be bound and activated by the RARβ-RXRβ heterodimer (Segars *et al*, 1993). atRA has also been found to induce the *de novo* expression of the transcription factor NF-κB, this transcription factor being involved in *trans*activating the expression of several haematopoiesis related genes including IL-2 and its receptor.

In a brief study, Heuer *et al* (1994) reported similar observations to those made in the present study. The system used by this group involved the culture of D3 ES cells in hanging drops in the presence varying concentrations of atRA and the absence of LIF for 0-3 days. The aggregates were then allowed to differentiate for 2 days in suspension culture in the absence of atRA. The number of embryoid bodies with blood islands were counted after incubation in semi-solid medium supplemented with leucocyte conditioned medium, consisting of 3U/ml of Epo. 10^{-9} M atRA made little or no difference to the percentage of blood islands, but 10^{-8} M reduced the number by approximately 50%. The percentage of embryoid bodies with blood islands was found to be reduced to less than 10% with a ten fold increase in atRA to 10^{-7} M. In the present study however, the number of CFU-A colonies was found to be reduced by concentrations of 10^{-10}-10^{-9} M, and a reduction of less 5% was achieved with 10^{-8} M atRA.

In the same study other cell types were also observed. A decrease in the number of myocardial cells was noted with increasing concentrations of atRA. The percentage of embryoid bodies producing skeletal muscle was found to increase at 10^{-8} M atRA with any further increases in concentration reducing this percentage. However, while myocardial, skeletal and blood cell types were reduced by 10^{-7}-10^{-6} M atRA, concentrations of between 10^{-8}-10^{-6} M induced nerve cell differentiation by almost 100%. Studies by other groups

where atRA has been found to induce embryoid bodies to differentiate towards neural lineages support these findings (Wobus *et al*, 1994; Bain *et al*, 1995). Similar findings were also reported earlier with EC cell lines (McBurney *et al*, 1982; Edwards and McBurney, 1983a; Husmann *et al*, 1989).

Findings by these groups could suggest that the decrease in the percentage of CFU-A colonies from embryoid bodies observed in the present study may not be directly due to the inhibition of haematopoiesis by atRA *per se*, but as a result of the morphogen inducing the specific differentiation of embryoid bodies towards one particular cell type, namely neural lineages. This hypothesis could be tested with *in vitro* assays designed for the detection of neural differentiation, and by gene expression studies.

Dose-response experiments with 13cRA show that this retinoid is also able to reduce the percentage of CFU-A colonies. However, it is found to be almost 100 fold less effective than atRA. Other studies with this retinoid have also found it to be less potent than atRA. Not only has 13cRA been found to be at least ten fold less effective than atRA in inducing differentiation of promyelocytic cells, both *in vivo* and *in vitro* (Chomienne *et al*, 1990), but also less effective on normal primitive human bone marrow haematopoietic progenitors (van Bockstaele *et al*, 1993).

Various groups have studied the effects of retinoids on normal haematopoiesis using bone marrow progenitors. In studies where mixed populations of human bone marrow cells, consisting of both primitive and committed haematopoietic cells, were used, together with only one colony stimulating factor (GM-CSF) to stimulate clonal proliferation, retinoids have been found to increases haematopoietic differentiation (Douer and Koeffler, 1982; Sakashita *et al*, 1993). However in studies where a purified populations of either primitive murine or human bone marrow cell populations, $Lin^{-}Sca1^{+}$ or $CD34^{+}$ cells respectively, were analysed, along with more than one colony stimulating factor to simulate clonal proliferation, which is thought to be more representative of the *in vivo* haematopoietic microenvironment, retinoids have been found to inhibit haematopoietic proliferation (van Bockstaele *et al*, 1993; Jacobson *et al*, 1994).

In studies where retinoic acid has been found to have multiple effects, primitive progenitor populations are still found to be inhibited whereas more committed progenitors have enhanced proliferation in response to retinoic acid, particularly granulocytic progenitors (Smeland *et al*, 1994; Zauli *et al*, 1995). Therefore the effects of retinoic acid on normal

haematopoietic cells appear to be dependent on the different culture conditions used as well as the cell types analysed.

These reports together with the findings that retinoic acid is able to stimulate the expression of GM-CSF and M-CSF in human bone marrow stromal cell lines (Nakajima *et al*, 1994), suggest that retinoic acid has a complicated role in normal haematopoiesis, with the response to the molecule probably being cell type dependent. These findings could also suggest that atRA may have an inhibitory effect on primitive haematopoietic stem cells derived from ES cells, but could also stimulate haematopoiesis in more mature progenitors. However treatment of embryoid bodies with retinoic acid during the later stages of differentiation does not appear to increase the number of CFU-A colonies (Figure 3.8).

Results of timed exposure experiments with atRA show that the reduction in the percentage of CFU-A colonies is temporally specific. The reduction is only observed if embryoid bodies are exposed to 10^{-8} M atRA between 0-3 days of differentiation, and for no less than 16 hours after being removed from LIF containing media (Figure 3.7 & 3.8). The percentage of CFU-A colonies are no different from the untreated sample if embryoid bodies are treated with atRA outside of this time frame, therefore the drug may be affecting a specific, as yet uncharacterised cell type.

Wobus *et al* (1994) have also found that cardiomyocyte differentiation of embryoid bodies in their culture system to be markedly reduced if they were treated with atRA (10^{-9}-10^{-6} M) during days 0-2 of differentiation. They suggest that this is due to the sensitivity of ES cells to the cytotoxic effects of atRA. The results of the timed exposure experiments in this study and the findings reported by Heuer *et al* (1994) discussed earlier, do not support this hypothesis. Furthermore reports by Chen and Gudas (1996) suggest that ES cells are able to tolerate these, and probably higher concentrations of exogenous retinoic acid since not only do the cells metabolise the drug within the first 2 hours of exposure, a function which appears to be part of their normal metabolism, but the molecule accumulates inside the differentiating cells bound to retinoid binding proteins.

Foetal calf serum (FCS), used for the culture of ES cells in this experimental system and others, contain retinoids as do the sera of other mammals. Although the retinoid content of FCS will vary from batch to batch the concentration has generally been found to be between 10^{-8} and 10^{-9} M (Fuchs and Green, 1981; Hagen *et al*; 1996). It must be noted that the concentrations of atRA used throughout this study are in addition to the serum retinoid levels and so the observations reported cannot be said to be representative of the 'normal'

effects of retinoids on embryoid body haematopoietic differentiation. If the normal effects of retinoids on ES cell/embryoid body differentiation were to be studied, either a serum-free culture system, such as reported by Johansson and Wiles (1995), or delipidised serum, would need to be used. However, this is not the concern of this study, the aims of which are to modulate embryoid body haematopoietic differentiation by exogenous factors.

Retinoids are oxidative agents and can therefore cause cellular and molecular damage and hence cell death. In order to test whether the effects observed with atRA were due to its oxidative potential the effect of iron (II) sulphate, another oxidative agent, on embryoid body differentiation was studied and the results compared with those observed after atRA treatment. Iron (II) sulphate was found to have very little or no effect on the percentage of embryoid body CFU-A colonies compared with untreated control samples, even at concentrations 1000 fold in excess of those used with atRA.

As an oxidative agent Fe^{2+} ions cause the production of superoxide radicals such as O_2^-, and iron (II) salts have been shown to cause oxidative DNA damage and lipid peroxidation in rat liver nuclei (Shires, 1982). However, the concentration of iron (II) salts used in the latter study (1.5×10^{-3} M and 7.5×10^{-4} M), were higher than those used in this study. Furthermore Fe_2^+ ions are bound by intracellular ferritin, which was also absent from the study by Shires (1982). Both these factors may have contributed to the lack of a marked effect on CFU-A numbers after iron (II) sulphate treatment of embryoid bodies. In order to determine whether oxidative damage can cause a reduction in the numbers of CFU-A colonies from embryoid bodies, the culture system needs to be saturated with iron. But this threshold is difficult to ascertain since overloading cells with iron, in turn results in apoferritin synthesis (Halliwell and Gutteridge, 1984).

Clearly however, the concentration of iron (II) sulphate, used in the experiments presented here, was far in excess of those of atRA. The potency of atRA at concentrations of 10^{-9} M and 10^{-8} M is most likely due to its action through its nuclear receptors and not as a result of oxidative damage. Moreover, even though there was a lack of CFU-A colonies, contracting muscle embryoid bodies were still observed at concentrations of 10^{-8} M atRA and above, which would not be expected if there was oxidative damage to the embryoid bodies.

Retinoids are known to exert their effects via their nuclear receptors which are ligand binding transcription factors that belong to the hormone nuclear receptor superfamily. Both families of retinoid receptors (RARs & RXRs) are not only expressed in EC cells but retinoic acid, at the concentrations similar to those used in this study, has also been shown

to induce EC cell differentiation through these receptors (Minucci *et al*, 1994; Roy *et al*, 1995). Detailed expression data for retinoid receptors in ES cells have not been published yet, but treatment of ES cells with retinoic acid has been shown to induce the expression of the receptors (Leroy *et al*, 1991; Chen *et al*, 1996). Thus it can be said that at the concentrations of atRA used in this study, atRA is probably acting via its cognate receptors. This assumption could be verified by the use of antibodies against one or more of the receptors, which could render them ineffective, or with the use of RARα 'knock-out' ES cell lines (Lufkin *et al*, 1993).

3.2(iii) Discussion of results of studies with mesoderm inducers.

Since factors such as bFGF and activin-1 induce mesoderm lineages, their addition to the culture media of differentiating ES cell aggregates would in theory indirectly enhance haematopoietic differentiation. Therefore, preliminary dose dependent studies with both the aforementioned factors were carried out. At the concentrations used, activin-1 appears to have very little or no effect on the percentage of CFU-A colonies, and bFGF appeared to reduce the percentage of colonies marginally. However, no definite conclusions can be drawn from these studies since further experiments are required to ascertain the reproducibility of the observations. Further experiments could not be carried out due to the limited availability of the factors.

One of the problems with using these factors in this culture system is the presence of serum in the media which consists of various unidentified proteins and growth factors that may mask the effects of any exogenous growth factors. In other studies where mesoderm factors, such as BMP-4, have been used to induce embryoid body haemaopoietic differentiation, defined serum-free media has been used (Johansson and Wiles, 1995). For the purposes of future experiments with these inducers it may be useful to use different concentration ranges to the ones looked at. Moreover recent studies in *Xenopus* have shown that the effects of activin-1 and bFGF are dependent on each other and therefore it may be useful to add both factors to the differentiating embryoid bodies, in order to induce noticeable effects (Cornell and Kimelman, 1994; Lebonne and Whitman, 1994). These experiments should also be coupled with gene expression studies to test whether the receptors for these factors are expressed in differentiating embryoid bodies.

3.4 Summary

When embryoid bodies are allowed to differentiate for 6 days in this culture system approximately 30-50% give rise to CFU-A colonies. In order to modulate this level of commitment for reconstitution and gene expression studies embryoid bodies were treated with various chemicals and mesoderm inducing factors during the earliest stages of differentiation, and the effects of the treatments assessed with the CFU-A assay. It was thus found that treatment of embryoid bodies with 1.0-1.4% DMSO increased the percentage of CFU-A colonies by more than 2 fold. It was furthermore found that the full 48 hour exposure was necessary to obtain the optimal effects. The treatment does not appear to alter the time course of embryoid body CFU-A differentiation and is also not thought to be due to the anti-oxidant properties of DMSO. Assays of disrupted DMSO treated embryoid bodies, once again showed a 2 fold increase in the percentage of CFU-A colonies, strongly suggesting that DMSO is enhancing haematopoietic commitment.

Treatment of embryoid bodies with 10^{-8} M atRA for the first 48 hours of differentiation however, was found to reduce the percentage of CFU-A colonies to less than 5%, an effect which is temporally dependent. No reduction in the percentage of CFU-A colonies was observed, relative to the untreated control samples, when embryoid bodies were exposed to atRA for less than 16 hours after being removed from LIF containing media, or if they were treated beyond the third day of differentiation. The drug is therefore not thought to act on committed haematopoietic cells. atRA treatment once again did not alter the time course of embryoid body CFU-A differentiation and the observations made are not thought to be as a result of the oxidative properties of atRA. The effects of DMSO and atRA on embryoid body gene expression patterns, over a time course of differentiation, were subsequently examined. These are discussed in Chapter 4.

4. Effects of Exogenous Factors on Gene Expression.

4.1 Introduction.

Results discussed in Chapter 3 clearly demonstrate that the number of CFU-A colonies can be modulated by the addition of chemical inducers to the differentiation environment of embryoid bodies. CFU-A assays of DMSO treated embryoid bodies, section 3.3(i), strongly suggest that the compound is enhancing haematopoietic commitment. The effects of atRA on embryoid body haematopoietic differentiation are however, somewhat more difficult to interpret, since the marked reduction in the number of CFU-A colonies after atRA treatment does not necessarily imply that haematopoiesis is inhibited.

To make a fuller assessment of the influence of these compounds and how they may be affecting embryoid body differentiation, their effects on gene expression was investigated. Using RT-PCR and Northern blot analysis the messenger RNA content of 1% DMSO or 10^{-8} M atRA treated embryoid bodies, together with untreated control samples were analysed over a time course of differentiation. The expression of most genes analysed in this study have been investigated by other groups, (Schmitt *et al*, 1991; Wiles *et al*, 1991; McClanahan *et al*, 1993; Keller *et al*, 1993; Hole *et al*, 1996).

The genes amplified by RT-PCR included the haematopoietic cell surface markers Sca-1 (Ly-6A/E) and CD45. Sca-1 is a cysteine rich cell surface protein expressed in primitive haematopoietic progenitors (van deRijn *et al*, 1989; Nishio *et al*, 1996). CD45 is a transmembrane spanning glycoprotein expressed in both mature and primitive haematopoietic cells; one of its splice variants gives rise to the B-cell specific marker, B220, which was also amplified (Saga *et al*, 1988; Saga *et al*, 1990). Other transcripts analysed included those for *c-kit* receptor and foetal β-globin, both of which have been shown to be expressed in embryoid bodies differentiating in this culture system (Hole *et al*, 1996).

RT-PCR reactions were also carried out for the transcripts of the chemokine MIP-1α and the intracellular haematopoietic cell phosphatase, HCP: two proteins implicated in the negative regulation of haematopoiesis (Dunlop *et al*, 1992; Yi *et al*, 1993), which could be up-regulated in embryoid bodies after atRA treatment. RT-PCR of GATA-1, and Northern blot analysis of GATA-2 and PU.1, three transcription factors known to be important in haematopoiesis were also performed (Pevney *et al*, 1995; Tsai *et al*, 1994; McKercher *et al*,

1996). The expression pattern of such markers should indicate whether treatment with either morphogen is acting to enhance or inhibit ES cell haematopoietic commitment.

Assuming that DMSO and atRA are acting to increase or decrease ES cell haematopoiesis respectively, they could be exerting their influence either at the stage of ES cell commitment to mesoderm, and/or mesoderm commitment to haematopoietic lineages. Therefore to determine whether DMSO is enhancing, and atRA diminishing mesoderm commitment, RT-PCR reactions for the mesoderm marker *brachyury* (Wilkinson *et al*, 1990), were also performed.

4.2 Results.

Total RNA was extracted from untreated embryoid bodies and those treated with 1% DMSO or 10^{-8} M atRA for the first 48 hours of differentiation. 1μg of each RNA sample was reverse transcribed to obtain cDNA. It is assumed that under optimum conditions each messenger RNA transcript is reverse transcribed into a single cDNA copy. Since 1/20th of the RT products were subsequently used for PCR reactions, approximately 50ng of total RNA was used for PCR amplification, of which only 2-3% consists of messenger RNA.

Before the amplification of RT products, the presence of genomic DNA contamination in RNA extracts was tested by PCR amplification of the β-actin gene, using 50ng of each total RNA sample. These PCR products were subsequently electrophoresed on an agarose gel and no DNA specific bands were observed. RT reactions were then PCR amplified for the β-actin transcript and samples of the reactions electrophoresed on an agarose gel. This gel was Southern blotted and hybridised with an [α-^{32}P]dCTP-labelled β-actin probe and the resulting hybridisation intensities were quantitated using a phosphor-imager. This was done to determine the variability between samples when a standard amount (1μl; approximately 50ng) of RT product is used for PCR amplification. The intensity of each band was derived by subtracting the background signal from the β-actin signal. The resulting intensities are shown in Table 4.1.

Sample	No. of days of differentiation	Intensity (Average pixel value)
Undifferentiated EFC-1	0	6.4
Untreated embryoid bodies	2	5.8
	4	10.2
	6	7.9
	8	7.0
1% DMSO treated embryoid bodies	2	8.2
	4	6.8
	6	11.9
	8	7.9
10^{-8} M atRA treated embryoid bodies	2	6.5
	4	10.3
	6	4.4
	8	6.9

Table 4.1. Intensity of β-actin RT-PCR products for samples of a time course study. The percentages were calculated by subtracting the background intensity from the product signals. All PCR reactions were were carried out for 30 cycles.

The lowest intensity of 4.4 was observed with the 6 day atRA sample, and the highest of 11.9 with 6 day DMSO treated sample. Assuming that β-actin gene expression is not affected by morphogen treatment, it can be said that the intensity of the samples follow a normal distribution with the intensities of most samples falling between 6-8. Given the number of variables that can affect any given PCR reaction, such as pipetting accuracy and reagent integrity, this level of spread in intensities between samples, in any given experiment can be tolerated. It should be noted that the RT-PCR experiments are no more than semi-quantitative in nature, and although the expression of one gene can be compared between samples, the level of expression of different genes cannot be compared. The results of subsequent RT-PCR experiments are summarised in Table 4.2. Gels from some experiments are illustrated in Figure 4.1a-c.

		Untreated				1% DMSO				10^{-8} M atRA			
		Time of differentiation (days)											
GENE	EFC-1	2	4	6	8	2	4	6	8	2	4	6	8
β-actin	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
*brachyury**	±	+	+++	±	+++	+++	+	+++	+++	+++	+++	-	+++
*HCP**	+++	+++	+++	+	+++	+++	+++	+++	+	+++	+++	+++	+++
GATA-1	+	±	-	++	+	-	-	+	-	+	-	-	-
Ly-6/Sca-1	+	-	++	++	+	±	+	++	++	+++	+++	±	++
c-kit	-	-	-	+	++	-	+	++	++	++	++	-	+
β-globin	-	+	-	-	-	-	++	+++	+++	-	-	++	-
*CD45**	-	-	-	-	±	-	-	+++	+	-	-	-	-
IL-3	-	-	-	-	-	-	-	-	-	-	-	-	-
MIP-1α	-	-	-	-	-	-	-	-	-	-	-	-	-
PU.1	-	-	-	-	-	-	-	-	+	-	-	-	-
GATA-2	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.2. Summary of RT-PCR (unshaded boxes) and Northern hybridisation (shaded boxes) analysis of embryoid body RNA extracts. 10μl of each RT-PCR product was electrophoresed on an agarose gel and the bands visualised with ethidium bromide staining except for * which were hybridised with the respective radiolabelled probes. Total RNA was extracted from 2, 4, 6 and 8 day old embryoid bodies cultured in the absence of morphogens or exposed to DMSO or atRA for the first 48 hours of differentiation. RNA extracts from undifferentiated ES cells (EFC-1) represents day 0. All PCR reactions were performed for 30 cycles. The levels of RT-PCR products were scored by eye using the following criterion: -, no signal; ±, trace signal; +, weak signal; ++, strong signal; +++, very strong signal. For Northern hybridisation analysis, 5μg of total RNA extract was electrophoresed on a 1% agarose/formaldehyde denaturing gel and blotted on to nitrocellulose membrane as described in section 2.3.4. The blots were consequently hybridised overnight with the appropriate [a-^{32}P]dCTP labelled probe. Bands were visualised by phospho-imaging.

4.2.1 Effects of DMSO on gene expression.

Results of CFU-A assays discussed in section 3.2, strongly suggest that DMSO is enhancing haematopoietic commitment. This effect can be exerted either at the point of ES cell commitment to mesoderm, or mesoderm commitment to haematopoietic lineages. In order to address this question RT-PCR was used to analyse the expression of the mesoderm marker *brachyury* in embryoid bodies treated with 1% DMSO for the first 48 hours of differentiation, over a time course of differentiation. Since no product for the gene was

detected after ethidium bromide staining, agarose gels were Southern blotted and hybridised with [α-^{32}P]dCTP labelled *brachyury* cDNA probe. Subsequently *brachyury* transcript was detected in nearly all samples. Only a weak signal was obtained from the day 6.5 p.c. mouse embryo sample, possibly due to a poor quality of RNA. At this level of analysis no marked increase in the level of *brachyury* transcription was observed with DMSO treated embryoid bodies compared to untreated samples. Total RNA samples were also used for Northern blot analysis of a second mesoderm marker, *gooscoid*, using a radioactively labelled genomic clone of the gene, containing an intronic sequence. The probe however hybridised to the ribosomal RNA fraction making the experiment uninformative with respect to *gooscoid* expression.

To further investigate the effects of morphogens on embryoid body differentiation several haematopoietic markers were also RT-PCR amplified. RT-PCR products of the intracellular phosphatase HCP, and the membrane bound phosphatase CD45, were analysed using Southern blot hybridisation due to poor visualisation of the products with ethidium bromide staining. Fragment of a cDNA clone was used as a probe for HCP, while total RNA extract from adult mouse spleen was used to obtain a probe for CD45 by RT-PCR. Consequently HCP was found to be expressed in all samples, and its level of expression was not found to be visibly altered after DMSO treatment of embryoid bodies. PCR amplification of the cDNA clone of the gene was used as a control for the reaction.

CD45 transcription levels were found to be markedly up-regulated post DMSO treatment. The CD45 gene is alternatively spliced, two splice variants of which were detected with the PCR reaction used in this study. The higher molecular weight band, (Figure 4.3b), indicates the transcript encoding the B220 cell surface marker. This product is not detected in untreated samples, but is found to be expressed in 6 and 8 day DMSO treated embryoid bodies. Similarly, the CD45 transcript was faintly visible in 6 and 8 day untreated embryoid bodies, but was found to be present with greater intensity at these time points after DMSO treatment.

Transcripts for the haematopoietic transcription factor GATA-1, were detected by RT-PCR in 6 and 8 day untreated embryoid bodies and also in 6 day DMSO treated sample. No distinct increase in the transcription level of GATA-1 was detected post DMSO treatment. Transcripts for GATA-1 were also detected in the day 13 p.c. mouse foetal liver control samples. Northern blot hybridisation of embryoid body total RNA extracts with radioactively labelled GATA-2 cDNA, revealed an absence of the transcript from all

samples. However, after Northern blot hybridisation of total RNA samples with PU.1 cDNA probe, a signal was detected in 8 day DMSO treated embryoid bodies, but no signal was detected with the untreated counterpart. The transcript for PU.1 was also found to be expressed in adult mouse bone marrow.

4.2.2 Effects of atRA on gene expression.

The CFU-A assay results observed with atRA treated embryoid bodies could be due to the inhibition of haematopoietic differentiation by the drug. As explained earlier such an effect could be exerted at at least two different stages of differentiation one of which is at the point of ES cell commitment to mesoderm. In order to assess whether atRA treatment results in the inhibition of mesoderm commitment, expression of *brachyury* in embryoid bodies treated with 10^{-8} M atRA for the first 48 hours of differentiation, over a time course of differentiation, was investigated. Subsequent to Southern blot analysis of the RT-PCR products *brachyury* transcripts were detected in the atRA treated samples. At this level of detection no marked reduction in *brachyury* transcription was detected post atRA treatment compared with the untreated counterparts. Although transcripts for this gene were not detected in 6 day atRA treated sample in repeated assays, it should also be pointed out that only a weak signal was detected for the transcript in 6 day untreated embryoid bodies (Figure 4.1a).

The intracellular phosphatase HCP and the chemokine MIP-1α have been implicated in haematopoietic inhibition. RT-PCR assays for the transcripts of both genes were carried out in order to assess whether either is up-regulated by atRA treatment. After Southern blot hybridisation no visible increase in the transcription of HCP was detected in atRA treated samples compared to their untreated counterparts. The transcripts were present in all samples at similar levels. RT-PCR product for MIP-1α was not detected in any of the reactions by ethidium bromide staining.

Transcripts for the haematopoietic markers CD45, PU.1 and GATA-2 were not detected in any of the atRA treated samples. PU.1 and GATA-2 were also not detected in untreated embryoid bodies after Northern blot hybridisation of total RNA samples. However, the B220 transcript variant of the CD45 gene was detected by RT-PCR in 8 day untreated embryoid bodies, but was found to be absent post atRA treatment.

Sca-1, c-kit receptor and GATA-1 transcripts were found to be expressed at distinctly higher levels in 2 and 4 day atRA treated embryoid bodies compared with the untreated samples of the same time points. GATA-1 was only expressed in 2 day atRA treated embryoid bodies and was not detected thereafter. Assessment of foetal β-globin transcript by RT-PCR showed the messenger RNA to be present in 6 day atRA treated embryoid bodies, but was not detected at the same time point in untreated samples. But the transcript was not expressed as strongly as in 6 day DMSO treated embryoid bodies.

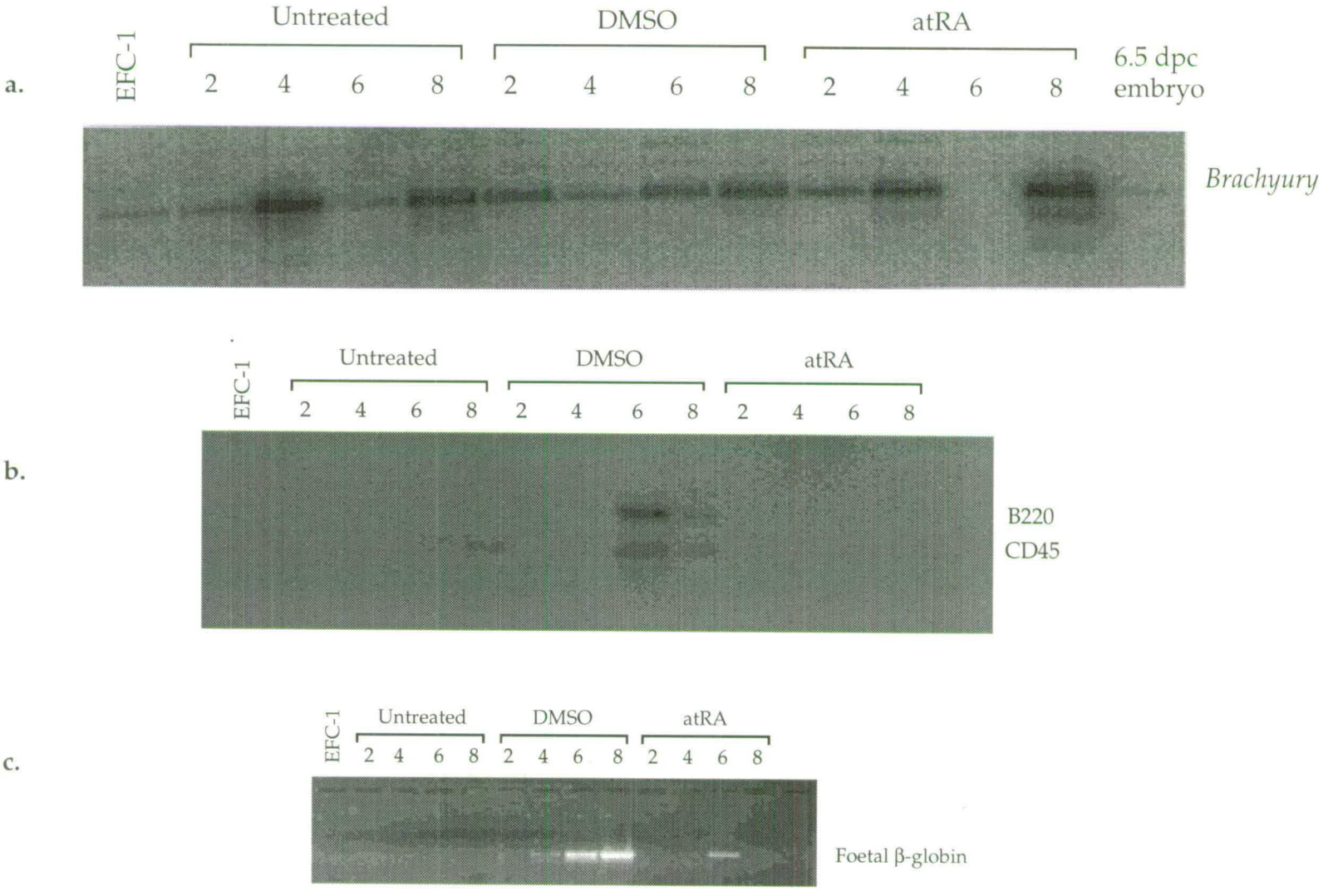

a.
EFC-1
Untreated
DMSO
atRA
2 4 6 8 2 4 6 8 2 4 6 8
6.5 dpc embryo
Brachyury
b.
EFC-1
Untreated
DMSO
atRA
2 4 6 8 2 4 6 8 2 4 6 8
B220
CD45
c.
EFC-1
Untreated
DMSO
atRA
2 4 6 8 2 4 6 8 2 4 6 8
Foetal β-globin

Figure 4.1. Results of RT-PCR assays. RT-PCR reactions were carried out with RNA extracted from untreated embryoid bodies, and those treated with 1% DMSO or 10^{-8} M atRA for the first 48 hours of differentiation. Embryoid bodies were allowed to differentiate for 2, 4, 6 and 8 days before total RNA extraction. Undifferentiated ES cells (EFC-1) were used as time zero control samples.

a.RT-PCR products for the *brachyury* transcript were Southern hybridised with a radioactively labelled cDNA probe of the gene and visualised with phospho- imaging.

b. RT-PCR products of the CD45 transcript were hybridised with a radioactively labelled probe of the transcript obtained by RT-PCR of adult mouse spleen RNA. The higher molecular weight product indicates the B220 splice variant.

c. Foetal β-globin RT-PCR products were resolved on an agarose gel and visualised with ethidium bromide staining.

4.3 Discussion

Results discussed in Chapter 3, showed that the percentage of CFU-A colonies could be increased by two fold after treatment of embryoid bodies with 1% DMSO for the first 48 hours of differentiation. However, treatment with 10^{-8} M atRA for the same period of differentiation reduced the percentage of CFU-A colonies to less than 5%. In order to assess whether DMSO and atRA are exerting their effects by enhancing and inhibiting mesoderm and/or haematopoietic lineage commitment, respectively, Northern blot and RT-PCR techniques were used to study the effects of these compounds on haematopoietic and mesoderm marker gene expression. Northern blot analysis is a more quantitative technique than RT-PCR since relative transcript numbers can be distorted during PCR amplification. RT-PCR was used in the majority of cases however, due to difficulties encountered with Northern blot analysis, such as poor detection of rare transcripts (e.g. PU.1), and non-specific hybridisation of probes to the ribosomal RNA fraction. As mentioned previously, results of RT-PCR reactions are not quantitative and therefore comparisons between experiments cannot be made.

Approximately 50ng of total RNA was used for PCR amplification only 2-3% of which comprises of messenger RNA. The RT product was initially used to amplify the transcript for β-actin to detect the presence of messenger RNA in the extract. These products were further blotted on to nitrocellulose filter, hybrised with the appropriate probe and the signals quantiated by phospho-imaging, in order to assess the variability in the reactions. Quantitation of the signals, showed the spread in percentage intensity between samples to be within a small range given the variety of factors that can affect PCR reactions.

To assess whether DMSO and atRA are influencing embryoid body haematopoiesis through the regulation of mesoderm commitment, RT-PCR reactions for the mesoderm marker *brachyury* were performed. Keller *et al* (1993) have reported the presence of this transcript in 4-6 day embryoid bodies. In this study transcripts for the transcription factor were detected to some extent in nearly all untreated embryo body samples (2-8 days). Trace signals for this transcript were also detected in ES cell RNA extracts which were not observed by Keller *et al* (1993). Although ES cells cultured in the presence of LIF are maintained in a largely undifferentiated state, a degree of differentiation nevertheless takes place. Furthermore, in the present study *brachyury* RT-PCR products were visualised by hybridisation with a radiolabelled cDNA probe of the gene. This is a more sensitive method of discerning transcripts than ethidium bromide staining used by Keller and co-

workers, and could account for the detection of *brachyury* in embryoid bodies at earlier time points as well as the ES cell sample.

At this level of analysis, transcription levels for *brachyury* in DMSO and atRA treated embryoid bodies were not found to be markedly different from those observed with untreated embryoid bodies. Therefore the increase in the number of CFU-A colonies after DMSO treatment, and the reduction after atRA treatment cannot be said to be due to either a marked enhancement or reduction in mesoderm commitment. This is supported by data from Vidricaire *et al* (1994) who have also found transcription level of the gene to be unaffected by DMSO in EC cell aggregates.

Transcripts of the putative negative regulator of haematopoiesis, HCP, were also detected in all samples, and its transcription levels was not found to be noticeably affected after DMSO or atRA treatment of embryoid bodies. The transcripts for HCP were also detected in ES cells after hybridisation of the RT-PCR products with a radiolabelled cDNA probe of the gene. This would therefore suggest that HCP is not a haematopoietic specific gene. It should however be noted that the detection of transcripts in any of these experiments does not necessarily indicate the presence of a functional protein. MIP-1α and IL-3 transcripts were not detected in any of the samples by ethidium bromide staining. Further analysis by cDNA hybridisation was not performed for either of these samples. Keller *et al* (1993) and Schmitt *et al* (1991) also failed to detect IL-3 transcripts in their experiments.

The expression pattern of Ly-6 (Sca-1) and c-kit receptor were found to be very similar during the time course of differentiation (table 4.2). In this study, c-kit receptor was found to be expressed in 6 and 8 day old embryoid bodies confirming observations reported by Keller *et al* (1993). Transcription of either c-kit receptor or Ly-6 were not found to be enhanced by DMSO treatment. However both transcripts were found to be elevated in 2 and 4 day atRA treated embryoid bodies in comparison to untreated samples at the same stage of differentiation. Mao *et al* (1996) have also recently reported the up-regulation of the human Ly-6 homologue, after atRA treatment of HL-60 and NB-4 cells, two promyelocytic cell lines.

Elevated levels of Ly-6 and c-kit receptor transcripts in 2 and 4 day atRA treated embryoid bodies, followed by a decrease in 6 and 8 day atRA treated samples, relative to their untreated counterparts, may suggest the drug is regulating the maturation of certain haematopoietic lineages. However, the significance of this hypothesis needs to be

determined with more detailed gene expression studies. The detection of transcripts for foetal β-globin in atRA treated samples, suggests the morphogen may not be inhibiting haematopoiesis completely. Labayya *et al*, (1994) have recently shown atRA to inhibit erythropoiesis in primitive haematopoietic progenitors. In the present study, atRA treatment takes place before haematopoietic progenitors can be detected, which is at day 4 of embryoid body differentiation. This could explain the difference between the findings of these two studies.

In the untreated embryoid bodies only the 2 day sample was found to express β-globin at low levels. This is in accordance with the findings reported by Schmitt *et al* (1991) who also detect low levels of this transcript at this time point. This group found the transcript to be expressed at higher levels in ES cells that were allowed to differentiate in excess of 8 days. The result of this experiment in the present study is however not in accordance with those reported by Hole *et al*, (1996) who found foetal β-globin to be expressed at high levels in 4-8 day embryoid bodies. This difference in findings could be due to a change in culture conditions between the time of the study by Hole and co-workers and the experiments presented here. A different batch of foetal calf serum is for instance known to have been used in this study.

The most striking aspect of this experiment however, is the marked increase in the level of foetal β-globin transcription in DMSO treated embryoid bodies. DMSO is known to cause globin messenger RNA accumulation in MEL cells (Ross *et al*, 1972). More recent studies have shown this accumulation to be dependent on haem synthesis (Fukuda *et al*, 1993; Fukuda *et al*, 1994). The ability of DMSO to induce β-globin transcription in embryoid bodies could suggest that the compound has some specificity for the erythroid differentiation pathway.

DMSO was also found to up-regulate CD45 and B220 transcript levels. The B220 transcript was not found to be expressed in untreated samples, but CD45 was weakly detected in 8 day untreated embryoid bodies. The same was reported by Hole *et al* (1996). Schmitt *et al* (1991) have detected both transcripts in ES cells which had differentiated for at least 16 days, by ethidium bromide staining of RT-PCR products. In the present study PCR products were hybridised with the homologous gene probes which may explain the detection of CD45 at an earlier time point in untreated samples. Both CD45 and B220 transcripts were found to be up-regulated in 6 and 8 day embryoid bodies post DMSO treatment. But neither transcript was detected in atRA treated embryoid bodies.

Transcripts of the GATA-1 transcription factor, which is known to be involved in erythroid differentiation (Pevney *et al*, 1991), were detected in 6 day DMSO treated samples, and in 6 and 8 day untreated embryoid bodies. Keller and co-workers have also reported the presence of this transcript in embryoid bodies during this stage of differentiation. In the atRA treated samples only 2 day embryoid bodies were found to express GATA-1. Labbaya *et al* (1994), have reported the down-regulation of GATA-1 in primitive human haemtopoietic progenitors after their exposure to atRA. GATA-1 transcription was also detected in ES cell extracts unlike the observations of Keller *et al*, (1993). As previously mentioned, ES cells undergo a certain degree of differentiation in the presence of LIF, and the culture conditions could furthermore be conducive to the expression genes normaly associated with differentiated or differentiating cells. But the detection of transcript does not necessarily indicate the presence of functional protein.

Northern hybridisation of embryoid body total RNA extracts with a radiolabelled cDNA GATA-2 probe did not detect transcripts for this gene. This could be due to the rarity of the transcript, but also, GATA-2 has been shown to be down regulated by GATA-1 (Weiss *et al*, 1994), the transcript for which was detected in the samples by RT-PCR. PU.1, a member of the ets family of transcription factors, has been found to play an important role in myeloid lineage differentiation (Zhang *et al*, 1994; Celada *et al*, 1996; McKercher *et al*, 1996). Therefore the presence of the transcript for this protein was also investigated. Embryoid body total RNA extracts were probed with a radiolabelled cDNA PU.1 probe. A weak signal was detected in 8 day DMSO treated embryoid bodies, but not in any of the other samples.

The up-regulation of transcripts for haematopoietic markers such as CD45, PU.1 and foetal β-globin, post DMSO treatment, would strongly suggest that the compound is enhancing haematopoietic lineage commitment and differentiation. The detection of the mesoderm marker *brachyury* in atRA treated embryoid bodies suggests that mesoderm differentiation in these samples is not inhibited, and it could be inferred that the drug affects a later stage of lineage commitment which ultimately results in the absence of CFU-A colonies. However whether atRA is inhibiting haematopoietic differentiation *per se*, is unclear, since foetal β-globin is found to be expressed. Further detailed gene expression studies are required to ascertain the effects of atRA on ES cell haematopoietic differentiation.

4.4 Summary

RT-PCR and Northern blot analysis was used to study the effects of DMSO and atRA on lineage commitment and haematopoietic gene expression in embryoid bodies. DMSO and atRA were not found to neither increase nor decrease the level of *brachyury* transcription in comparison with untreated samples. On the basis of this result it could be assumed that neither morphogen is affecting embryoid body haematopoiesis through the regulation of mesoderm commitment. Treatment of embryoid bodies with 1% DMSO during the first 48 hours of differentiation was found to increase foetal β-globin, CD45 and PU.1 transcript expression, but did not markedly alter Ly-6, c-kit, HCP, and GATA-1 transcript levels compared to untreated control samples.

Transcripts for PU.1 and CD45 were not detected in embryoid bodies treated with 10^{-8} M atRA for the first 48 hours of differentiaion. But foetal β-globin transcripts were detected in 6 day atRA treated samples. GATA-1, Ly-6 and c-kit receptor were also detected in atRA treated embryoid bodies but these were found to be expressed at higher levels earlier in the time course compared with the untreated control samples.

On the basis of these findings it can be concluded that DMSO acts to enhance haematopoietic lineage commitment and differentiation. But in order to unequivocally determine whether atRA is inhibiting haematopoietic differentiation further gene expression studies are required.

5. Differential Protein Expression Analysis of Embryoid Bodies.

5.1 Introduction.

Results discussed in Chapter 3 demonstrated that DMSO can increase the percentage of CFU-A colonies from embryoid bodies by approximately 2-fold, this effect being exerted within the first 48 hours of differentiation. RT-PCR studies showed that the transcription level of various haematopoietic markers are enhanced post DMSO treatment. Although atRA treated embryoid bodies were found to express foetal β-globin, suggesting the drug may not be inhibiting haematopoietic commitment, assay results clearly demonstrated that atRA is acting to reduce CFU-A differentiation in embryoid bodies treated during the first 72 hours of differentiation.

The contrast in the effects of DMSO and atRA on embryoid body CFU-A differentiation provides a useful means of studying differential gene expression and could be employed in identifying factors that may be involved in ES cell commitment to HSCs, and in the *in vitro* differentiation of HSCs to various haematopoietic lineages. Differential library screening of treated and untreated embryoid body cDNA extracts is one method whereby the effects of DMSO and atRA can be utilised to this end. However, like RT-PCR and Northern blot analysis, this technique only detects the presence of transcripts which may not necessarily be translated to functional protein. This shortfall can however be overcome by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis of protein extracts, which provides a means of assessing gene transcription and translation. This method can also be indicative of the activity of certain proteins, since many proteins are activated by translational modifications, such as glycosylation and phosphorylation which can be detected by 2D-PAGE analysis.

Computer assisted 2D-PAGE analysis has in recent years gained popularity and facilitated the study of protein expression during cell growth and differentiation. Software packages, such as PDQuest™ and Phoretix™ allow the quantitative and qualitative evaluation of any given protein, and provide a systematic means of comparing and contrasting protein expression levels over a time course of differentiation and under different growth conditions.

The ability to assess temporal changes in protein expression has made 2D-PAGE analysis a productive method for developmental biology studies (Shi *et al*, 1994). The technique has been used for studying various aspects of murine embryogenesis including: differential protein expression studies of the three primary germ layers (Latham *et al*, 1993); the transition from maternal to embryonic protein expression (Flach *et al*, 1982; Poueymirou *et al*, 1987; Latham *et al*, 1991; Latham *et al*, 1992; Latham *et al*, 1994); and genetic imprinting (Latham and Solter, 1991a; Bowden *et al*, 1996).

Conditions in the cell culture environment directly influence protein expression. By altering growth conditions and/or exposing cells to various environmental stresses and differentiation inducing factors, various groups have adopted computer aided 2D-PAGE analysis as a means of studying differential protein expression. These studies include the comparison of protein expression patterns between normal and virally transformed human keratinocytes (Celis and Olsen, 1994), comparisons of normal murine hepatocytes and those exposed to ionising radiation (Giometti *et al*, 1992), and differential protein expression studies of human germ cell-tumour derived cell lines before and after induction with retinoic acid (Damjanov *et al*, 1993).

It is apparent from the above examples, that 2D-PAGE analysis provides a useful means of studying embryoid body haematopoietic differentiation, a temporally regulated process (Schmitt *et al*, 1991; Hole *et al*, 1996), which can also be influenced by exogenous factors, such as DMSO and atRA. Using 2D-PAGE analysis protein expression patterns between untreated, DMSO and atRA treated embryoid bodies over a time course of differentiation, were studied. The PDQuest™ software system was employed to identify proteins which might play an important role in ES cell haematopoietic commitment and differentiation. The details and results of this analysis are outlined in the following sections.

5.2 Results

Untreated embryoid bodies, and those treated with 1% DMSO or 10^{-8} M atRA for the first 48 hours of differentiation, were pulse labelled with L-[^{35}S]methionine and subsequent 2D-gels were analysed using the PDQuest™ software system. In order to minimise experimental error, all embryoid body samples were labelled at the same time, and protein extracts were resolved on 2D-PAGE gels simultaneously.

The PDQuest™ software system has been described in detail previously (Garrels, 1989). In brief, autoradiographs of the gels were scanned, and images calibrated with the calibration strip. Background noise and streaking were removed before spots in both dimensions were fitted to a Gaussian distribution. The 2D-gel of 6 day untreated embryoid bodies contained the largest number of spots (923). This gel was therefore used as the template for the construction of a standard protein map, which contains all spots in the matchset (Figure 5.1). Matched spots were manually landmarked to enabled orientation and compensation for stretching of gels. Automated spot detection was then carried out, followed by manual editing and matching.

A standard spot number (SSP) was allocated to each spot in the completed standard protein map as a means of identification, and the intensity of the spots normalised to parts per million (p.p.m.), this being calulated from the amount of radiolabelled cell extract loaded on each gel. The molecular weight (Mr) in kilo-Daltons (kDa) and isoelectric point (pI) of each spot was calculated with reference to proteins with known Mr and pI. The intensity of each spot is expressed in pixel density units (PDU), this being a function of the spot intensity, number of pixels covered and the normalisation factor. The software can locate proteins which are up- or down-regulated by 5 fold, and those which are switched-on (present) or -off (absent) between specific gels. Therefore by posing questions that can be answered in these terms, Boolean sets containing elements, i.e. proteins, satisfying specific parameters, were created and proteins fulfilling certain criteria identified. The accuracy of the Boolean set analysis was tested by posing a series of 'control questions' to which the answer was expected to be zero (Table 5.8).

5.2.1 Protein expression patterns in untreated embryoid bodies.

Initially comparisons between 2-dimensional protein gels of undifferentiated ES cells and 2 day embryoid bodies were performed. The data shows a large number of changes in protein expression taking place during this early stage of the differentiation programme. 345 proteins were found to be switched on during this phase of differentiation. An additional 145 spots were up-regulated and only 6 down-regulated.

Switching-on or -off and up- or down-regulation of protein expression was found to continue throughout the time course of embryoid body differentiation (Table 5.1). Such temporal changes in protein expression are illustrated by spot number 1403, representing a protein of pI 4.4 and Mr 38.2 kDa. The intensity of this protein increases by almost 5 fold, from 15.8

PDU in 2 day untreated embryoid bodies, to 74.9 PDU in 8 day untreated embryoid bodies. In contrast, after being up-regulated by more than 35 fold from 6.3 PDU in undifferentiated ES cells, to 226.3 PDU in 2 day embryoid bodies, spot number 8513 is then steadily down-regulated across the time course of embryoid body differentiation (Table 5.4).

Boolean-sets of proteins:	**Number of spots in set**
up-regulated from 2C to 4C	21
up-regulated from 4C to 6C	9
down-regulated from 6C to 8C	23
switched-off in EFC-1, on in 2C *and* up-regulated in 4C	9
switched-off in 2C, on in 4C *and* up-regulated from 4C to 6C	1
switched-on from 4C to 6C *and* switched-off from 6C to 8C	297

Table 5.1. Boolean sets of spots created for the analysis of protein expression patterns in untreated embryoid bodies. 2-dimensional protein gels of undifferentiated ES cells (EFC-1), and those for 2, 4, 6 & 8 day untreated control (C) embryoid bodies were compared and the number of spots in each of the sets, determined.

For the purposes of identifying proteins involved in ES cell haematopoietic commitment and differentiation, various criteria were considered. During the time course of untreated embryoid body differentiation, the number of CFU-A colonies gradually increased, with the largest number of CFU-A colonies being detected at around the 6th day of differentiation and decreased thereafter. Assuming that the expression pattern of haematopoietic related proteins reflects this observation, the CFU-A differentiation profile can be used as the basis for identifying proteins of interest. As shown in Table 5.1, there are proteins which are up-regulated from 2 to 4 to 6 days, and some which are down-regulated from 6 to 8 days. But none of these proteins were found to be common between all 4 sets. However, 297 spots were found to be switched-on in embryoid bodies between days 4 and 6, and down-regulated between days 6 to 8.

Proteins such as 1305, which are highly expressed in 6 and 8 day embryoid bodies, may be markers of differentiated cells, whereas protein spots which are switched on during the first 2 days of differentiation and down-regulated thereafter, (such as spot 3109), or switched-off, (such as spot 5005), may be representative of proteins involved in the early stages of ES cell commitment, among which proteins required for haematopoietic commitment may be included.

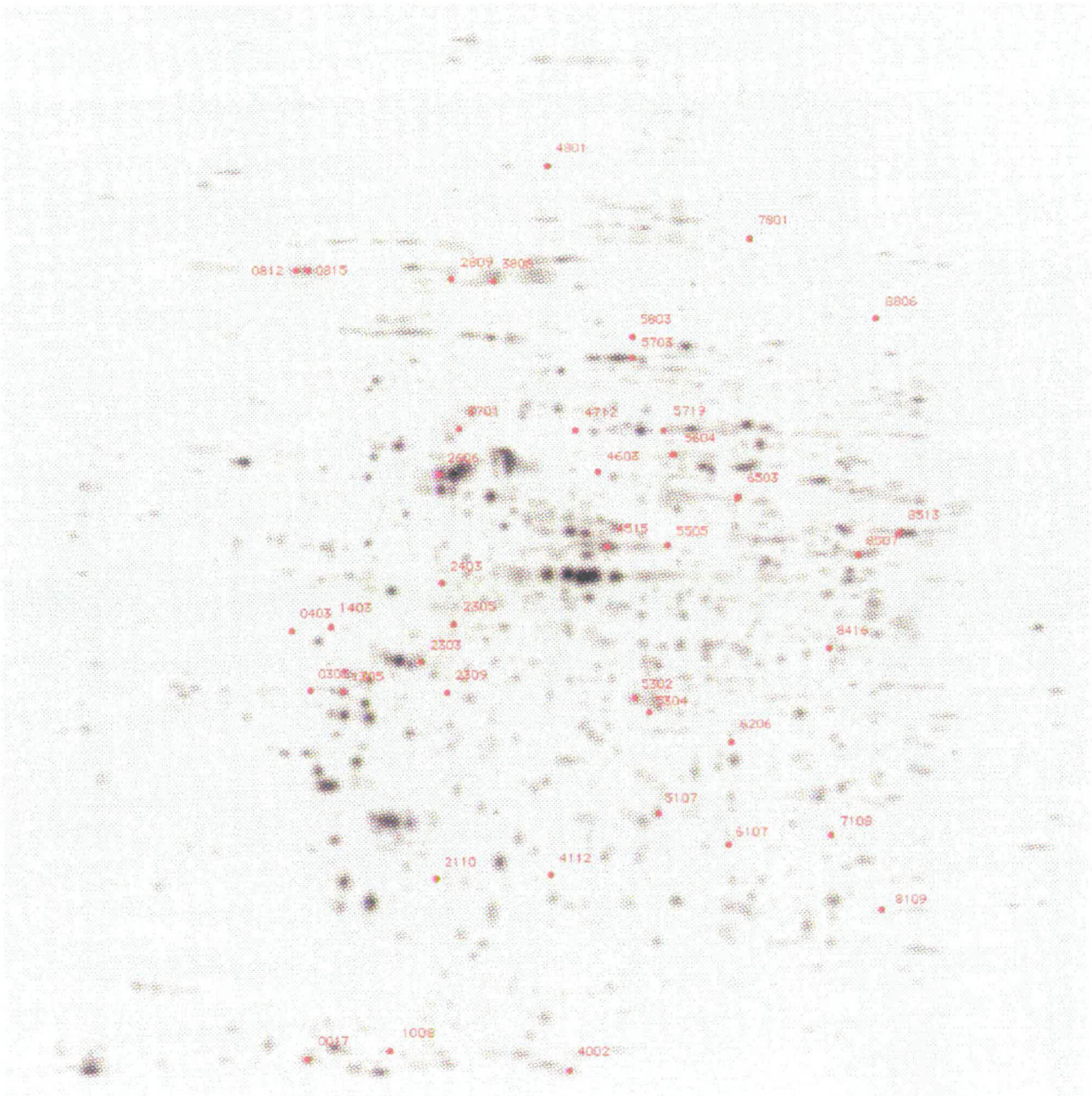

4801
7901
0812
0815
2809
3806
8806
5803
5703
8703
4712
5717
5604
2606
4603
8503
8513
8507
5505
2403
0403
1403
2305
8416
2303
0305
2309
5302
5304
8206
5107
7109
6107
2110
4112
8109
0017
1009
4002

Figure 5.1. Standard protein map. The 2D-PAGE gel of 6 day untreated embryoid bodies was used to create a standard protein map which is a composite of all the protein gels in the matchset and therefore contains all the spots from the 13 gels analysed. The red spots indicate the position of proteins of interest, i.e. proteins which fulfil one or more aspect(s) of the criteria used to define proteins that could potentially play a role in haematopoietic commitment/differentiation (see main text). The SSP numbers for each protein is given to the right of each spot.

5.2.2 Embryoid body protein expression is modified by DMSO.

Protein expression in embryoid bodies treated with 1% DMSO for the first 48 hours of differentiation was found to vary over the time course of differentiation. The protein expression patterns in these embryoid bodies were also found to differ from that of untreated samples; this is demonstrated in Tables 5.2 & 5.3. A larger number of spots were found to be switched on from day 2 to day 4 of embryoid body differentiation after DMSO treatment, compared with the untreated samples at the same phase of differentiation. However, fewer spots were found to be switched on from day 4 to day 6, and from day 6 to day 8 in DMSO treated embryoid bodies, compared with the same gels in the untreated samples.

No. of proteins switched-on:	Untreated	1% DMSO
from EFC-1 to 2D	345	210
from 2D to 4D	21	117
from 4D to 6D	346	205
from 6D to 8D	354	203

Table 5.2. Comparison of the number of proteins being switched-on from EFC-1 to 2, 2 to 4, 4 to 6, and 6 to 8 days in untreated and 1% DMSO (D) treated embryoid body samples. All embryoid body samples were labelled with L-[^{35}S] methionine simultaneously.

Comparison of the protein expression profiles in time course experiments of untreated embryoid bodies with those of their DMSO treated counterparts, also shows that not only does DMSO up-regulate the expression of some proteins, but it also induces the expression of additional proteins, (Table 5.3). Some proteins such as 1217, representing a protein of pI 4.4 and Mr 27.9 kDa, were found to be consistently up-regulated after DMSO treatment, throughout the time course of differentiation (Table 5.4).

Of the proteins whose expression was switched-on in 2 day embryoid bodies as a result of DMSO treatment, 16 were also switched-on in the 4 day samples following DMSO treatment (Figure 5.2a). Ten protein-spots were also found to be switched-on in both 6 day and 8 day embryoid bodies as a result of DMSO treatment (Figure 5.2b). However, since no proteins were found to be common to all four sets, it can be assumed that ES cells do not possess genes which are specifically induced by DMSO. Proteins were also found to be down-regulated or switched-off as a result of DMSO treatment, as shown in Table 5.3.

	Switched-on	Switched-off	Up-regulated	Down-regulated
From 2C to 2D	55	130	14	102
From 4C to 4D	220	120	6	75
From 6C to 6D	154	385	18	113
From 8C to 8D	114	130	3	32

Table 5.3. Numbers of proteins being switched-on/off or up/down-regulated as a result of DMSO treatment. 2-dimensional protein gels of untreated control (C) and DMSO (D) treated embryoid bodies at various days of differentiation were compared and the numbers of protein-spots either up/down-regulated or switched-on/off as result of DMSO treatment at particular time points determined.

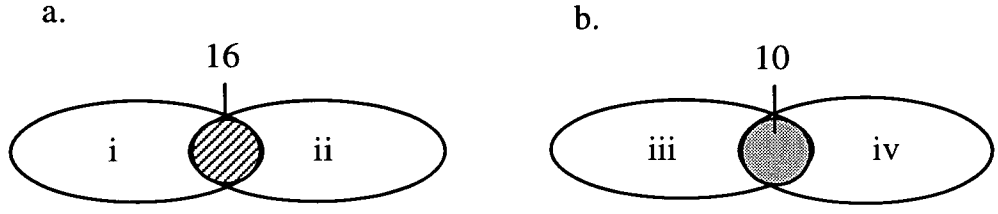


Figure 5.2. Common proteins shared between sets of proteins switched-on following DMSO treatment. The numbers 16 and 10, indicated in (a) and (b) respectively, refer to spots shared between the following sets:

i. Spots switched-on from 2 day untreated samples to 2 day DMSO treated samples
ii. Spots switched-on from 4 day untreated samples to 4 day DMSO treated samples
iii. Spots switched-on from 6 day untreated samples to 6 day DMSO treated samples
iv. Spots switched-on form 8 day untreated samples to 8 day DMSO treated samples

For the purposes of identifying proteins involved in ES cell haematopoietic commitment and differentiation, similar criteria to those mentioned in section 5.2.1 were considered. Since twice as many embryoid bodies have been found to give rise to CFU-A colonies after exposure to 1% DMSO during the first 48 hours of differentiation, proteins of interest may be among those either up-regulated or switched-on, upon DMSO treatment (Table 5.3).

If proteins switched-on in the early stages of embryoid body differentaition, and switched-off or down-regulated thereafter, are representative of proteins involved in the early stages of ES cell haematopoietic commitment, proteins such as 5005 may be of particular interest. Spot 5005 is a protein of pI 5.3 and Mr 17.2 kDa. It is switched-off in ES cells but switched-on in 2 day untreated embryoid bodies, and is switched-off thereafter. In 2 day DMSO treated embryoid bodies the PDU value for this spot is increased by more than 2 fold compared with untreated samples (Table 5.4). Other proteins such as 1305, which are up-regulated throughout the time course of untreated embryoid body

			Spot Intensities (PDU)												
			Untreated					1% DMSO				10^{-8} M atR			
SSP	pI	Mr	EFC-1	2	4	6	8	2	4	6	8	2	4	6	8
1217	4.4	27.9	349.6	403.1	429.7	383.0	176.3	258.3	1156.7	1451.0	1304.7	1712.7	1345.9	1209.4	791.7
1305	4.4	33.5	104.3	87.2	170.8	303.0	333.6	28.9	254.9	246.2	373.8	201.1	60.1	47.5	51.7
1403	4.4	38.2	14.8	15.8	-	60.1	74.9	24.9	42.8	51.9	96.8	-	-	-	-
2110	4.8	23.5	-	-	-	49.3	84.8	-	37.1	75.2	97.8	29.6	-	-	-
2303	4.7	35.2	-	33.6	-	45.7	168.6	-	-	162.0	179.8	-	-	-	-
2405	4.8	38.5	38.2	70.3	61.2	83.4	-	26.0	-	-	-	44.7	158.9	55.7	95.5
2606	4.8	55.2	287	668.9	1129.7	578.6	266	847.9	539.1	325.5	310.6	340.8	1996.5	1146.1	620.9
3109	5.0	22.3	-	257.7	165.1	132.2	-	165.0	137.9	-	-	-	-	85.7	-
3312	5.1	32.6	-	36.1	30.3	42.2	44.4	47.5	-	-	-	-	129.4	47.5	76.6
4112	5.1	23.9	28.7	72.1	32.3	19.7	37.0	437.0	23.0	23.5	42.2	-	171.3	227.8	576.3
4801	5.1	108.7	-	-	-	54.6	-	-	19.1	61.1	-	238.3	-	-	-
5005	5.3	17.2	-	79.4	-	-	-	199.0	-	-	-	-	-	111.7	-
7404	6.4	42.6	10.9	83.6	225.7	54.3	-	124.4	159.9	-	50.7	-	-	74.3	51.7
8513	7.1	47.9	6.3	226.3	287.2	200.7	147.8	130.7	261.7	31.7	152.1	22.7	30.0	199.6	422.8

Table 5.4 . Protein spots of interest and their intensities. The above table shows some protein-spots which may involved in ES cell haematopoietic commitment and/or differentiation, and variations in their expression patterns with respect to time and treatment (see text). The spots are identified by their standard spot numbers (SSP). The values for their pI and Mr (kDa) were calculated with reference to those for known proteins. The intensities are expressed in pixel density units (PDU). The samples are undifferentiated ES cells (EFC-1); 2, 4, 6 & 8 embryoid bodies either untreated or treated with 1% DMSO or 10^{-8} M atRA for the first 48 hours of differentiation. (-) denotes: no detectable intensity.

differentiation, and are further up-regulated post DMSO treatment, could be involved in embryoid body haematopoietic differentiation.

5.2.3 Embryoid body protein expression is modified by atRA.

Expression patterns of proteins in embryoid bodies treated with 10^{-8} M atRA during the first 48 hours of differentiation, are also found to vary over the time course investigated. Protein expression profiles in these embryoid bodies differ not only from that of untreated embryoid bodies, but also from DMSO treated samples. Comparisons between 2-dimensional protein gels of untreated and atRA treated embryoid bodies, show that atRA can up- or down-regulate the expression of some proteins while it can switched-on or -off the expression of others (Table 5.5). For example, protein spot 2606 (pI 4.8, Mr 55.2 kDa), is up-regulated in atRA treated samples compared with untreated counterparts, and continues to be up-regulated throughout the time course of differentiation. Spot 1305 is up-regulated by more than 2-fold in 2 day atRA treated embryoid bodies, compared to untreated samples but is down-regulated thereafter during the time course of differentiation. In contrast, some protein spots displayed a sharp and temporally specific expression pattern in atRA treated embryoid bodies, such as 3109 and 5005, both of which are only expressed during the 6th day of differentiation (Table 5.4).

	Switched-on	Switched-off
From EFC-1 to 2A	345	230
From 2A to 4A	171	398
From 4A to 6A	261	91
from 6A to 8A	255	155

Table 5.5. Changes in protein expression across the time course of atRA (A) treated embryoid body differentiation. 2-dimensional gels between undifferentiated ES cells (EFC-1) & 2 day, 2 & 4 day, 4 & 6 day, 6 & 8 day embryoid bodies were compared and the number of protein spots being switched-on or -off, recorded.

	Switched-on	Switched-off	Up-regulated	Down-regulated
From 2C to 2A	285	329	108	47
From 4C to 4A	139	159	113	25
From 6C to 6A	76	76	46	38
From 8C to 8A	273	92	131	22

Table 5.6. Comparison between untreated control (C) and atRA (A) treated embryoid bodies. 2-dimensional protein gels between 2, 4, 6 & 8 day embryoid bodies were compared and the number of protein spots switched-on/off or up/down-regulated between samples recorded.

After treatment with 10^{-8} M atRA during the first 48 hours of differentiation, the number of embryoid bodies giving rise to CFU-A colonies is markedly reduced. Assuming that the expression pattern of haematopoietic related proteins reflect the results of CFU-A assays, effects of atRA can be used to identify proteins of interest. Therefore protein spots which are either down-regulated or switched-off after atRA treatment, in comparison to untreated samples, are of particular interest. Examples of such proteins include spot 1305, which is gradually down-regulated through the time course in atRA treated embryoid bodies, and spot 1403, which is not expressed at any time point in atRA treated samples.

However, results of CFU-A assays of embryoid bodies after time course experiments show that a very small proportion of 6 and 8 day atRA treated embryoid bodies give rise to haematopoietic colonies (Figure 3.8). Therefore, proteins that could potentially be involved in haematopoiesis may be switched-off or down-regulated in 2 and 4 day atRA treated embryoid bodies but could be switched-on or up-regulated in 6 and 8 day samples, in comparison to untreated samples. Protein spot 7404 (pI 6.4, Mr 42.6 kDa) fulfils such criteria. This protein is not expressed in 2 and 4 day atRA treated embryoid bodies but is expressed in 6 and 8 day samples. Furthermore, in untreated samples, 7404 is most highly expressed in 4 day embryoid bodies, and down-regulated thereafter, a profile which could be indicative of a protein involved in the early stages of ES cell differentiation.

5.2.4 Use of DMSO and atRA for the selection of haematopoietic related proteins.

By exploiting the opposing effects of DMSO and atRA on the CFU-A differentiation of embryoid bodies, a set of criteria for identifying haematopoietic related proteins from 2-dimensional gels can be defined. Using this approach the Boolean sets defined in the

previous sections were superimposed for further selection of proteins that may be involved in the haematopoietic commitment and/or differentiation of ES cells. Two main criteria for the selection of such proteins were defined; these are outlined below.

Data obtained from CFU-A analysis, discussed in chapter 3, show that the number of CFU-A colonies from untreated and DMSO treated embryoid bodies gradually increases between the 2nd and 6th day of differentiation. DMSO treatment increases this number of CFU-A colonies by about 2-fold (Figure 3.2). Although the number of CFU-A colonies obtained from atRA treated embryoid bodies is markedly reduced in comparison to untreated samples, it nevertheless increases marginally over the time course of differentiation (Figure 3.6). In theory, the expression pattern of the protein-spots which reflect these observations could help identify proteins involved in the differentiation of haematopoietic progenitors. Such a criterion is represented by Figure 5.3a.

Figure 5.3. Superimposition of sets to define proteins involved in (a) the differentiation of haematopoietic progenitors (shaded) and (b) commitment of ES cells to haematopoietic lineages (shaded). The sets are:

i. Proteins up-regulated throughout the time course of untreated embryoid body differentiation
ii. Proteins up-regulated with DMSO treatment and up-regulated throughout the time course of DMSO treated embryoid body differentiation
iii. Proteins down-regulated with atRA treatment and up-regulated over the time course of atRA treated embryoid body differentiation
iv. Proteins not expressed in EFC-1
v. Proteins up-regulated throughout the time course of untreated embryoid body differentiation
vi. Proteins up-regulated with DMSO treatment but down-regulated throughout the time course of DMSO treated embryoid body differentiation
vii. Proteins down-regulated with atRA and up-regulated over the time course of atRA treated embryoid body differentiaion

As previously mentioned, proteins which are either switched-off or are expressed at low levels in undifferentiated ES cells, but which are expressed during the early stages of embryoid body differentiation, and thereafter switched-off or down-regulated, could potentially be involved in the early stages of ES cell commitment. In theory proteins which could be involved in the early stages of ES cell haematopoietic commitment would

also have this profile but in addition they would be up-regulated after DMSO treatment and down-regulated after atRA treatment. Such a criterion is represented in Figure 5.3b.

As demonstrated by Table 5.7, when various Boolean sets are superimposed, the number of common spots is rapidly reduced. No protein-spots were found to fulfil either of the stringent criteria described in Figure 5.3a or b. But a number of proteins were found which displayed an expression profile that satisfied some aspects of these criteria, and which warranted further analysis (Table 5.7). Amongst these, are proteins the expression patterns of which are outlined in Table 5.4 & Figure 5.4.

Other sets of proteins which may also be of interest are those which are not expressed in atRA treated embryoid bodies, but which are expressed in untreated and DMSO treated samples, such as protein spot 2405. Assuming that atRA is inhibiting the differentiation of certain haematopoietic lineages, proteins which are only expressed in atRA treated embryoid bodies and not expressed or are expressed at low levels, in untreated or DMSO treated samples, such as protein spot 4112, could potentially be involved in the inhibition of such differentiation.

Boolean set of proteins:	**Number of spots in set**
up-regulated from 2A to 2D	12
down-regulated from 2A to 2D	74
up-regulated from 2A to 2D *and* present in 2C	12
up-regulated from 6A to 6D *and* present in 6C	16
up-regulated from 2A to 2D *and* present in 6C	12
up-regulated from 2A to 2D, present in 4C *and* up-regulated from 4A to 4D	1
up-regulated from 2A to 2D, present in 4C *and* up-regulated from 6A to 6D	0
on in 6D *and* off in 6A	210
on in 8D *and* off in 8A	75
on in 4D,off in 4A *and* on in 6D, off in 6A	42
on in 4D, on in 6D, off in 4A, off in 6A *and* up-regulated from 4D to 6D	2

Table 5.7. Sets defined for the identification of haematopoietic related proteins. 2-dimensional protein gels of 2, 4, 6 & 8 day untreated control (C), DMSO (D), and atRA (A) treated embryoid bodies were compared for the identification of haematopoietic related proteins.

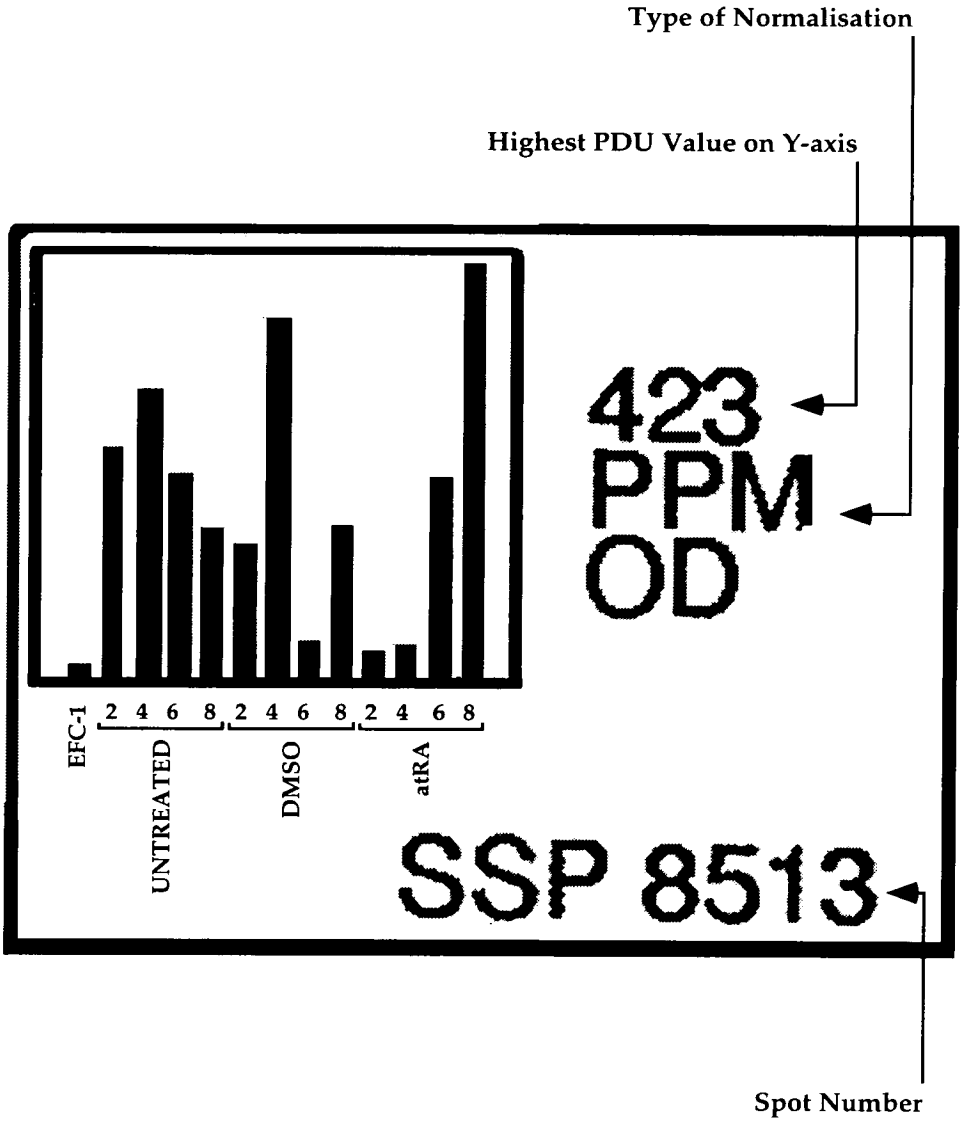
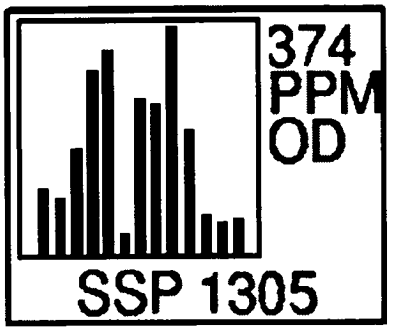


Figure 5.4. Expression profiles of eight proteins of interest (opposite). The key for the graphs is shown above. The PDU value is given on the y-axis and the the sample on the x-axis. The samples include undifferentiated ES cells (EFC-1); 2, 4, 6 & 8 day untreated, 1% DMSO treated and 10^{-8} M atRA treated embryoid bodies. See Table 5.4 for details.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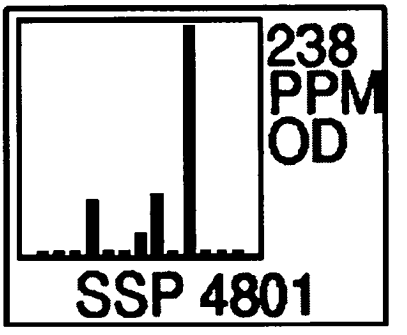
PPM
OD
SSP 1305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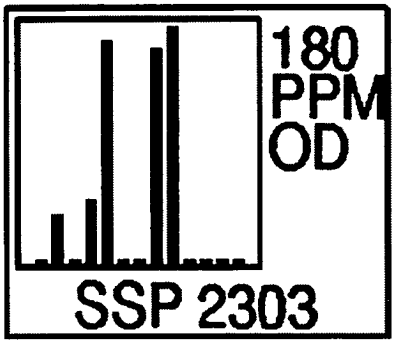
PPM
OD
SSP 4801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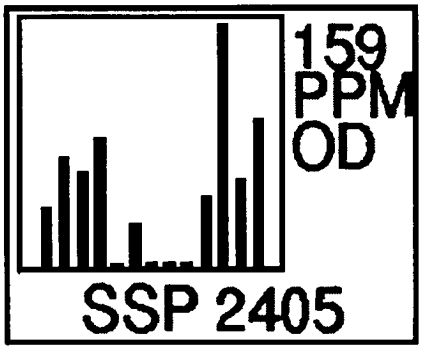
PPM
OD
SSP 2303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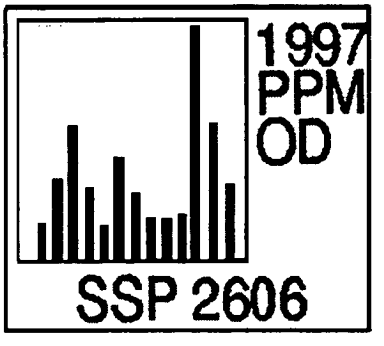
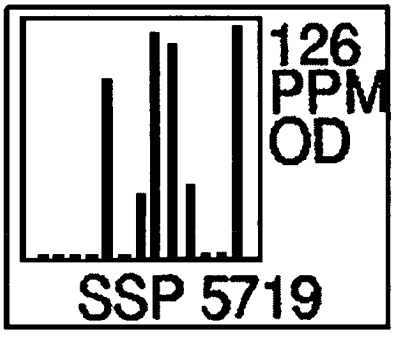
PPM
OD
SSP 2405


1997
PPM
OD
SSP 2606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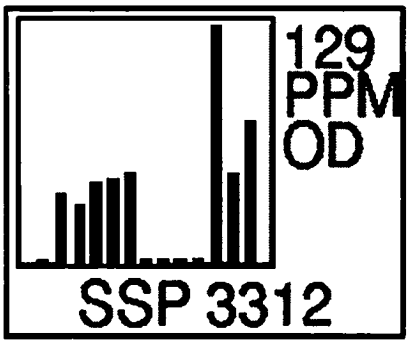
PPM
OD
SSP 5719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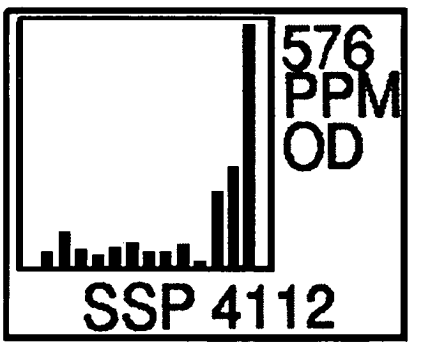
576
PPM
OD
SSP 4112

Boolean sets of proteins:
switched-off in EFC-1, on in 2C *and* off in 2C, on in 4C
switched-off in 2C, on in 2D *and* on in 2C off in 2A
switched-off in 8C ,on in 8D *and* on in 8C, off in 8D
switched-on in 4D, off in 4A *and* off in 4D, on in 4A

Table 5.8. Control questions posed at intervals during the analysis to check the efficacy of the Boolean sets. The expected answer in each case is zero since the sets in each example are mutually exclusive. The correct answer was given in each case. 2-dimensional protein gels of undifferentiated ES cells (EFC-1), 2, 4, 6 & 8 day untreated control (C), DMSO (D) and atRA (A) treated embryoid bodies were compared.

5.2.5. Identification of β-tubulin by Western blotting

The estimation of a value for the Mr and pI of each protein-spot on the standard map, should in theory enable the identification of proteins after comparison of these values with those of other 2D-PAGE databases. However, a variety of factors affect the resolution of proteins in either dimension during electrophoresis, such as electrophoresis parameters and IEF gel equilibriation. Due to such variation identification of individual proteins by inter-laboratory comparisons of 2-dimensional protein databases, can be difficult and may be unreliable.

Despite these drawbacks, attempts were made to identify developmentally regulated protein-spots, by visual comparison of the gels in this matchset with those of 6.5 and 7.5 day p.c. murine embryos (K. Latham, Temple University School of Medicine, Philadelphia, pers. com.). Although it is difficult to accurately identify the counterpart of any given protein-spot on other gels, this sort of comparison could be useful for determining which spots may be of interest for further study. Spot 2303, for instance, is thought to be a protein whose expression is elevated in endoderm relative to ectoderm; spot 1305 was identified as being in the region of proteins that have transiently elevated expression at the 2-cell stage; and spot 5505 could be a protein that is highly expressed in 6.5 day embryos and in ectoderm.

In addition, spot 2606 was thought to have a mobility pattern characteristic of β-tubulin. In order to ascertain this, Western blot analysis was carried out. The highest PDU value for spot 2606 was observed in 4 day atRA treated embryoid bodies while the lowest was in 8 day untreated embryoid bodies. In order to be able to observe the difference in expression

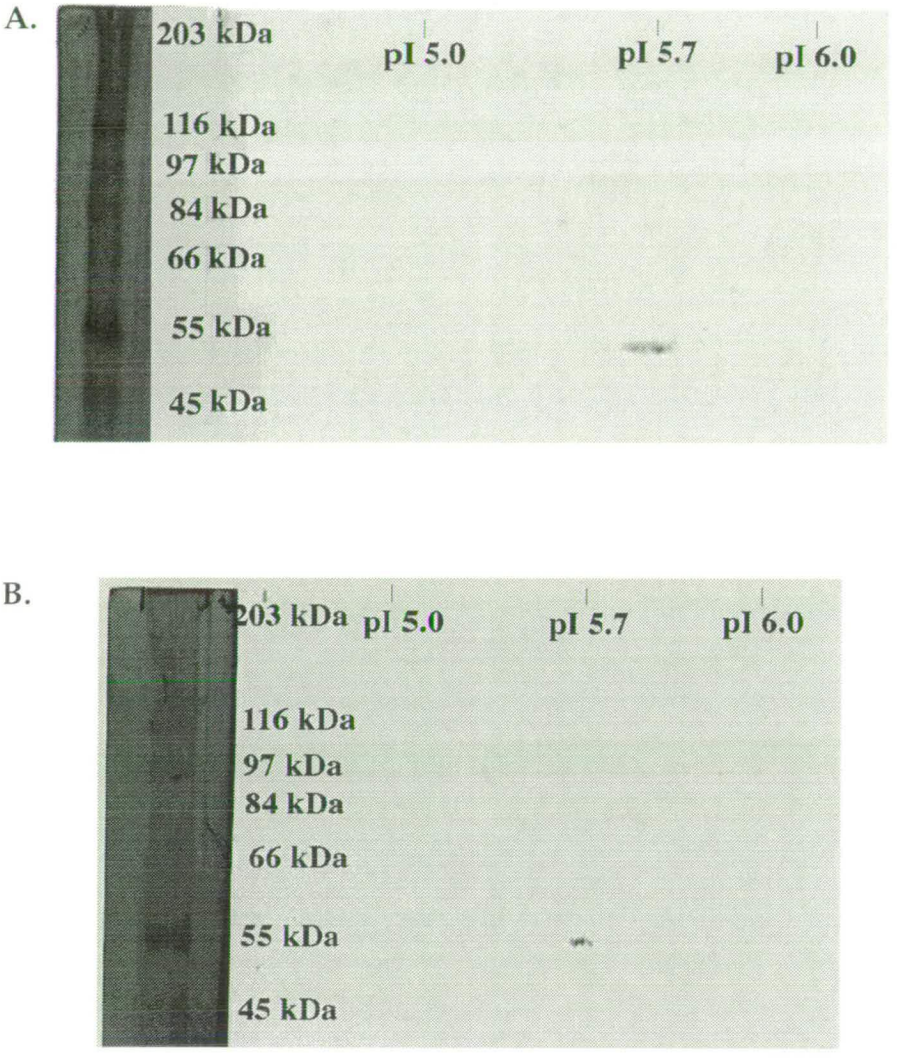

Figure 5.5. Western blot analysis of β-tubulin. 0.5mg of protein extract from 4 day atRA treated (A) and 8 day untreated (B) embryoid bodies were electrophoresed in two dimensions and western blotted onto a PVDF membranes. The membranes were then probed with monoclonal antibody against β-tubulin. The spots detected have a pI of approximately 5.7 and molecular weight of 55 kDa.

levels, 0.5mg of protein extracts from these two samples were resolved on 2D-PAGE gels and Western blotted onto PVDF membranes. The membranes were probed with a mouse IgG monoclonal anti-β-tubulin antibody. The secondary antibody against the mouse IgG was conjugated to horse radish peroxidase, enabling spot detection with a chromogenic reagent. A spot was detected with both 8 day untreated and 4 day atRA treated samples both of which were approximately 55 kDa in weight, the same Mr as that calculated for spot 2606 (Figure 5.5). However, the pI values were found to be more basic than that of spot 2606. Both spots were estimated to have a pI of approximately 5.7, spot 2606 having an estimated pI of 4.8. β-tubulin is known to have different isoforms with varying pI values. Although the spots detected by Western blot analysis had different pI to that of spot 2606, since the same spot was detected in both samples with a monoclonal-antibody specific for β-tubulin, and the spot was found to have the same Mr as spot 2606, it is very likely that this represents a β-tubulin isoform. This experiment does not conclusively identify spot 2606 as β-tubulin. Further immunological experiments would be required with another β-tubulin specific antibody to ascertain this.

5.3 Discussion

The molecular mechanisms controlling and contributing to the haematopoietic commitment and differentiation of ES cells are poorly understood. One of the first steps in deciphering this process is to identify genes which are either switched-on/off and/or up/down-regulated at various stages of differentiation. Differential cDNA library screening offers one means by which such genes can be identified. However as previously mentioned this method gives little indication as to whether the transcripts from which cDNA clones are derived are actually translated to functional protein. To overcome this, protein expression patterns in cells can be analysed directly; however, a means of screening for proteins of interest is necessary.

Since protein expression patterns change over a given period of differentiation, many groups have used such temporal changes to identify proteins that regulate such processes as embryogenesis (Latham *et al*, 1991; Shi *et al*, 1994). Alternatively, alterations in tissue culture conditions and exposure of cells to environmental stresses can also change protein expression patterns. Other groups have used such techniques as viral transformation and retinoic acid treatment as ways of assessing qualitative and quantitative changes in protein expression patterns, in comparisons with untreated samples (Celis and Olsen, 1994, Damjanov *et al*, 1993).

CFU-A assay studies have shown that embryoid body haematopoietic differentiation is a temporally regulated process (Hole *et al*, 1996). Furthermore as shown throughout the course of this study, this differentiation can be modulated by DMSO and atRA in diametrically opposing ways. Time and morphogen treatment were therefore used as means of screening for haematopoietic related proteins. L[^{35}S] methionine labelled protein extracts from undifferentiated ES cells, untreated embryoid bodies and those treated with either DMSO or atRA, over a time course of differentiation were analysed. Total protein extracts were resolved in 2-dimensions according to charge and molecular weight and the resulting 2D-PAGE gels subjected to analysis with the PDQuest™ software.

The analysis demonstrated that protein expression patterns changed over the time course of embryoid body differentiation and with respect to treatment. Both DMSO and atRA were found to switch-on/off and/or down/up-regulate protein expression (Tables 5.2, 5.3, 5.5 & 5.6). The ability of atRA to down-regulate or switch-off protein expression is particularly interesting, since retinoids and their receptors are most widely associated

with *trans*activation of gene expression, although they are also known to down-regulate the expression of a number of genes particularly metalloproteases (Nicholson *et al*, 1987).

Based on the opposing effects of DMSO and atRA on the CFU-A differentiation of embryoid bodies, two main criteria were defined for identifying proteins involved either in ES cell commitment to haematopoietic progenitors, or the differentiation of haematopoietic progenitors to terminally differentiated lineages (Figures 5.3a & b). However no proteins were found to fulfil either of these stringent criteria. This could be due to several reasons. For example, both criteria assume that atRA is inhibiting haematopoiesis, an assumption which may not be strictly true, since RT-PCR data have shown atRA treated embryoid bodies can express haematopoietic marker transcripts, (Table 4.2). This could be taken into account for the outlining of more detailed, criteria at the expense of creating complicated sets which are difficult to decipher. Also to incorporate this into the model for selecting proteins of interest it would be necessary to know the manner in which atRA was affecting embryoid body differentiation.

Not all proteins present in a cell will be represented on a given 2D-PAGE gel, since most but not all mature proteins contain methionine residues, unlabelled proteins will consequently not be represented on gels. PDU values may also not be representative of actual protein expression levels, since some proteins may be highly expressed but have few methionine residues, or proteins which are methionine rich may be expressed at low levels. These shortfalls could be overcome by using more than one radiolabelled amino acid residue in labelling experiments. Another drawback with 2D-PAGE analysis is that it may not be sensitive enough to detect proteins which are expressed at low levels, but which are key regulators of commitment and differentiation, such as transcription factors. Labelling with more than one radiolabelled amino acid would also aid the detection of such proteins on gels. An alternative way of representing the total protein content of a cell is by silver staining, as used by Burggraf and Lottspeich (1995), for the construction of 2D-PAGE protein databases from various human cell lines. However once again the technique has similar limitations to radiolabelling since some proteins will silver stain more easily than others.

Despite the absence of proteins which fulfilled all the the criteria outlined in Figures 5.3a & b, some proteins were found to satisfy aspects of these criteria, either with respect to their expression patterns across the time course of differentiation, and/or the way in which their expression was affected by either DMSO or atRA treatment (Table 5.4; Figures 5.1 & 5.4). For instance spot 3109 could be indicative of a protein involved in the

early stages of ES cell commitment to haematopoietic lineages, since it was not expressed in undifferentiated ES cells, but was expressed in 2 day untreated and DMSO treated embryoid bodies, and thereafter down-regulated over the time course. Spot 2110 however could be representative of a protein involved in the differentiation of committed haematopoietic progenitors. This protein was not expressed in undifferentiated ES cells, but was switched-on in differentiating embryoid bodies and up-regulated throughout the time course of both untreated and DMSO treated samples, while it was only expressed in 2 day atRA treated embryoid bodies.

In this experiment only 2D-PAGE gels of embryoid bodies treated with DMSO or atRA for the first 48 hours of differentiation were analysed. For further 'fine-tuning' of Boolean sets for the identification of haematopoietic related proteins, it would be useful to produce 2D-PAGE gels from other types of experiments. For example timed morphogen exposure experiments, outlined in Figures 3.3, 3.7 & 3.8, would also be useful sources of information. Since atRA appears to have little effect on the CFU-A differentiation of embryoid bodies after 72 hours (Figure 3.8), proteins related to CFU-A differentiation should in theory be switched-off during the 1st, 2nd and 3rd day of atRA exposure, but switched-on thereafter. Furthermore, timed DMSO exposure experiments suggest that DMSO acts in a cumulative fashion (Figure 3.3b), and therefore proteins involved in haematopoietic differentiation could also be up-regulated as embryoid bodies were exposed to DMSO for longer periods of time.

As previously mentioned, identification of specific protein spots by inter-laboratory comparisons of 2D-PAGE gels are not particularly practical or reliable for various reasons. There are many factors which can influence the resolution of proteins in either gel direction (Duncan and Hershey, 1984). Although most laboratories follow standard protocol procedures, specific practices can alter the outcome of 2-dimensional gels. For instance ampholytes from different manufacturers, and different gel electrophoresis times between laboratories, will affect the resolution of the gels. Thus a protein such as actin may have an apparent pI of 5.4 under certain electrophoresis conditions but a pI of 5.5 in others. In addition to this the 2D-gel protein-pattern obtained from different cell types will inevitably differ, making comparisons and matching of spots difficult. The specific post-translational modification that a given protein may be subjected to in different cell types could also vary, making its specific identification difficult. Nevertheless, attempts were made to compare the gels in the present study with those of other databases, particularly those from day 6.5 and 7.5 p.c. murine embryos. Consequently protein-spots

such as 2303 and 5505 were suggested to be developmentally regulated (K. Latham, Temple University School of Medicine, Philadelphia, pers. com.).

The mobility pattern of spot 2606 was also thought to resemble that of β-tubulin. To confirm this, Western blots of 2D-gels of 8 day untreated and 4 day atRA treated embryoid bodies were probed with a mouse monoclonal antibody against β-tubulin. These samples were chosen since spot 2606 was found to have the lowest and highest PDU values in these samples respectively. A spot of approximately 55 kDa was observed with both samples, which corresponds to the molecular weight estimated for spot 2606 (Table 5.4; Figure 5.4). However the pI of the spot on both membranes was found to be approximately 5.7, more basic than that estimated for spot 2606. However, β-tubulin has been shown to have many isoforms with different pI values (Linhartová *et al*, 1993). This experiment therefore does not determine whether spot 2606 is β-tubulin. But the fact that a monoclonal antibody was used, together with the detection of the same spot in both samples strongly suggests that the antibody may be detecting a particular isoform of β-tubulin. The use of another antibody specific for β-tubulin or radio-immunoprecipitation should help clarify this question.

It is interesting to note that the expression of the protein spot thought to be β-tubulin is up-regulated after atRA treatment, in comparison with either untreated or DMSO treated samples (Table 5.4; Figure 5.4). Studies with atRA have shown that it induces ES cells to differentiate towards neural lineages (Wobus *et al*, 1994; Bain *et al*, 1995). Since class III β-tubulin is specifically expressed in neural tissue, it would be interesting to use a monoclonal antibody against this particular isoform in order to test whether it would bind to spot 2606. Spot 0305 has also been suggested to be a tropomyosin isoform, however further studies of this protein spot could not be carried out due to the unavailability of the antibody.

Recently Bowden *et al* (1996) have published 2D-PAGE gels of two ES cell lines, different from the cell line analysed here. Although their database does not include differentiated embryoid bodies, it would nevertheless be useful to compare these gels with those from this study for the purposes of identifying specific proteins. Ultimately, the only way of identifying specific proteins is by physical means as opposed to comparative methods. One way of achieving this is by physical removal of the spot of interest from the gel, cleavage of the protein by enzymatic digestion or alkylation, and N-terminal sequencing of the fragments. These amino acid sequences could then be checked against protein sequence databases and homologous or identical sequences distinguished (Merrick *et al*, 1994).

A more rapid method of identifying proteins from 2D-PAGE gels has been developed in recent years which not only eliminates the requirement for N-terminal sequencing but also requires only picomolar amounts of protein. This method involves the tryptic digestion of protein spots either after removal from gels or directly on a Western blot membrane. The masses of the peptide fragments generated are then determined by mass spectrometry. Various computer programs have been developed which search protein databases and match the peptides derived, with those obtained from a given protein under similar cleavage conditions. A number of groups are using this new technology for identifying proteins from 2D-PAGE gels (Henzel *et al*, 1993; Ji *et al*, 1994; Arnott *et al*, 1996). This technology coupled with the use of DMSO and atRA as a means of screening, is a potentially efficient method of identifying proteins involved in the commitment and differentiation of ES cells to haematopoietic lineages.

Post-translational modification is also known to play an important part in regulating protein function (Howlett, 1986). DMSO has been shown to release intracellular calcium stores (Morley and Whitfield, 1993), one function of which is the activation of protein kinases and phosphatases that modify other proteins, either activating them or sequestering their actions. Using ^{32}P-labelling of proteins it would be valuable to determine to what extent DMSO influences the phosphorylation or dephosphorylation of embryoid body proteins. Other modifications to the present 2D-PAGE studies may include the extraction and analysis of specific protein fractions such as the cell-membrane, cytosolic and nuclear fractions. This kind of analysis can provide a much more detailed profile of protein expression patterns.

5.4 Summary

Studies of 2D-PAGE gels of untreated, DMSO or atRA treated embryoid bodies, have shown that protein expression patterns can be regulated both temporally and with respect to treatment. Following the observations made with CFU-A assays of untreated and morphogen treated embryoid bodies, two main criteria were outlined for the identification of haematopoietically related proteins. However no proteins were identified which fulfilled these stringent criteria. Since some proteins satisfied certain aspects of the specifications and these have been designated for future studies. No specific proteins could be definitively identified, at this level of analysis, but spots 2606 and 0305 have been suggested to be β-tubulin and tropomyosin respectively. These studies have therefore demonstrated that the opposing effects of DMSO and atRA on embryoid body CFU-A differentiation can be used to screen for proteins that may be involved in either the haematopoietic commitment of ES cells or differentiation of committed haematopoietic progenitors.

6. Summary of Conclusions.

The intricate cellular and molecular events which control and contribute to haematopoietic commitment and differentiation, during embryogenesis and adult life, remain largely elusive. Despite the difficulties in studying such a complex process, a variety of different techniques have been employed in an attempt to unravel the complexity of this fundamental biological process, some of which have already been discussed in Chapter 1. In recent years ES cell lines have in particular proved an important and productive *in vitro* source for studying haematopoiesis. One of the limiting steps in understanding the mechanisms of haematopoiesis has been the identification of genes involved in directing and controling the process. Using ES cells and their differentiated aggregates, the main aim and proposal of this project was to devise a strategy for identifying genes that could be involved in the very early stages of haematopoietic commitment and differentiation.

Using the CFU-A assay as a means of studying the haematopoietic potential of embryoid bodies, it was found that in the absence of cytokines or stromal cell lines in the culture system, approximately 30-40% of embryoid bodies could give rise to CFU-A colonies. For the purposes of devising a method of screening for genes involved in haematopoiesis, to the exclusion of the much larger pool of unrelated genes, it was deemed desirable to alter and modulate this level of haematopoietic commitment. This was achieved by the addition of chemical inducers, namely DMSO and atRA, known to affect the differentiation of not only ES and EC cell lines but also several leukaemic cell lines *in vitro* and *in vivo*.

It was consequently found that exposure to 1% DMSO during the first 48 hours of differentiation increased the number of embryoid bodies giving rise to CFU-A colonies by 2 fold. Assays of single cell suspensions derived from DMSO treated embryoid bodies were also found to produce twice as many CFU-A colonies as their untreated counterparts. These findings were furthermore found to be reproducible. This together with the fact that no significant difference between the colony sizes of DMSO treated and untreated embryoid bodies was observed, indicate that DMSO is acting to enhance haematopoietic commitment as opposed to the treatment causing an expansion of committed cell lines. RT-PCR studies furthermore demonstrated that the expression of various haematopoietic related genes, such as foetal β-globin, PU.1 and CD45, was elevated in DMSO treated embryoid bodies.

Conversely it was found that treatment with atRA during the first 48 hours of differentiation markedly reduced the number of CFU-A colonies from both intact and disrupted embryoid bodies. These results were once again found to be highly reproducible. This effect however, was only found to be exerted within a specific time frame. The drug had the described effect when embryoid bodies were cultured in its presence for longer than 16 hours, and appeared to have little or no effect beyond 72 hours of differentiation. Whether the absence of CFU-A colonies is due to haematopoietic inhibition by atRA however remains elusive. The embryoid bodies continue to express the mesoderm marker *brachyury*, along with haematopoietic related genes foetal β-globin and GATA-1. It would be interesting to use other colony forming unit assays such as BFU-E (erythroid burst forming unit), to determine whether atRA treated embryoid bodies are able to produce haematopoietic colonies under different assay conditions.

As discussed in Chapter 3, several groups have recently reported atRA to be an inducer of neural lineages from ES cell lines (Wobus *et al*, 1994; Bain *et al*, 1995). Consequently the absence of CFU-A colonies within the specified time frame could be due to the induction of neural lineages and not the direct inhibition of haematopoiesis. Alternatively atRA could be acting on a specific cell type, possibly a very primitive haematopoietic cell type, (such as those recently described by Kennedy *et al* (1997)), present within the 16 to 72 hour time frame which is atRA sensitive, but after further differentiation becomes more committed and is rendered atRA insensitive.

This hypothesis is corroborated by the finding that reconstitution of haematopoietically compromised hosts with ES derived progeny, is best achieved by embryoid bodies that have been allowed to differentiate for no longer than 4 days (Hole *et al*, 1996). Furthermore various workers have shown that atRA does inhibit differentiation of primitive haematopoietic cells from the bone marrow. Whatever the manner in which atRA is acting to produce the results reported, it is evident from the marked reduction in the number CFU-A colonies that it is clearly affecting ES cell haematopoietic differentiation at some level, implying that the expression of haematopoietic related genes is also affected, this being confirmed to some extent by the RT-PCR experiments (Chapter 4).

Having modulated the level of embryoid body haematopoiesis by the use of exogenous factors, which was the initial aim of the project, the findings could then be used for the purposes of screening for haematopoietic related genes. By exploiting the opposing effects of DMSO and atRA several techniques for screening of genes were feasable. The method

adopted upon consideration was that of 2-dimensional protein gel analysis. As discussed in Chapter 5, the technique provides both qualitative and quantitative measurements of protein expression, and has been utilised by various groups for differential protein expression studies in a range of biological systems. Since embryoid body haematopoietic differentiation was found to be affected both temporally and by the culture conditions used, this method was deemed apt as a means of screening for proteins which could potentially be involved not only in the early stages of haematopoietic commitment but also in the differentiation of progenitor cells.

Various criteria based on the results of the tissue culture studies were therefore defined. Several proteins were found to satisfy particular aspects of these criteria, such as being up-regulated or switched-on by DMSO or down-regulated or switched-off by atRA, and therefore their physical parameters were determined. The limitation with the use of 2-dimensional protein gels however is the actual identification of the proteins of interest. Antibodies for proteins known to be involved in haematopoiesis could be used for immunoprecipitation experiments, however for the purposes of identifying novel proteins one of two options are available. N-terminal sequencing of proteins isolated from acrylamide gels is one option, however this is somewhat time consuming and slow. The technique which is currently being developed by various workers and which would be more suitable for large screening projects, involves the enzymatic digestion of protein of interest followed by analysis of the fragments by mass spectrometry. Although these options could not be explored in this study through lack of time and resources, the protein database created, represent a rich source of information and possibilities for future work.

One of the disadvantages with using 2-dimensional protein gels is that some proteins which may play key roles in haematopoiesis, but which are expressed at low levels, such as transcription factors, may not be detected. Another option therefore which is currently being explored in conjunction with the Beatson Institute in Glasgow is the construction of cDNA libraries from DMSO and atRA treated embryoid bodies which could then be used for differential library screening. This technique has already been used for generating a large number of clones which are differentially expressed between day 3 and 5 embryoid bodies. Using these temporal variations alongside the opposing effects of DMSO and atRA an efficient screening method could be devised for the identification of haematopoietic related genes.

The main proposals and intentions of the project have been successfuly addressed. The basic level of embryoid body haematopoiesis, as detected by the CFU-A assay, was

modulated with the use of chemical induction, and these results were then employed to devise strategies for screening of haematopoietic related genes/proteins. However, some questions concerning the exact nature of the way in which the chemicals are affecting haematopoiesis, or the exact cell lineage being affected remain unanswered.

The data presented in the thesis highlight one of the main disadvantages of using potentially pleiotropic inducers such as DMSO and atRA and although much is now known about the signalling mechanisms of retinoids, the method of action of DMSO remains largely elusive. Regulation of the level of embryoid body haematopoiesis with cytokines has also been explored. However, the use of sera in the culture media could at least theoretically distort the effects of these, thus requiring the same clarifications about the exact nature of any observable effects, as are demanded with the use of chemical inducers.

Many possibilities and avenues are presented by the results obtained and the techniques explored. The use of DMSO and atRA in assisting the isolation and identification of either novel cDNA clones or proteins involved in haematopoieis can, not only contribute to understanding the key events of the process *in vitro*, and with the use of the reconstitution experiments *in vivo*; but also in answering the question of how these chemicals regulate the complex cascade of biological events that control haematopoiesis.

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Homeyra Doostdar-Smith for everything.

Appendices

Appendix I - Abbreviations.

AGM	-	Aorta, gonads and mesonephros
AMV	-	Avian myeloblastosis virus
atRA	-	all *trans*-retinoic acid
bFGF	-	Basic fibroblast growth factor
BSA	-	Bovine serum albumin
cDNA	-	Complementary DNA
13cRA	-	13 - *cis* retinoic acid
CFU-A	-	Colony forming unit assay
CFU-S	-	Colony forming unit-spleen
cm	-	Centimetre
CPM	-	Counts per minute
CSF - 1	-	Colony stimulating factor - 1
dATP	-	2'-deoxyadenosine 5'-triphosphate
dCTP	-	2'-deoxycytidine 5'-triphosphate
[α-^{32}P]- dCTP	-	dCTP labelled with 32-phosphorous at the α–position
DEPC	-	Diethyl pyrocarbonate
DMSO	-	Dimethyl sulphoxide
D-MEM	-	Dulbecco - MEM
DNA	-	Deoxyribonucleic acid
dGTP	-	2'-deoxyguanosine 5'-triphosphate
dNTP	-	2'-deoxynucleotide 5'-triphosphate
DTT	-	Dithiothreitol
dTTP	-	2'-deoxythymidine 5'-triphophate
EB/EBs	-	Embryoid body/Embryoid bodies
EDTA	-	Ethylenediaminetetra - acetic acid
ES	-	Embryonic stem (cell)
FCS	-	Foetal calf serum
g	-	Relative centrifugal force
GM - CSF	-	Granulocyte/macrophage - colony stimulating factor
G-MEM	-	Glasgow - MEM
HRP	-	Horseradish peroxidase
HCP	-	Haematopoietic cell phosphotase
HSC/HSCs	-	Haematopoietic stem cell/haematopoietic stem cells

ICM		Inner cell mass
IEF	-	Isoelectric focusing
kDa	-	kilo-Dalton
L-[^{35}S]-methionine	-	L- methionine labelled with35-sulphur
LD	-	Lethal Dose
LIF	-	Leukaemia inhibitory factor
MAP	-	Mitogen activated protein
MEM	-	Minimal essential medium
MIP	-	Macrophage inhibitory protein
M	-	Molar
mM	-	Milli-molar
µM	-	Micro-molar
µCi	-	MicroCurie
µl	-	Microlitre
mCi	-	MilliCuries
ml	-	Millilitre(s)
MOPS	-	4 - Morpholinepropanesulphonic acid
ng	-	Nanograms
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
p.c.	-	Post coitum
PCR	-	Polymerase chain reaction
PDU	-	Pixel density unit
PGCs	-	Primordial germ cells
p.p.m	-	Parts per million
PVDF	-	Polyvinylidene difluoride
RNA	-	Ribonucleic acid
RT-PCR	-	Reverse transcript-PCR
SCF	-	Stem cell factor
s.d.	-	Standard deviation
SDS	-	Sodium dodecyl sulphate
SSC	-	Sodium sulphate/Sodium citrate
TBE	-	Tris/boric acid/EDTA electrophoresis buffer
TE	-	10mM Tris (pH7.5)/0.1mM EDTA
TEMED	-	NNN'N'- Tetramethylethylenediamine
Tris	-	Tris (hydroxymethyl)-methylamine
TVP	-	Trypsin/varsene (EDTA)/PBS

Appendix II - Suppliers and Addresses.

Aldrich Chemical Company, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL, UK.

Amersham, Buckinghamshire, UK.

Advanced Protein Products Ltd, West Midlands, UK.

Biotecs, Bournmouth, UK.

Bioline, 16 The Edge Business Centre, Humber Road, London, NW2 6EW, UK.

Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD, UK.

BOC Gases, Leith, Edinburgh, UK.

Boehringer Mannheim, Bell Lane, Lewes, East Sussex, BN7 1LG, UK.

Cruachem, Glasgow, UK.

Difco, East Molesey, Surrey, UK.

Fisons, Bishops Meadow Road, Loughborough, Leics, LE11 0RG, UK.

Gibco BRL, Life Technologies Ltd., Trident House, P.O. Box 35, Renfrew Road, Paisley, PA3 4AF, UK.

GlobePharm Ltd., P.O. Box 89c, Esher, Surrey, KT19 9MD, UK.

Law Hospital, Lanarkshire, UK.

Merck-BDH, Merck House, Poole, Dorset, BH15 1TD, UK.

Oxoid, Basingstoke, Hants, UK.

Pharmacia Biotech AB, Uppsala, Sweden.

Promega UK Ltd., Delta House, Chilworth Research Centre, Southampton, SO16 7NS, UK.

Sigma, Fancy Road, Poole, Dorset, BH17 7NH, UK.