Gene expression in early haematopoietic development.

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"We are each at a point of our own transformation."

(Opus d.13; 4/1994)
ALAN DAVIE, painter *1920

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ABSTRACT

The developmental origin of haematopoietic stem cells (HSCs) has been the focus of much interest because of their biological and clinical significance. Although our understanding of haematopoietic development has been advanced by the identification of an intraembryonic source of HSCs, the molecular events that control development, self-renewal and commitment of HSCs remain to be determined. The rarity of HSCs *in vivo*, their incompletely defined phenotype, their refractoriness to transgenesis, and the importance of understanding HSC biology in relation to the haematopoietic microenvironment, make purification of these cells difficult. Alternative approaches to their study have been sought. The study of murine embryonic stem (ES) cells is one such approach. Under appropriate culture conditions, ES cells will spontaneously differentiate *in vitro* into a range of embryonic lineages, including the haematopoietic lineage. All haematopoietic lineages can result from ES cell differentiation including transplantable HSCs. The relative ease of transgenesis in ES cells has been exploited for the analysis of gene function and the identification of novel genes, by the use of gene targeting and gene trapping methodology, respectively. Conventional analysis of mutated ES cells is based on the production of chimeric embryos for *in vivo* studies. However, *in vitro* differentiation of mutated ES cells can provide an alternative and complementary approach to *in vivo* analysis. In particular an *in vitro* strategy for the screening of ES cell gene trap libraries could restrict the number of gene trap clones to be analyzed subsequently in chimeric embryos *in vivo*.

Based on an established ES cell system for *in vitro* haematopoiesis, part of the present project has been the assessment of an *in vitro* prescreening strategy of a gene trap library for the identification of genes that may be involved in early haematopoietic differentiation. This was achieved by monitoring the temporal expression of a ß-gal reporter gene in established gene trap lines after induction of haematopoietic differentiation by a morphogenic factor. The gene trap cell lines for use in this study were selected on the basis of their spatial expression patterns in chimeric embryos. The potential application of this strategy on a large scale has been tested by the simplification of the culture procedures that support haematopoietic differentiation.

The integration of gene trap constructs into the ES cell genome facilitates the identification of the trapped endogenous gene but also allows the use of *in situ* hybridization for the analysis of reporter gene expression. Spatial analysis of the co-ordinate expression of

reporter gene and haematopoietic marker genes e.g. globin, was attempted as an alternative *in vitro* screening strategy for the ES cell gene trap library, to that outlined above. The importance of the haematopoietic microenvironment is well accepted and temporal gene expression during haematopoietic differentiation of EBs has been extensively studied. However, these studies give no information about the spatial relationship of haematopoietic and microenvironmental cells expressing specific transcripts. Spatial expression of haematopoietic marker genes during the time course of haematopoietic differentiation of ES cells has been investigated by whole-mount *in situ* hybridization and *in situ* hybridization of sectioned EBs. In the first instance whole-mount *in situ* hybridization was combined with colorimetric signal detection. Data from these experiments demonstrated the need to obtain a more detailed picture of gene expression patterns. The attempt to take advantage of non-invasive optical sectioning by combining whole-mount *in situ* hybridization with fluorescent signal detection by confocal laser scanning microscopy was not successful but *in situ* hybridization of wax-sectioned EBs with haematopoietic marker genes did reveal details of spatial gene expression patterns at a cellular level. Furthermore, the opposing effects of DMSO and RA on haematopoietic differentiation were analyzed at the cellular level by investigating their effects on globin expression.

The present study demonstrates the potential of *in vitro* differentiation combined with exposure to haematopoiesis inducing morphogenic factors as an *in vitro* prescreening strategy for gene trap cell lines, that should allow one to identify 'haematopoietic' trapped genes. The spatial analysis of gene expression in haematopoietic EBs clearly illustrates the importance of co-ordinate gene expression analysis for the reconstruction of the molecular sequence of expression which accompanies haematopoietic differentiation.

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ABBREVIATIONS

A	Absorbance
AGM	Aorta-gonad-mesonephros
AP	Alkaline phosphatase
APAAP	Alkaline phosphatase anti-alkaline phosphatase
BBR	Boehringer blocking reagents
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bHLH	basic helix-loop-helix
bp	base pairs
cDNA	complementary DNA
CFC	Colony-forming cell
CFU-A	Colony-forming unit A
CFU-C	Colony-forming unit culture
CFU-G	Colony-forming unit granulocyte
CFU-GM	Colony-forming unit granulocyte/macrophage
CFU-S	Colony-forming unit spleen
Cl	Chlorine
cm	centimetre
CSF	Colony-stimulating factor
DAB	Diamino benzidine
DEPC	Diethylpyrocarbonate
DIA	Differentiation inhibitory activity
DIF	Differentiation medium
DIG	Digoxigenin
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acids
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide-5'-triphosphate
dpc	days post coitum
EBs	Embryoid bodies
EC cells	Embryonal carcinoma cells
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetra-acetic acid

Epo	Erythropoietin
EpoR	Erythropoietin-receptor
ES cells	Embryonic stem cells
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FL	Fetal liver
5-FU	5-Fluorouracil
ß-gal	ß-Galactosidase
g	grams
G-CSF	Granulocyte colony-stimulating factor
G-CSF-R	Granulocyte colony-stimulating factor-receptor
GFP	Green fluorescence protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GM-CSF-R	Granulocyte-macrophage colony-stimulating factor receptor
GMEM	Glasgow's modified Eagle's medium
h	hours
HPP-CFC	High proliferative potential colony-forming cell
HSCs	Haematopoietic stem cells
ICM	Inner cell mass
IgM	Immunoglobulin M
IL	Interleukin
IL-2R	Interleukin-2-receptor
JAK	Janus-associated kinase
K	Potassium
kb	kilobases
l	litre
LCR	Locus control region
Li	Lithium
LIF	Leukemia inhibitory factor
LIF-R	Leukemia inhibitory factor-receptor
Lin	Lineage specific marker
lo	Low
LSM	Laser scanning microscope

LTBMC	Long term bone marrow culture
LTC-IC	Long-term culture inititating cell
LTR	Long-term repopulating
M	Molar
μM	micro Molar
MAB	Malic acid buffer
M-CSF	Macrophage colony-stimulating factor
M-CSF-R	Macrophage colony-stimulating factor-receptor
MeOH	Methanol
Mg	Magnesium
MHC	Major histocompatibility complex
min	minutes
MIP-1	Macrophage inflammatory protein-1
ml	milli Litre
mM	milli Molar
mRNA	messenger RNA
NBT	4-nitroblue tetrazolium chloride
ng	nano grams
nm	nano metre
ONPG	o-nitrophenyl-ß-D-galactopyranoside
OSM	Oncostatin M
PBS	Phosphate buffered saline
pc	post coitum
PCR	Polymerase chain reaction
pg	pico grams
POD	Peroxidase
P-Sp	Paraaortic splanchnopleura
RA	Retinoic acid
RACE	Rapid amplification of cDNA ends
Rh	Rhodamine
RNA	Ribonucleic acids
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse-transcriptase-dependent PCR
SCF	Stem cell factor
SCL	Stem cell leukemia

sec	seconds
SMA	Single cell multipotential assay
ssDNA	sheared herring sperm DNA
TCR	T-cell receptor
TESPA	3-aminopropyl-triethoxysilane
TGF-ß1	Transforming growth factor-ß1
TNF	Tumor necrosis factor
TNF-R	Tumor necrosis factor-receptor
TVP	Trypsin solution
tRNA	transfer RNA
U	units
UV	ultraviolet
V	voltage
% (v/v)	ml per 100 ml
WGA	Wheat germ agglutinin
W locus	white spotting locus
% (w/v)	grams weight per 100 ml
YS	Yolk sac

1. INTRODUCTION

1.1 Development of mammalian embryos

During mammalian development three consecutive developmental stages are classified; the embryonic, the fetal and the adult stage, each of which expresses distinctive features. Whereas the embryonic and the fetal stages are temporary, the adult stage is characterized by specialized, terminally differentiated cells that maintain the organism throughout adult life.

Early post-implantation development proceeds by the establishment of the anterior-posterior body axis and the process of gastrulation, which generates the three definitive germ layers - the ectoderm, the mesoderm and the endoderm. The subsequent development of the organism depends on the interactions between the three germ layers, which are determined for different fates in the organization of the organism. The principal body plan of the mouse is established with these early events, and the later stages of embryogenesis are characterized mainly by organogenesis, growth and differentiation processes. As a general rule of development, the body plan is first formed in miniature and then maintained as the embryo grows (Gossler and Balling, 1992).

It is during the early stage of embryonic development where cells lose their individual totipotency and become determined for their fate, often long before overt differentiation occurs. As development proceeds cell-cell interactions become more complex and the cells differentiate in a precise pattern to a multicellular organism with the consequence that only few cells maintain pluripotent characteristics in the adult organism. In concert with the individual 'differentiation programme' of cells, cells change their pattern of gene expression during development. The major events of early mammalian development can thus be summarized as cell lineage specification, segmentation and regional specialization along the body axis. A detailed summary of mouse development has been described by Hogan et al. (1994).

Tissue maintenance and renewal in the adult body is essential and is performed by the interaction of a variety of cell types of each tissue. New differentiated cells can be produced during adult life either by the simple duplication of existing differentiated cells, which divide to give pairs of daughter cells of the same type, or they can be generated from relatively undifferentiated stem cells, by a process that involves a change of cell phenotype.

Stem cells, by general definition, are not terminally differentiated and have the ability to divide throughout the lifetime of the organism, yielding some progeny that differentiate to one of various lineages and others that remain stem cells. However, the assignment of stem cells is not to carry out the differentiated function but rather to produce cells that will. Consequently, stem cells often have a nondescript appearance, making them difficult to identify. Due to the inability of adult tissue to produce cells of such importance, the ontogeny and commitment of stem cells during embryogenesis is of particular interest. Somatic stem cells, e.g. epidermal stem cells, epithelium stem cells and haematopoietic stem cells, all play a critical role in the maintenance of organ systems in the adult mammal. One organ system in mammals that is studied intensively is the haematopoietic system with its self-renewal and differentiation properties.

1.2 Mammalian Haematopoiesis

1.2.1 The Haematopoietic System

The established mammalian haematopoietic system can be viewed as a dynamic hierarchy of pluripotent, committed and maturing cell populations continually differentiating into at least eight different cell lineages: lymphocytes, erythrocytes, platelets, macrophages, neutrophils, eosinophils, basophils and mast cells. The haematopoietic system can be considered as a spectrum of progressive differentiation and self renewal with terminally differentiated cells at one extreme and pluripotent haematopoietic stem cells (HSCs) at the other (Figure 1.1).

These cells are all located in a complex network of haematopoietic tissues.
The stem cells are the foundation of the haematopoietic system and are responsible for replacing terminally differentiated cells throughout the life span of the animal, since terminally differentiated cells within the haematopoietic system have finite life spans.
Much is known about the mature cell populations of the established haematopoietic system. However, little is known about the embryonic origin of the haematopoietic system and how or if the initiator cells of the embryonic blood system are related to those producing the adult haematopoietic system.
Haematopoietic activity during early development is derived from cells of the mesodermal germ layer formed during gastrulation. The commitment of mesoderm cells to haematopoiesis results in the restriction of their differentiation potential to haematopoietic lineages (Hogan et al, 1994).
The sequence of events leading from these committed mesoderm cells to the definitive haematopoietic system is a complex process and much is still a matter of debate. Furthermore, changing demands (spatial and temporal) of the developing embryo on the haematopoietic system during different developmental stages aggravate analysis. However, the understanding of haematopoiesis has significantly advanced in recent years by refined experimental technologies and the identification of an intraembryonic source of haematopoietic activity during mammalian embryogenesis (Godin et al, 1993; Medvinsky et al, 1993). Nevertheless, many questions remain to be answered. An overview of the developmental processes leading to the establishment of the murine haematopoietic system, of the spectrum of cells in the haematopoietic system, of the molecular control of haematopoiesis, and of methods and models for studying haematopoiesis is outlined in the following sections.

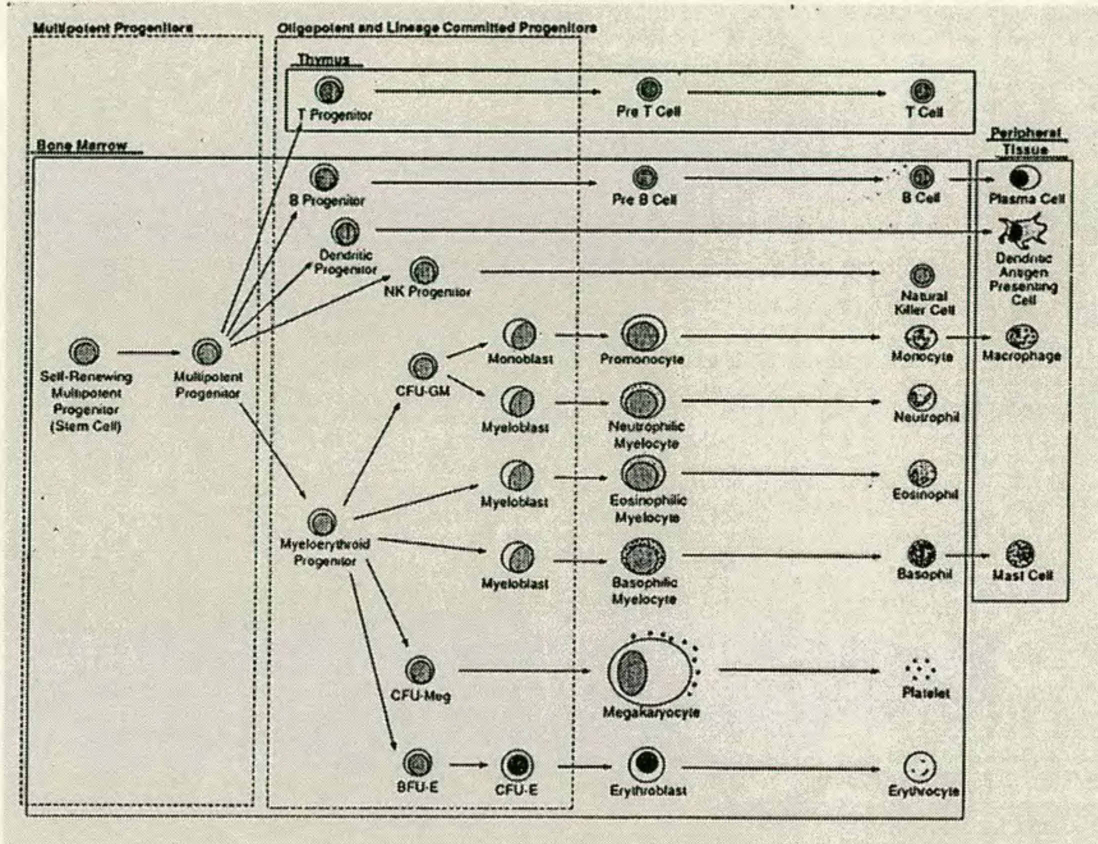

Figure 1.1: The hierarchical order of the haematopoietic system. A model of lineage commitment and differentiation from HSCs in relation to haematopoietic tissues (Morrison et al, 1995).

1.2.2 Ontogeny of the murine haematopoietic system

In the mouse, the earliest site where haematopoietic activity can be observed is the yolk sac at day 7 of gestation. Primitive erythrocytes, derived from extraembryonic mesodermal cells of the yolk sac form aggregates, termed blood islands, which are the first morphologically recognizable haematopoietic cells (Moore and Metcalf, 1970). Later at midgestation, haematopoiesis shifts into the embryo proper to the fetal liver, the thymus and the spleen, and shortly before birth, to the bone marrow. The fetal liver is the major source of haematopoietic activity in the embryo and the bone marrow becomes the major site of haematopoiesis during adult life (Johnson and Moore, 1975).

This sequence of haematopoietic events has long been considered as a reflection of the migration of pluripotent HSCs from the yolk sac to the sites of definitive haematopoiesis. However, evidence in support of this notion was circumstantial and not conclusive to answer questions challenging the concept of a singular origin of the haematopoietic system within the yolk sac.

Arguments in favour of the possibility of a more potent haematopoietic tissue within the mammalian embryo as a source of definitive HSCs were originally based on evolutionary and comparative studies of mammalian development to other vertebrate species (Zon, 1995). Similarities in mesoderm formation in many vertebrate species ranging from fish, amphibians and birds to the mouse have been well documented, suggesting valid comparisons of mammalian developmental haematopoiesis with that of non-mammalian vertebrates (Smith and Albano, 1993).

Non-mammalian animal models are well acknowledged as useful models for the understanding of initial events in mammalian development and controversy of the yolk sac concept in mammals for definitive haematopoiesis was strengthened by progress in evolutionary studies. It was argued that if definitive haematopoiesis in mammals originates in the yolk sac, mammals would be strikingly distinct from other vertebrate species. Furthermore, considering the conserved mechanisms for other

organ systems between different species e.g. the nervous system (Hogan et al, 1994), the mammalian haematopoietic system would be a remarkable exception from otherwise closely related organisms.

Developmental studies of haematopoiesis in non-mammalian vertebrates revealed two sites of haematopoietic activity during embryonic development, an extraembryonic and an intraembryonic site. The relative accessibility of avian and amphibian embryos during development enabled the use of experimental strategies which are not suitable for mammalian embryos because of their *in utero* development.

Grafting experiments in avian and amphibian embryos identified the yolk sac in birds and the ventral tissue, the yolk sac analogue in amphibians, as the source of primitive haematopoiesis, which is of transient nature, and an intraembryonic mesoderm derived region, containing the dorsal aorta, as the source of definitive haematopoiesis (Dieterlen-Lievre, 1975). Generation of chick-chick and chick-quail chimeras provided evidence that the initiation of definitive haematopoiesis exclusively arises from the dorsal aorta in avian embryos.

Grafting experiments in frog, *Xenopus*, revealed similar findings, with adult haematopoiesis arising essentially from an intraembryonic region and not the ventral blood islands, which are functionally and positionally equivalent to the mammalian yolk sac and the amphibian and avian counterparts (Tavassoli, 1991).

As a result of the restriction to functional assays for developmental studies in mammals, the origin of definitive haematopoiesis remained still obscure, long after the discovery of the haematopoietic origin in other vertebrate species.

However, recent developmental studies in the mouse, re-examining the possibility of an analogous intraembryonic source of HSCs indicate that similar to avians, mammals possess a potent haematopoietic tissue within the embryo that is responsible for the initiation of definitive haematopoiesis (Godin et al, 1993; Medvinsky et al, 1993). Using functional colony assays and transplantation assays, it was shown that the ontogenetic origin of definitive HSCs is more likely to lie in the intraembryonic site

of haematopoiesis, suggesting homologous migration of HSCs as known from other vertebrate species (Medvinsky and Dzierzak, 1996). The presence of two mesodermally derived haematopoietic regions functioning at pre-liver stages is suggestive that two different origins for HSCs exist, with two distinct classes of stem cells, that may or may not arise independently. Observations that early embryonic cells of the yolk sac are quite different from those differentiating from bone marrow cells, e.g. erythroid cells, led to the perception that an early embryonic (primitive) lineage is replaced by a later adult (definitive) lineage about the time fetal liver becomes a haematopoietic organ.

1.2.3 Anatomical sites of haematopoiesis

During mammalian development, several haematopoietic sites within the embryo are observed. Haematopoietic activity sequentially progresses during embryonic development from the yolk sac (Moore and Metcalf, 1970) and para-aortic mesoderm (Godin et al, 1993; Medvinsky et al, 1993) to fetal liver (Johnson and Moore, 1975), thymus and spleen (Moore and Owen, 1967), and bone marrow (Johnson and Moore, 1975), which becomes the major site of haematopoiesis during adult life. These haematopoietic tissues serve as generators and/or reservoirs of haematopoietic activity with each tissue displaying a characteristic composition of haematopoietic cell types, as each tissue contains a distinct population of blood cells (Morrison et al, 1995). The following sections summarize some of the characteristic features of the haematopoietic tissues involved in the establishment of definitive haematopoiesis in the bone marrow of adult mammals.

1.2.4 The yolk sac, extraembryonic site of haematopoiesis

The yolk sac forms during gastrulation, which begins at 6.5 days post coitum (dpc) in mouse development and is an extraembryonic tissue composed of endodermal

and mesodermal derivates. The endodermal cells of the yolk sac arise from primitive endoderm, whereas the mesoderm cells descend from primitive ectoderm which migrate beneath the visceral endoderm between 7 and 7.5 dpc (Hogan et al, 1994). The mesoderm of the visceral yolk sac is the first site within the developing embryo where morphologically discernible haematopoiesis in form of blood islands can be found (Moore and Metcalf, 1970).

Blood islands begin to form at around 7 dpc and are cell aggregates consisting of primitive nucleated erythrocytes surrounded by a loose network of endothelial cells (Tavassoli, 1991). As the blood island develops, the haematopoietic progenitors ultimately differentiate into erythroblasts surrounded by the endothelial cell layer and supported for growth by an endodermal cell layer (Zon, 1995).

The peripheral endothelial cells form the first vascular structures that surround the inner blood cells (reviewed by Wagner, 1980). The close developmental association of the haematopoietic and endothelial lineages within blood islands has led to the hypothesis that they arise from a common precursor the 'haemangioblast', since both lineages are mesodermal derivatives.

Support for the concept of the existence of a 'haemangioblast' comes from observations that the haematopoietic and endothelial cell lineages share expression of a number of different genes (Yamaguchi et al, 1993; Kallianpur et al, 1994; Young et al, 1995; Carmeliet et al, 1996).

Targeted mouse mutants affecting both the haematopoietic and vascular system are indicative for the existence of a common precursor. Mouse embryos homozygous for disruption of the *flk-1* gene, lacking a functional receptor tyrosine kinase Flk-1, die between 8-9.5 dpc and lack vascular cells and blood islands in the yolk sac (Shalaby et al, 1995; 1997). These findings are consistent with a primary role for *flk-1* in maintenance/formation of embryonic blood cell production, although it has been suggested that the haematopoietic defect may occur secondary to the failure to produce endothelial cells for a vascular microenvironment.

Another example are mice lacking the gene for transforming growth factor-ß1 (TGF-ß1). The yolk sac of homozygous TGF-ß1$^{-/-}$ embryos displays defective endothelial differentiation and a much reduced number of erythrocytes (Dickson et al, 1995). Further evidence, indicating a dependence of blood island haematopoiesis on the endothelial and endodermal cell layers comes from *in vitro* studies (Auerbach et al, 1996; Yoder et al, 1995).
Co-culturing experiments suggest that endothelial and endodermal cell lineages provide a supportive microenvironment for haematopoiesis and vasculogenesis. Murine bone marrow progenitors co-cultured with yolk sac endoderm derived cell lines (Yoder et al, 1995) or yolk sac HSCs with a yolk sac endothelial cell layer (Auerbach et al, 1996) showed increased haematopoietic growth and proliferative potential. Visceral yolk sac explants from 7.5 dpc can give rise to haematopoietic cells in the absence of the endodermal layer, in an *in vitro* culture system, but a failure in angiogenesis was observed (Palis et al, 1995). Although consistent with the concept of a putative common progenitor for haematopoietic and endothelial lineages, these observations can not prove its existence.

Yolk sac haematopoiesis in blood islands produces a limited range of haematopoietic cell lineages and consists almost exclusively of erythrocytes and some macrophages (Moore and Metcalf, 1970; Gordon et al, 1992). Embryonic erythrocytes are large in size, compared to their counterparts in definitive haematopoiesis, remain nucleated throughout their lifespan and express embryonic globins. In contrast, definitive haematopoiesis in fetal liver and bone marrow produces all cell types of the haematopoietic cell lineages, both lymphoid and myeloid, and the erythrocytes produced are enucleated and express adult type globins. Although primitive nucleated erythrocytes are the predominant cell type during primitive haematopoiesis, *in vitro* analysis showed that 7 dpc yolk sac progenitor cells can give rise to granulocytes and macrophages, as detected by colony-forming unit granulocyte/macrophage (CFU-GM)

analysis. The number of CFU-GM progenitors has been found to grow in direct proportion, as the yolk sac increases in cellularity from 8-9 dpc (Moore and Metcalf, 1970).

Similar to the morphological heterogeneity of primitive and definitive erythrocytes, it has been found that fetal macrophages show distinct ultrastructural characteristics depending on their developmental stage (reviewed by Naito et al, 1996).

Although primitive haematopoiesis and committed progenitors can be detected in the yolk sac as early as 7-8.5 dpc, haematopoietic stem cell activity in yolk sac is not detectable until late 9 dpc, after circulation is initiated (Müller et al, 1994). This time discrepancy, committed progenitors being present in the yolk sac before primitive progenitors with HSC activity are detectable, added controversy to the hypothesis that the yolk sac is the site where definitive haematopoiesis originates.

Experimental evidence which added to this debate was obtained from systematic analysis of the haematopoietic activity in yolk sac during mouse development (Medvinsky et al, 1993; Müller et al, 1994). Using the colony-forming unit-spleen (CFU-S) assay for the identification of early haematopoietic cells it was found that significant numbers of CFU-S progenitors are not detectable until late 9 dpc in yolk sac (Medvinsky et al, 1993) and long term repopulating (LTR) HSCs, as analyzed by haematopoietic reconstitution, can not be detected in the yolk sac until 11 dpc (Müller et al, 1994).

Reports of the presence of HSC at earlier stages than 11 dpc during development were diverse and not consistent from different groups. One group reported the presence of lymphoid precursors in 8-8.5 dpc yolk sac but not in the embryo proper by testing reconstitution of adult irradiated SCID mice or *in vitro* cultures on thymic endothelium for T cells or on fetal liver stromal cell lines for B cells (Palacios and Imhof, 1993). Others reported the detection of lymphoid and myeloid potential in both yolk sac and embryo proper at 9 dpc after *in vivo* and *in vitro* analysis (Huang and Auerbach, 1993).

A variety of experimental *in vitro* approaches, addressing the supposingly restricted differentiation potential of early haematopoietic yolk sac progenitors, found that culture requirements for *in vitro* differentiation of yolk sac progenitors differ for each haematopoietic lineage. For example, T cell differentiation of yolk sac progenitors was observed in fetal thymic organ culture; B cell differentiation by stromal co-culture assays; myeloid differentiation occurred best in a semisolid culture environment; and erythropoiesis occurred best in cultures provided with erythropoietin as the principal growth factor (reviewed by Auerbach et al, 1996). These conditional microenvironment requirements made it impossible to test individual cells for haematopoietic pluripotentiality prior to clonal proliferation, but allowed the characterization of different cell lineages derived from yolk sac progenitors. Despite the developmental potential of haematopoietic yolk sac progenitors *in vitro*, *in vivo* differentiation in yolk sac is mostly restricted to the erythroid lineage (Moore and Metcalf, 1970). The yolk sac becomes extensively vascularized and the extraembryonic circulation becomes directly linked to that of the embryo at 8.5 dpc (Cumano et al, 1996). With the connection to the intraembryonic sites established haematopoietic cells can circulate intravascularly between intraembryonic and extraembryonic sites. Haematopoiesis in the yolk sac continues until ~13 dpc when the yolk sac begins to degenerate. It is thought that yolk sac embryonic haematopoiesis is no longer required as other intraembryonic sites of haematopoiesis are established (Dzierzak and Medvinsky, 1995) and that haematopoiesis in yolk sac serves for the growth and survival of the early developing embryo.

1.2.5 Intraembryonic haematopoiesis

Based on the presence of an intraembryonic source of definitive haematopoiesis in non-mammalian vertebrates, investigations searching for HSC activity associated with analogous mammalian dorsal regions were performed (Godin et al, 1993; Medvinsky et al, 1993).

Analysis of the intraembryonic mesoderm in mouse identified the dorsal region of the mesoderm, which includes the splanchnic mesoderm, dorsal aorta, genital ridges/gonads and pro/mesonephros, surrounding mesenchyme and some intermediate mesoderm, as a source of definitive haematopoiesis. At early stages of gestation (8.5 dpc) this haematopoietic region consists of the caudal splanchnic mesoderm and the endoderm of the developing gut end and endothelium of arteries, termed as the paraaortic splanchnopleura (P-Sp) (Godin et al, 1993; 1995). At a slightly later stage 9 to 10.5 dpc, this region comprises the aorta, gonads and mesonephros, termed the aorta-gonad-mesonephros (AGM) region (Medvinsky et al, 1993). The AGM region is derived from the P-Sp and the relevant rudiments which have begun organogenesis. These intraembryonic haematopoietic sites (P-Sp and AGM) do not contain erythropoietic foci as observed in yolk sac and thus functional assays were used to identify their haematopoietic nature (Godin et al, 1993; Medvinsky et al, 1993). Using transplantation methodology and *in vivo* assays, the CFU-S assay and the long term repopulating HSC assay, the presence of definitive haematopoietic progenitors was detected (Godin et al, 1993; Medvinsky et al, 1993; Müller et al, 1994). Grafting intraembryonic splanchnopleura from 8.5 to 9 dpc (10 to 18 somites stage) mouse embryos under the kidney capsule of adult immuno deficient SCID mice and subsequent analysis of the host haematopoietic tissues showed the presence of donor derived lymphoid cells (Godin et al, 1993). Immunoglobulin M (IgM), IgM-secreting plasma cells and the B1a cell subset (CD5 expressing B cells) were detected, thus demonstrating the existence of B-cell progenitors in the P-Sp. However, B1a lymphocytes are a primitive subset of B-cells and no maturation was detectable.

Systematic examination of mouse embryos for CFU-S activity from 8 to 10.5 dpc was performed by Medvinsky et al (1993). In the CFU-S assay, the cells to be tested are injected into lethally irradiated recipients and macroscopic colonies on the surface of their spleen counted after 8-15 days (Till and McCulloch, 1966). The numbers of

CFU-S colonies present in a cell population are a readout for early haematopoietic progenitor cells and are widely used as a reliable mean to measure haematopoiesis. CFU-S progenitors are rather immature cells within the haematopoietic hierarchy of the adult mouse with high proliferative potential but represent a nonhomogenous population. Although CFU-S are not pluripotent HSCs, HSCs can be separated from CFU-S colonies (Jones et al, 1990), the development of the CFU-S compartment during ontogeny is thought to reflect crucial steps in the establishment of the definitive haematopoietic system.

Progenitor CFU-S activities in the AGM, yolk sac and fetal liver were compared for the production of *in vivo* macroscopic colonies on the spleen at 8 days (CFU-S_8) and 11 days (CFU-S_{11}) posttransplantation. Donor derived CFU-S_8 colonies, rather mature progenitors in the haematopoietic hierarchy, were detected from 9 dpc AGM regions, which is reported to be the earliest time point where the AGM region can be accurately dissected from surrounding tissues. The frequency of CFU-S_8 activity in AGM regions continued to rise to a maximum on day 10 of gestation, at a frequency equivalent to that found in the adult bone marrow, and then fell rapidly by 11 dpc. The decline in CFU-S_8 activity in the AGM region coincided with an increase in CFU-S_8 activity in fetal liver, where no detectable CFU-S_8 activity occurs until the end of 10 dpc.

Analysis for the presence of more primitive progenitors, CFU-S_{11}, showed that donor-derived CFU-S_{11} activity at statistically significant numbers can only be found in the AGM regions at day 10 of gestation. Significant CFU-S_{11} activity derived from yolk sac cells or fetal liver cells was detected only after 10 dpc.

To test whether cells of the AGM region possess definitive HSC activity and to determine the order of appearance of HSCs in the developing embryo, experiments testing for the long term repopulation potential of cells of the AGM region were performed (Müller et al, 1994). It was found that the AGM region at 10 dpc is the

first site within the developing embryo where cells with HSC activity, capable of complete haematopoietic multilineage long term reconstitution of a lethally irradiated adult recipient, are present. Serial secondary and tertiary transplantation showed consistent high level repopulation of all haematopoietic lineages and proved that AGM derived haematopoietic cells bear characteristics of adult bone marrow HSCs (Müller et al, 1994).

To further analyse the precise localization of the CFU-S activity in the AGM region of day 10 mouse embryos, dissection of the AGM region into their dorsal aorta and uro-genital ridges components was carried out (Medvinsky et al, 1996). This finer dissection of the AGM region showed the presence of CFU-S both around the dorsal aorta and in the uro-genital ridges but no significant differences in CFU-S activity was found.

Comparative morphological analysis of CFU-S colonies revealed a close resemblance between AGM-derived CFU-S colonies and the more definitive colonies from fetal liver, but not yolk sac derived CFU-S. However, difficulties in obtaining pure tissues from the AGM region at earlier stages were reported to restrict the analysis of the CFU-S distribution inside this region, thus the identification of the origin of the HSCs found in the AGM region remained unclear (Medvinsky et al, 1996).

In vitro experiments that analysed the differentiation potential of the P-Sp used a culture system that allows the proliferation of uncommitted precursors and their differentiation into the myeloid and lymphoid pathways (Godin et al, 1995).

This study suggested the simultaneous emergence of multipotent haematopoietic progenitors in the yolk sac and the P-Sp, beginning at 8.5 dpc. However, since a set of *in vitro* culture conditions was used for analysis, suitable to assess specific lineage differentiation but not multilineage differentiation potential, it remained unclear whether these cells possess self-renewal and long-term repopulating potential.

The identification of the haematopoietic pre-liver intraembryonic P-Sp/AGM region

in the mouse embryo was a major breakthrough for the study of mammalian haematopoietic development (Godin et al, 1993; Medvinsky et al, 1993). However, the site where definitive HSCs originate remained obscure since active interchange of cells between the yolk sac and the embryo proper via circulation could not be excluded. The blood connection between yolk sac and the embryo becomes established at the 8-somites stage (8.5 dpc), allowing haematopoietic precursors to pass through the circulation from one site to the other (Cumano et al, 1996). Although compatible when compared to the avian model for haematopoiesis, the observations of intraembryonic haematopoiesis in the mouse embryo were not conclusive, whether progenitors appeared in parallel in the AGM region and the yolk sac or whether the AGM region seeded the yolk sac or the opposite. To elucidate whether the AGM region is an intraembryonic site where HSC differentiate and proliferate on their migration to the developing fetal liver or whether it is an independent haematopoietic organ and source of HSCs further analysis was required.

By investigating the haematopoietic progenitors in the pre-circulation embryo two recent studies largely advanced the search for the origin of definitive HSCs (Cumano et al, 1996; Medvinsky et al, 1996). A series of experiments which directly addressed the lymphoid differentiation potential before circulation between the yolk sac and P-Sp were performed by Cumano et al (1996). Yolk sac and caudal P-Sp were dissected at different stages before and after the onset of circulation at 8.5 dpc and cultured intact in organotypic conditions *in vitro* for two days. The cells were then dispersed and expanded for further three days, at which time portions of each clone were assayed in separate *in vitro* cultures optimized for the growth of B cells, T cells and erythroid and myeloid cells. It was found that before the onset of circulation, all the lymphoid progenitors resided in the P-Sp region, but erythroid and myeloid progenitor cells resided in both embryonic tissues. The differentiation potential of the progenitor cells was not altered when yolk sac and P-Sp cells were mixed together during culture,

indicating that the limitation of yolk sac cells is intrinsic and may result from possible environmental influences acting earlier at precursor emergence. Lymphoid potential in the yolk sac was only observed following the onset of blood circulation between the yolk sac and the embryo body. The number of yolk sac derived myeloid and erythroid progenitors was lower and multilineage differentiation occurred less frequently compared with clones of P-Sp origin. The cytological and culture analyses of yolk sac and P-Sp cells were confirmed by gene expression patterns. Lymphoid specific transcripts e.g. RAG-1 were not detectable in yolk sac cells. In contrast, P-Sp derived cells expressed RAG-1 as well as B and T cell specific genes. HSCs activity as analyzed by CFU-S and LTR-HSC activity was not found in cultured cells from day 8.5 P-Sp or yolk sac. The failure to detect CFU-S and LTR-HSC activity was explained that very early progenitors might not be able to home and settle in the adult environment in which they were transferred. Previous reports had reported that CFU-S appeared in both intra- and extraembryonic locations only after the 27-somite stage (late 9.5 dpc) and LTR-HSC after 10 dpc (Müller et al, 1994, Medvinsky et al, 1996).

Another approach, using a novel *in vitro* organ culture system, re-examined the presence of CFU-S and LTR-HSCs in day 9 to day 11 AGM region, yolk sac and fetal liver (Medvinsky and Dzierzak, 1996). Explanted organs and tissues were cultured intact for 2-3 days in novel *in vitro* organ culture conditions, dissociated and then assayed for CFU-S and LTR-HSC activity by injection of cells into lethally irradiated mice. The additional organ culture step allowed to culture isolated embryonic tissues separately from other tissues eliminating the possibility of cellular exchange between haematopoietic sites but preserved the potentially important cellular microenvironment of the explant. Largely enhanced numbers of CFU-S activity and donor-derived long term reconstitution, compared to previously performed similar studies without organotypic culture (Medvinsky et al, 1993), were observed from

10 dpc and 11 dpc AGM regions but not 9 dpc. Since the haematopoietic progenitors within the AGM regions were unable to emigrate and disseminate, it was thought they accumulate *in situ* and surpass the numbers that can be observed in uncultured AGM region (Medvinsky et al, 1996). The delay of onset and low numbers of CFU-S activity in isolated cultured yolk sac and fetal liver was thought to strongly indicate autonomous and exclusive initiation of definitive haematopoiesis within the AGM region at 10 dpc. Also suggesting colonization of yolk sac and fetal liver by AGM generated LTR-HSCs and CFU-S progenitors, which would be much like the colonization of haematopoietic organs observed in avian embryos.

These observations of CFU-S development in 9 to 11 day mouse embryos led to the modification of the classical view of haematopoietic development, in which stem cells were thought to arise in the yolk sac and sequentially colonize the haematopoietic organs (Moore and Metcalf, 1970). The modified model suggested for mammalian haematopoiesis (Dzierzak and Medvinsky, 1995) proposes that fetal liver colonization occurs in two successive waves, with the first wave consisting of committed CFU-culture (CFU-C) produced in the yolk sac entering at 9 dpc and the second wave at around 10 dpc containing primitive CFU-S and LTR-HSC derived from the AGM region. The reverse order of haematopoietic activity within the embryo, with differentiated cells and progenitors detected at 7-8.5 dpc and fully potent adult repopulating HSCs not found until 10 dpc, is thought to reflect a primitive embryonic hierarchy of haematopoietic development which is in reverse orientation to that of the adult. It was further postulated that these two waves of fetal liver colonizing haematopoietic cells may reflect primitive and definitive stem cell activities and might be analogous to the two separable activities observed in the avian and amphibian systems. This model is consistent with the conserved vertebrate mechanism, whereby the initial extraembryonic-derived haematopoietic progenitors are replaced by a second and stable population of definitive HSC derived from a dorsal mesoderm intraembryonic region (reviewed by Zon, 1995).

1.2.6 Definitive haematopoiesis

The fetal liver develops into the major site of definitive haematopoiesis during mid- and late-gestation of mouse development and is responsible for the transfer of haematopoiesis into thymus, spleen and bone marrow (Morrison et al, 1995). The liver rudiment begins to form at late 9 dpc and by 10.5 dpc definitive fetal liver haematopoiesis is established (Johnson and Jones, 1973) resulting in a gradual shift of haematopoietic activity from the yolk sac and the AGM region to the fetal liver. The liver rudiment does not initiate haematopoiesis *in situ* but is colonized by founder haematopoietic cells generated at earlier haematopoietic sites within the conceptus (Moore and Metcalf, 1970; Johnson and Moore, 1975).

Erythrocytes and haematopoietic progenitors first enter the liver at 9 dpc (Houssaint, 1981). The developing erythroid lineage consists of small enucleated cells producing adult globins, which are characteristics for definitive haematopoiesis. Myeloid CFU-S appear also at 9 dpc and macrophages and B cells are present at 10-11 dpc (Dzierzak and Medvinsky, 1995). Although these differentiated haematopoietic lineages are found early in liver development, the more primitive cell types, CFU-S progenitors and LTR-HSCs are not detectable before 10.5 to 11 dpc (Medvinsky et al. 1996; Müller et al, 1994). Fetal liver colonization with definitive haematopoietic progenitors and HSCs occurs at 10.5 dpc and 11 dpc (Dzierzak and Medvinsky, 1995; Delassus and Cumano, 1996).

The colonization of haematopoietic organs is thought to occur via circulation. This notion is supported by observations that fetal blood at 10 dpc is rich in progenitor cells, in contrast to circulating blood in adult animals which contains few immature haematopoietic cells (Delassus and Cumano, 1996). The fetal liver is thought to serve as a reservoir for LTR-HSCs derived from pre-liver haematopoiesis, which provides a supportive microenvironment for the expansion of LTR-HSCs required for subsequent initiating adult haematopoiesis in bone marrow. The expansion of

haematopoiesis in the fetal liver, which results in the presence of a relatively high frequency of LTR-HSCs, is associated with the decline of haematopoietic activity in the yolk sac and the AGM region (Medvinsky et al, 1996). Although the haematopoietic activity in fetal liver declines during postnatal life, the liver does not lose its haematopoietic potential entirely. The liver in adult mammals can regain haematopoietic activity in abnormal situations and compensate for diminished bone marrow haematopoiesis. Experimental evidence for the haematopoietic potential of adult liver has been obtained by the haematopoietic reconstitution of lethally irradiated recipient mice with cells derived from the liver of adult donor mice (Taniguchi et al, 1996; Watanabe et al, 1996). During adult life, haematopoiesis takes place in the bone marrow. The embryonic thymus is colonized with haematopoietic precursors starting at 10.5 dpc, somewhat later than fetal liver colonization, and during late fetal and early postnatal life haematopoiesis moves to spleen and bone marrow (Moore and Owen, 1975). The development of the fetal thymus and the fetal spleen mark the emergence of lymphoid cells. During adult life, the thymus is important for the generation of mature T-lymphocytes. In mouse, the spleen is initiated to haematopoiesis at 15 dpc of gestation and continues to be haematopoietic for the entire period of gestation and some weeks thereafter. Although the haematopoietic activity in spleen declines after the postnatal stage, in the murine system, the spleen never completely loses its haematopoietic function and remains a site of erythropoiesis throughout adult life. The colonization of bone marrow with haematopoietic progenitors begins to take place around 16 dpc of development (Ogawa et al, 1988), which is the last and final haematopoietic organ to be activated for haematopoiesis during development. During mouse development, bone marrow haematopoiesis is limited to granulopoiesis but gains full haematopoietic potential after birth and provides all the haematopoietic lineages during adult life (Tavassoli, 1991). In normal adult mammals almost all haematopoietic activity occurs in the bone marrow, which is the only site where myelopoiesis, erythropoiesis and lymphopoiesis proceed simultaneously.

1.2.7 Haematopoietic cells: Developmental stage dependent differences

The haematopoietic system consists of a large spectrum of different cell types for each developmental stage. The haematopoietic cell population is a dynamic pool of cells, with constant morphological and functional changing cells and with each developmental stage having distinct cell types. Corresponding to the changing demands on the haematopoietic system in the developing embryo, distinct cell types exist for each ontogenetic stage until the permanent steady-state haematopoietic system within the adult bone marrow cavities is established. In mammals, the maternal haematopoietic system provides immune function and the supply of oxygen for the developing embryo and the developing haematopoietic system of the embryo only produces cells that serve its own need.

Morphological and functional heterogeneity between embryonic/fetal and adult-type haematopoiesis has been described for different haematopoietic lineages. The most obvious example being the formation of nucleated erythrocytes in the yolk sac expressing fetal-type globin in contrast to enucleated erythrocytes in the adult animal. Morphological heterogeneity has also been observed for macrophages between the fetal and the adult stage (reviewed by Naito et al, 1996).
The use of molecular biological techniques such as specific antibodies and cloned gene probes made it possible to analyse the molecular basis of ontogenetic differences between the various haematopoietic cell types and their precursors. Molecular differences for a variety of haematopoietic cell types have been uncovered, including the observations of a developmental switch in B-cell and T-cell lineages (Ikuta and Weissman, 1993). At the level of the HSCs, the expression of the surface antigen AA4.1 has been recognized as an ontogenetic stage-specific marker. Yolk sac HSCs and fetal liver HSCs have been characterized as positive for AA4.1 (Huang and Auerbach, 1993; Jordan et al, 1990) whereas AGM-derived HSCs and bone marrow HSCs are negative for AA4.1 (Sanchez et al, 1996; Spangrude et al, 1995).

Although fetal and adult HSCs can be enriched by selecting for the same combination of surface markers, differences in their developmental potential can be observed at the level of molecular analysis (Spangrude et al, 1991). The differential expression of ß-globin genes in the various erythrocyte precursor populations is among the best studied difference in gene expression between embryo-derived and adult haematopoietic cells (Bonifer et al, 1998).

The molecular analysis between developmental stages of haematopoiesis has largely advanced with the establishment of the gene targeting methodology. Several knockout mice, deficient in a specific regulatory molecule have been described in which the mutation affects haematopoiesis at various developmental stages. For instance, mouse embryos homozygous deficient in the transcription factor gene *c-myb* initially develop normally but die by 15 dpc from severe anaemia (Mucenski et al, 1991). These knockout embryos produce primitive erythroid cells in the yolk sac but have defective haematopoiesis in the fetal liver. Other targeted mouse mutants that exclusively affect definitive haematopoiesis are core binding factor alpha2 (Cbfa2/AML1) and erythroid Krüppel-like factor (Eklf) (Okuda et al, 1996; Nuez et al, 1995). Mutation in either of these genes leads to a defect in definitive haematopoiesis, with mutation of the AML1 gene affecting all lineages of fetal liver haematopoiesis and mutation of Eklf gene blocking definitive erythropoiesis.

Some mutations have been found to differentially affect yolk sac, fetal liver and bone marrow haematopoiesis e.g. *Gata2* has profound effects on fetal liver haematopoiesis and some effects on yolk sac haematopoiesis (Tsai et al, 1994). Mutation of the *Gata2* transcription factor gene affects yolk sac and fetal liver haematopoiesis to a different degree. Whereas fetal liver contains virtually no multipotent progenitor cells some are present in yolk sac but at reduced numbers.

1.2.8 Cells of the established haematopoietic system

Mammalian haematopoietic cells have finite life spans, considerably shorter than the life span of the organism and the maintenance of constant numbers of functional cells in the peripheral blood is achieved by the proliferation and differentiation of precursor cells which are located primarily in the bone marrow.

At steady state, the cells of the haematopoietic system can be broadly divided into three types of cell populations: multipotent HSCs, committed progenitor cells and maturing/mature cells. The haematopoietic progenitor cells and mature cells are all derived from HSCs which are established during embryogenesis. HSCs are a cell population of limited number which remain quiescent until induced by signalling events for the production of mature blood cells which leads to the daily production of millions of blood cells (Ogawa, 1993).

Figure 1.1 shows an schematic overview of the cells and the hierarchical structure of the adult haematopoietic system.

1.2.9 Haematopoietic stem cells

Haematopoietic stem cells (HSCs) are the foundation of the haematopoietic system and are responsible for the maintenance of the haematopoietic system throughout the life span of an animal. HSCs are defined as the cell population of the haematopoietic system with multilineage differentiation potential and self-maintenance ability. This circumscribes the ability of HSCs to give rise to all haematopoietic cell types and to self-renew for the subsistence of a steady-state in HSC numbers. HSCs are rare in numbers, occurring with a frequency of approximately 1 per 10^5 nucleated cells in whole bone marrow in the adult mouse (Micklem et al, 1987; Harrison et al, 1989). In steady-state haematopoiesis the majority of HSCs are thought to stay in the metabolically quiescent phase of the cell cycle, the G_0 phase, and only a few stem cell clones contribute by clonogenic expansion to the daily blood cell production (Lajtha, 1979). The concept of cell cycle dormancy of HSCs, thought to be responsible for

their indistinguishable phenotype and their radioresistance, is supported by observations that brief exposure of *in vitro* bone marrow cells to radioactivity does not reduce the number of multipotential progenitors (Hara and Ogawa, 1978). Furthermore longterm reconstituting HSCs were found to be refractory to retroviral infection (Williams et al, 1984). Examination of the cell cycle distribution of multipotent progenitors indicated that longterm reconstituting stem cells are generally not mitotically active (Morrison and Weissman, 1994). Due to the quiescent state of most HSCs during normal haematopoiesis and their rarity in cell number it has been difficult to characterize and isolate these cells.

Many different *in vivo* and *in vitro* functional assays have been used to determine lineage potential of haematopoietic progenitors and HSCs. *In vitro* assays to detect committed single and multilineage progenitors include the colony forming unit, culture assay (CFU-C) (Metcalf, 1984), stromal co-culture assays (Godin et al, 1995) or fetal thymic organ culture systems (Liu and Auerbach, 1991). An *in vivo* assay for multipotent progenitors is the colony forming unit spleen (CFU-S) which was the original assay for the search of HSCs (Till and McCulloch, 1961). This assay involves transplantation of haematopoietic progenitors yielding macroscopic colonies, in form of nodules of haematopoietic cells in the spleens of lethally irradiated mouse recipients between 8 to 13 days after injection (Moore and Metcalf, 1970; Medvinsky et al, 1993). CFU-S progenitors are not HSCs but can give rise to myeloid and erythroid lineages, have some degree of self-renewal capability and were found to account for the initial haematopoietic reconstituting activity and short-term repopulation in lethally irradiated recipients (Jones et al, 1989). CFU-S progenitors and HSCs can be separated by centrifugal elutriation, which is based on differences in cell size and density (Jones et al, 1990).

A two step *in vitro* culture system with stromal cells and growth factors, the single cell multipotential assay (SMA), was developed for measuring the multipotential activity of a single cell (Godin et al, 1995). The first step of this culture system is

to induce clonal expansion of haematopoietic progenitors and in the second step clones of progenitors are tested for lineage differentiation capacity. *In vitro* CFU and co-culturing assays generally detect cells nearing the final stages of differentiation. Haematopoietic progenitor cells in these assays are not required to migrate or home as they must in some *in vivo* assays.

The ultimate assay for the most primitive HSCs measures the long-term multilineage repopulation of the haematopoietic system of lethally irradiated or genetically haematopoiesis deficient mouse recipients (Moore and Metcalf, 1970; Müller et al, 1994). In this *in vivo* assay, the full potential of the HSC is analyzed but requires 4-6 months before the stable engraftment by a stem cell can be measured.

Many characteristics attributed to HSCs have been derived from studies using functional *in vitro* and *in vivo* assays as described above. Simultaneously to functional analysis, efforts have increased for the enrichment and isolation of HSCs in cell populations of haematopoietic progenitors. Although functional assays have proven an indispensable tool for the analysis of HSC characteristics they are of limited use for the isolation of HSCs. Functional capabilities of HSCs can only be assessed by testing the abilities of the cells, which itself alters their characteristics during the assay procedure, thus reflecting on the HSCs present at the beginning of the analysis.

Many approaches for the enrichment and isolation of HSCs are based on the isolation of rare cells by fluorescence-activated cell sorting (FACS). Fluorescein-conjugated antisera were used to identify cell surface marker expression patterns and FACS analysis was used to enrich haematopoietic cells for HSCs. Spangrude et al (1988) isolated a phenotypically defined multipotent progenitor population which was shown to be highly enriched in HSCs. Bone marrow cells isolated by combination of Thy-1 antigen expression at low levels, high level expression of the stem cell antigen-1 (Sca-1) and the absence or low expression of specific lineage markers found on mature blood cells ($Lin^{-/lo}$) were found to be a rich source of HSCs.

The Thy-1^{lo}Sca-1^+Lin$^{-/lo}$ cell population was shown to be capable of long-term multilineage reconstitution of lethally irradiated mice. On average 1 out of 20 intravenously injected cells from this population was found to give rise to multipotent progenitor activity (Spangrude et al, 1988; Smith et al, 1991).
However, upon closer examination the Thy-1^{lo}Sca-1^+Lin$^{-/lo}$ population was found to be heterogeneous with respect to functional activity and found to consist of longterm and transient repopulating cells (Smith et al, 1991). Further phenotypic analysis found that the Thy-1^{lo}Sca-1^+ cell population can be divided into three subpopulations based on heterogeneity in low level of lineage specific marker expression (Morrison and Weissman, 1994). However, each of these subpopulations was found to be highly enriched for progenitor activity and despite being a heterogeneous population the Thy-1^{lo}Sca-1^+Lin$^{-/lo}$ cell population is used as the basic population for further HSC enrichment and analysis (Ikuta and Weissman, 1992). For instance, a subpopulation of Thy-1^{lo}Sca-1^+Lin$^-$ cells staining low for Rhodamine 123 (Rh123^{lo}) has been found to be an apparently pure population of long-term repopulation progenitors (Spangrude et al, 1995).

Other surface antigen markers have been used for phenotypical characterization and identification of HSCs. Two antigens closely associated with HSCs are the receptor tyrosine kinase c-kit (Okada et al, 1991) and the cell surface glycoprotein CD34 (Krause et al, 1994). Adult LTR-HSCs in bone marrow have been described to be Lin$^-$c-kit$^+$Sca-1^+CD$34^{-/lo}$ (Osawa et al, 1996), whereas LTR-HSCs in the AGM region and fetal liver were reported to be c-kit$^+$CD34^+ (Sanchez et al, 1996). The transient phenotype of HSCs during various developmental stages and transitory localization further complicates the isolation of HSCs. Observations have been reported that the level of antigen expression can be dependent on the developmental stage of the HSC. For instance, CD34 is reported to be selectively expressed within the haematopoietic system on stem and progenitor cells but expression of CD34 is

lost as the cells mature (Krause et al, 1994). Some surface antigens designated to HSCs are also expressed by non-haematopoietic cells, e.g. CD34 is also expressed in vascular endothelial cells (Fina et al, 1990). Expression of c-kit is broadly distributed within the hierarchy of haematopoietic cells and is also found in other tissues (Broudy, 1997).

Currently, there are no specific phenotypic antigens unique to definitive HSCs. Thus, combinations of antigens combined with enrichment techniques which take advantage of physical characteristics of HSCs unrelated to antigen expression are used for the enrichment and isolation of HSCs.

One of the many methodologies used for HSCs enrichment is based on the uptake of fluorescent dyes. Fluorescence dyes such as the nucleic acid dye Hoechst 33342 or the vital fluorochrome Rhodamine-123 (Rh123) have been proven useful for HSC enrichment. Hoechst 33342 hardly stains the most quiescent HSCs (Neben et al, 1991), whereas Rh123 accumulates in mitochondria. It has been shown that enriched progenitor fractions can be further subdivided with respect to Rh123 uptake (Spangrude and Johnson, 1990). The $Rh123^{lo}$ fraction has been observed to be more enriched for long-term multipotent progenitors than the $Rh123^{med/hi}$ (Spangrude and Johnson, 1990) and it has been reported that long-term and transiently reconstituting multipotent progenitors can be separated based on Rh123 uptake (Zijlmans et al, 1995). Quiescent primitive HSCs have been shown to be resistant to cytotoxic agents such as 5-fluorouracil (5-FU) and this property has been exploited to enrich HSCs by exclusion of all other cells (Hodgson and Bradley, 1979). HSCs have also been enriched based on the level of wheat germ agglutinin binding (WGA) (Jurecic et al, 1993) and on the basis of size and density by counter flow centrifugal elutriation (Jones et al, 1990). Often two or more techniques are combined for the enrichment of HSCs, e.g. centrifugal elutriation of cells sorted on the basis of Rh123 cell sorting resulted in a substantial enrichment of HSCs (Ploemacher and Brons, 1989).

The study of the fundamental mechanisms of self-renewal and differentiation ideally requires analysis either of an absolute pure population of HSCs or analysis of individual HSCs independently of the accompanying cells in the sample. It is therefore critical that HSC populations are pure or nearly pure, if population level characteristics such as cell cycle distribution and radioprotective capacity are to be interpretable. Retroviral labelling of individual stem cell clones allowed insight into clonal behaviour and systematic dynamics of stem cells and entire reconstitution of the haematopoietic system *in vivo* (Capel et al, 1990; Jordan and Lemischka, 1990). Analysis of retrovirally marked haematopoietic progenitor clones *in vivo* found that molecular marking appears not to alter the behaviour of the stem cell population. Genetically marked cells with HSC properties such as ability to reconstitute lethally irradiated mice, contribution to haematopoiesis over a long-term, ability to give rise to CFU-S and CFU-C, and expression of haematopoietic surface markers, have been produced by retroviral labelling (Wong et al, 1994). Retroviral marking of HSCs makes it possible to uniquely mark and simultaneously follow multiple progenitor clones, based on retroviral integration sites.

A number of models have been proposed for the conceptional organization of the haematopoietic system and although there are data supporting all aspects of these models non can be considered conclusive (Loeffler and Potten, 1997). Nonetheless, these models continue to provide the foundations for current concepts of HSC self-renewal and differentiation. Whereas early model systems tended to view HSCs in isolation, as techniques for studying HSCs became more precise aspects of the influence of microenvironmental differences on self-renewal and differentiation were included.
At present there is no experimental way to decide if a given haematopoietic cell in a functional mammalian tissue is a HSC or not, there are also no unique morphological

criteria to identify HSCs, thus most of the present knowledge of HSC commitment and self-renewal is based on the population approach.

Two frequently used models based on a stochastic concept or a deterministic concept are applied for the description of self-renewal and differentiation of HSC. In both models regulatory influences of the haematopoietic microenvironment by its generation of cytokines are included. The first model suggests that self-renewal and commitment of HSCs and pluripotential progenitors are stochastic processes but survival and proliferation of committed progenitors is regulated by cytokines (Ogawa, 1993).

This stochastic model is based on the formation of multilineage colonies with replating potential in methylcellulose. The production of secondary blast cell colonies was postulated as a self-renewal process and the generation of secondary multilineage colonies as differentiation. The distribution of colony types generated by multipotential progenitors was interpreted as consistent with stochastic mechanisms of stem cell renewal and commitment (Ogawa, 1993).

In contrast, the second frequently used model suggests instructive processes for HSC self-renewal and differentiation. This deterministic model is based on the purification of three multipotent populations with distinct self-renewal potentials (Morrison and Weissman, 1994). These populations have been reported to form a lineage of multipotent progenitors from long-term self-renewing stem cells to the most mature multipotent progenitor population (Morrison et al, 1997). The fact that self-renewal potential of particular multipotent progenitors based on surface marker expression can be predicted is interpreted to indicate that self-renewal is deterministic. Although self-renewal is explained to be determined by intrinsic factors, microenvironmental factors are thought to influence proliferation and survival of committed progenitors.

The necessity for new and well defined concepts that are amenable to experimental analysis is discussed to a great extent on concepts generally applicable to all stem cell systems by Loeffler and Potten (1997).

1.2.10 Haematopoietic progenitor cells and mature blood cells

The immediate progeny of differentiating multipotent HSCs are haematopoietic progenitor cells which possess limited further differentiation potential. In contrast to HSCs which are quiescent under normal conditions, progeny of HSCs undergo significant clonal expansion. This expansion accounts for the majority of the functional cells of the haematopoietic system owing to the fact that the mature terminally differentiated cells have only a modest proliferative capacity. The commitment of haematopoietic cells to various cell lineages occurs as multipotent progenitors progress through an irreversible descending hierarchy of differentiation steps that eventually results in the production of mature haematopoietic cells. The sequential development of progenitor cells and mature cells from HSCs is illustrated in Figure 1.1 (Hierarchical model of haematopoiesis).

Haematopoietic progenitors, also termed lineage-restricted or committed progenitors, are detectable by their ability to give rise to colonies of morphologically recognizable haematopoietic cells in semisolid clonal cell assays. Relatively primitive progenitor cells with high proliferative and some renewal capability that can be cloned in soft agar or methylcellulose are the colony-forming unit-blast (CFU-blast) (Suda et al, 1983) and the high proliferative potential colony-forming cell (HPP-CFC) (Bradley and Hodgson, 1979). The long-term culture initiating cell (LTC-IC) is another primitive high-renewal cell that has been defined by its growth on irradiated adherent cells in long-term marrow cultures (Ploemacher et al, 1989).

As progenitor cells mature they become restricted to a single cell lineage - erythrocytes, platelets, neutrophils, macrophages, eosinophils, basophils, mast cells or lymphoid cells. Functional *in vitro* assays, the colony forming unit (CFU) assays, for committed single- and multi-lineage progenitors lead to mature blood cell production. The progenitor cells are characterized by the cell lineage(s) they form in the colony forming unit assay, e.g. CFU-G, CFU-GEMM. These culture assays led to the identification of close cell lineage relationships between populations,

in some cases sharing a common progenitor cell, e.g. neutrophils and mononuclear phagocytes are closely related (Wright and Lord, 1992). Progenitor cells have little, if any, capacity to self-renewal but are committed to a programme of differentiation and maturation with any proliferative capacity serving to amplify the population prior to the terminal maturation of the cells. Most progenitors are unipotential cells, generating colonies of maturing/mature cells of a single lineage. The survival and proliferation of progenitor cells *in vitro* is dependent on the presence of cytokines (Han and Caen, 1994).

Whilst differentiation can be defined as a qualitative change in the cellular phenotype, maturation can be regarded as a quantitative change in the cellular phenotype leading to functional competence (Loeffler and Potten, 1997).

Maturing and mature cells of the haematopoietic system encompass the majority (~95%) of the haematopoietic cells which reflects the proliferative amplification of the differentiated cells as they mature to become functional cells (Wright and Lord, 1992). Mature haematopoietic cells have distinctive morphological characteristics and are responsive and adaptive to a variety of stress.

Haematopoiesis - commitment of HSCs, differentiation of haematopoietic progenitors and maturation occurs in the bone marrow environment in association with the stromal cells of the marrow but when mature, cells leave the marrow and enter the peripheral blood circulation. Mature haematopoietic cells localize and function in varied microenvironments and are able to interact with a spectrum of non-haematopoietic cell types.

1.2.11 The haematopoietic microenvironment

The steady-state production of haematopoietic cells depends to a large extent on interactions between haematopoietic progenitors cells and their microenvironment. The haematopoietic microenvironment is a highly organized structure that influences the location and physiology of HSCs and all other haematopoietic cells. The stroma

in bone marrow is a network of stromal cells (endothelial cells, fibroblasts, macrophages, adipocytes) and extracellular matrix (collagen, laminin, fibronectin proteoglycans), whereas the stroma of other haematopoietic sites is less complex (Allen et al, 1990). Differentiation, proliferation and maturation of haematopoietic cells are regulated and influenced by interactions with non-haematopoietic cells, mediated directly by cell-cell contact and/or secreted regulatory molecules acting in a positive or negative manner (Clark et al, 1992).

Haematopoietic cells removed from the body can be maintained for short periods of time in the presence of growth factors, but in the absence of growth factors the survival of these cells rapidly declines *ex vivo*. However, cultured in association with marrow-derived stromal cells, haematopoietic cells will proliferate and differentiate *in vitro* in the absence of exogenous factors (Dexter et al, 1977). These long term bone marrow culture (LTBMC) conditions maintain stem cell replication and commitment to differentiation for many weeks *in vitro*. The *in vitro* LTBMC system appears to closely reproduce the conditions observed *in vivo* and has been useful in defining cell populations and associations between haematopoietic and stromal cells. In LTBMC systems an adherent cell layer, consisting of different cell types provides both, the physical support and the haematopoietic cytokines (soluble and cell-associated proteins) necessary for the growth of haematopoietic progenitors. Separation of the haematopoietic cells from the stromal cells or the use of conditioned medium, harvested from LTBMC was found to result in reduced haematopoiesis *in vitro*, suggesting that close proximity or contact between haematopoietic and stromal cells is required for haematopoiesis (Clark et al, 1992).

Haematopoietic cells can bind avidly to stromal cells via adhesion proteins and possibly via membrane cytokines and interactions occur via factors secreted by stromal cells and via cell-cell contacts (Ohneda and Bautch, 1997). Cells of the haematopoietic microenvironment are a major source of haematopoietic cytokines supporting growth and differentiation of haematopoietic cells. Microenvironmental cells have been found

to produce haematopoietic stimulators e.g. GM-CSF, G-CSF, M-CSF, SCF, IL3, and inhibitors e.g. TNF-alpha, TGF-ß and IFN. Some of these are produced only by activated microenvironmental cells; thus they have been postulated to play a major role on blood cell production under situations of acute stress, such as infection. Although haematopoiesis is recapitulated *in vitro* using stromal cells from the bone marrow in LTBMC, the heterogeneity of the stromal cells in these cultures complicates the dissection of the role of specific stromal cell types in haematopoiesis.

1.2.12 The molecular control of haematopoiesis

Haematopoiesis is controlled by the dynamic balance of co-operative actions of various regulators exerting positive and/or negative effects on cells of the haematopoietic system. The diverse mechanisms controlling stem cell self-renewal, proliferation, commitment, differentiation and maturation within the haematopoietic system are interactive and able to response to changing conditions.
Whereas much is known about molecular control mechanisms in progenitor and mature cells, little is known how HSCs decide between self-renewal versus commitment. Lineage commitment occurs as a progressive lineage restriction of multipotent progenitors through an irreversible descending hierarchy of differentiation steps that eventually results in the production of mature haematopoietic cells.
As haematopoietic lineage commitment proceeds there is a concomitant loss in the capacity of committed progenitors to differentiate to other cell lineages.
Two main models to explain the controlling mechanisms of lineage commitment and haematopoietic differentiation have been proposed. One model suggests control of lineage commitment by extrinsic regulators such as haematopoietic cytokines and/or interactions with stromal microenvironment (Metcalf, 1991). Others propose that cytokines have only a minor role in determining cell lineage commitment and suggest a model, whereby intrinsic regulators direct self-renewal and lineage commitment

of HSCs and progenitor cells and cytokines regulate proliferation and survival of already committed cell types (Ogawa, 1993).

Whichever model of haematopoietic lineage commitment is correct, cytokines and transcription factors play important roles in controlling mechanisms of the haematopoietic system. Analysis of both, cytokines and transcription factors has widely relied on *in vitro* studies. However, new insight and conceptional understanding of haematopoiesis has emerged using targeted mutagenesis in mice allowing functional analysis *in vivo*.

Some of the recent understanding of the regulatory effects of cytokines, cytokine receptors and transcription factors is outlined in the sections below.

1.2.13 Cytokines in haematopoiesis

A variety of molecules recognized to have regulatory effects in the haematopoietic system have been identified, including the colony stimulating factors (CSFs), the interleukins (ILs) and haematopoietic growth factors, together generally referred to as cytokines. Cytokines are soluble molecules produced by haematopoietic cells and cells of the stromal microenvironment with a wide range of bioactivities for controlling the various pathways for lineage commitment, proliferation, differentiation and functional activity of the haematopoietic system. Many cytokines were originally identified as soluble mediators in LTBMC systems and subsequently isolated. LTBMC systems attempt to provide the required microenvironment of cell-cell interaction, as well as soluble and membrane-bound mediators necessary for haematopoiesis (Dexter et al, 1977). Cytokines can be produced by multiple cell types, both haematopoietic and non-haematopoietic cells, however, stromal cells of the haematopoietic microenvironment have been identified as the main source of cytokines. Initially the CSFs were defined by their ability to induce clonal proliferation and differentiation, and characterized by the types of colonies formed from bone marrow cells in soft agar assays (Nicola, 1989).

Most cytokines are pleiotropic and have multiple biological functions at various levels of cell differentiation and on multiple lineages, however, some functions may predominant and be recognized as the main characteristic of a cytokine. Cytokines described to act in a relative lineage specific manner are erythropoietin (Epo) and macrophage-CSF (M-CSF). Epo is responsible for maintenance and control of erythropoiesis and induces erythrocyte production (Spivak, 1989). M-CSF is considered to be for specific macrophage/monocyte lineages (Bajorin et al, 1991). Most cytokines acting at later stages of lineage development show lineage specificity of some sort, supporting proliferation and maturation of highly committed progenitors. In contrast, cytokines acting early in lineage development are relative non-specific factors e.g. IL-6, IL-3, SCF, GM-CSF.

Functional pleiotropy (multiple biological actions) is observed for most cytokines. Examples for multilineage cytokines are IL-3, also referred to as multi-CSF, and IL-6. IL-3 stimulates the growth and differentiation of multipotent progenitors, B-cell precursors, and myeloid progenitors, including those of erythroid, mast cell and, granulocyte lineages. However, it has been reported that IL-3 acts best in synergy with a combination of other factors and IL-3 alone is less effective (Yonemura et al, 1992). IL-6 is a cytokine with multiple activities that is produced by various types of lymphoid and non-lymphoid cells, e.g. B-cells, monocytes, macrophages, megakaryocytes, eosinophils, fibroblasts, stromal cells (Han and Caen, 1994). IL-6 induces B-cell maturation and immunoglobulin secretion but has also been shown to enhance megakaryocyte development and proliferation of multipotential progenitors. IL-6 also interacts synergistically with other cytokines to stimulate myeloid proliferation (Veiby et al, 1997).

Some cytokines have been found to be able to act in a direct and/or indirect manner by either affecting progenitor cells directly or by inducing the production of other cytokines. GM-CSF stimulates the proliferation of granulocyte, macrophage, eosinophil and basophil progenitors, but also exerts a variety of direct or indirect

effects on mature cells including neutrophils, eosinophils, monocytes and macrophages at concentrations below that required to promote proliferation of progenitor cells (Rapoport et al, 1992). Another cytokine able to act in a direct and/or indirect way is IL-1. IL-1 is reported to have direct effects on early progenitor cells, to act synergistically with many other cytokines and to induce the production of other cytokines. IL-1 has also been reported to improve haematopoietic recovery from cytotoxic drug damage *in vivo* and the survival of mice after irradiation or bone marrow transplantation. However, these effects may be triggered by the ability of IL-1 to induce the production of a range of cytokines, such as GM-CSF, G-CSF, M-CSF and IL-6, in monocytes, fibroblasts, endothelial and other cell types. The ability of IL-1 to induce cells to produce cytokines makes the interpretation of direct effects of IL-1 complex. Effects of IL-1 are probably a result from acting directly on early progenitors and by interacting synergistically with other cytokines (Heyworth et al, 1997). Some cytokines show highly conserved sequence homology between species, e.g. IL-5 and G-CSF. Mouse IL-5 and human IL-5 have a sequence homology of 77% at the DNA level and 70% at the protein level. The amino acid sequences of mouse and human G-CSF exhibit more than 70% homology and have been reported to highly cross-react between species (Han and Caen, 1994).

G-CSF acts primarily to stimulate proliferation, differentiation and maturation of committed neutrophil progenitor cells. However, in combination with IL-3, G-CSF can enhance the proliferation of multipotent haematopoietic progenitors and megakaryocyte progenitors (Ikebuchi et al, 1988).

Stem cell factor (SCF) is a cytokine with its most prominent actions on HSCs and primitive haematopoietic progenitors (Williams et al, 1990). However, *in vitro* studies have shown that SCF can also act on precursor cells and mature cells, showing a broad range of activities. SCF alone has limited capacity to stimulate proliferation, however, in combination with other cytokines it has been found to have a potent

costimulatory effect. It enhances myeloid and erythroid colony growth when combined with GM-CSF, G-CSF, IL-3 or Epo and it acts in synergy with IL-3 and GM-CSF to stimulate megakaryocytopoiesis (Han and Caen, 1994).

Functional analysis of cytokines *in vitro* and *in vivo* has given a large amount of information about the regulatory effects of cytokines in haematopoiesis. Although it may appear that the haematopoietic system exhibits a high degree of redundancy in regulatory factors, with many similar or overlapping actions from different regulators, the complex interactions of cytokine influenced activities does not allow conclusions to be drawn about the direct effects of any cytokine. The most definitive approach to establishing the function of a particular regulator is by analysing animals in which the gene in question has been deleted or functionally inactivated. Naturally occurring mutations in the Sl locus, which encodes SCF, were among the first *in vivo* models for cytokines. Mutations at the Sl locus result in defective haematopoietic, melanocyte and gonadal development. The haematopoietic phenotype in homozygous mice is characterized by severe macrocytic anaemia. *In vitro* studies revealed that the defect in the Sl mutation was due to a stromal cell microenvironment that could not support haematopoiesis, however, haematopoiesis can occur in the absence of functional SCF (Galli et al, 1994). Another mouse mutation in a cytokine gene that has arisen spontaneously results in a deficiency of M-CSF (Yoshida et al, 1990). Mice harbouring a homozygous mutation for the gene (op) encoding M-CSF exhibit a major deficiency in macrophage-derived osteoclasts and partial deficiencies in other macrophage populations. These abnormalities have been found to be 'correctable' by the injection of M-CSF (Wictor-Jedrzjczak et al, 1991).
A number of targeted mutations in cytokines has been produced, e.g. GM-CSF, G-CSF, IL-3 (Lieschke et al, 1994a; Dranhoff and Mulligan, 1994; Hera and Miyajima, 1995) which were anticipated to produce definitive answers to their molecular function. However, this has not proved to be the case, since some

animal models produced for cytokines thought to be critical to haematopoiesis display relatively little or no haematopoietic phenotype, e.g. GM-CSF, IL-3 (Dranhoff and Mulligan, 1994; Hera and Miyajima, 1995).
Nevertheless, animal models with phenotypes more in line with the predicted effects have also been obtained. For instance, G-CSF knockout mice have a deficiency in granulocyte and macrophage progenitors and impaired neutrophil mobility (Lieschke et al, 1994a). To address this apparent redundancy in cytokines on a genetic level, generation of mice deficient in two or more cytokines, double and multiple knockouts, will be required. For instance, a study has been reported analysing mice deficient in GM-CSF and M-CSF. These mice are osteopetrotic and have lung disease (Lieschke et al, 1994b).

Not all cytokines involved in the control of haematopoiesis have stimulatory effects on haematopoietic cells. Inhibitory and bifunctional cytokines of haematopoietic precursors have been described and characterized on the basis of their inhibitory effects on progenitors at different stages e.g. transforming growth factor-ß (TGF-ß1), macrophage inflammatory protein 1alpha (MIP-1alpha) (Keller et al, 1988; Dunlop et al, 1992). The actions of TGF-ß1 and MIP-1alpha appear to be dependent upon the developmental status of the cell, cell type, growth conditions and presence of other factors. TGF-ß1 is able to inhibit HSCs and primitive progenitors but stimulates or has no effect on more mature progenitors (Keller et al, 1990). MIP-1alpha protein was purified for its ability to inhibit haematopoietic progenitor cell proliferation *in vitro*, however, in combination with other cytokines MIP-1alpha has been reported to promote colony growth. TGF-ß1 and MIP-1 alpha appear to inhibit the cell cycling of the primitive cells but can act in a growth stimulatory manner on the more mature progenitor cell.

It is apparent that a balance between stimulatory and inhibitory growth factors is essential for the maintenance of the homeostasis associated with normal haematopoiesis. The effect of cytokines is not only dependent on their concentration but also on the responsiveness of their potential target cells e.g. the presence of specific cytokine receptors for the initiation of responsive effects after cytokines bind to their specific receptor.

1.2.14 Cytokine receptors in haematopoiesis

Cytokines act by binding to their specific cytokine receptors expressed on the cell surface which determine the responsiveness of target cells to cytokines. Cytokine receptors are cell surface glycoproteins that serve the dual function of recognizing their cognate ligands among a variety of other factors and of initiating a series of cellular signals that ultimately lead to multiple cellular functions.

Two distinct families of related receptors exist for cytokines with members of these superfamilies defined by structural criteria. Members of the cytokine receptor superfamily are defined by structural motifs in the exoplasmic domain and by the absence of catalytic activity in the cytosolic segment. Members include the receptors for Epo, G-CSF, GM-CSF, LIF, IL-2, IL-3, IL-4, IL-5, IL-6, and IL-7 (Banzan, 1990). Members of this superfamily are divided into subfamilies based on oligomeric structure and shared components of the receptors.

Other cytokine receptors have classical transmembrane tyrosine kinase activity belonging to the receptor tyrosine kinase superfamily. These include the receptors for SCF, termed c-kit receptor (Chabot et al, 1988) and for M-CSF, termed c-fms receptor (Sherr et al, 1985). The cytoplasmic domains of receptor tyrosine kinases contain a highly conserved catalytic kinase whereas the extracellular region is specific for their cognate ligands. Receptor tyrosine kinases dimerize upon ligand binding followed by auto-phosphorylation which is an essential step for the activation of signaling pathways.

In contrast to the tyrosine kinase-containing receptors, the cytoplasmic domains of members of the cytokine receptor superfamily lack a conserved motif associated with apparent enzymatic activity and do not show extensive similarity. These receptors participate in receptor signal transduction by interacting with and activating cytosol-localized proteins. Cytokine receptor signal transduction is initiated by ligand-mediated receptor oligomerization, with some members of the family active as homodimers, e.g. EpoR and G-CSF-R, whereas others are active as heterodimers, e.g. IL-3R, IL-5R, and GM-CSF-R, or as hetero-oligomers, e.g. IL-6R, LIF-R, IL-2R, and IL-4R. Receptor dimerization has been described to be essential for signal transduction (Watowich et al, 1994). Single chain receptors such as EpoR and G-CSF-R form homodimers in response to ligand binding, whereas the majority of cytokine receptors function as heterodimers or hetero-oligomers sharing common receptor signaling subunits within subfamilies. Receptors for IL-3, IL-5 and GM-CSF are hetero-dimers which share a common membrane bound chain, the common ß-subunit ($ß_C$), but have ligand-specific alpha-subunits. The alpha-subunits bind ligand in the absence of the ß-subunit, but oligomerization with the ß-subunit is required for high-affinity ligand binding and for cell proliferation. The high-affinity receptors for other cytokines also have distinct and common receptor components that oligomerize in response to ligand binding to initiate signaling e.g. IL-6, LIF, IL-2 and IL-4.

Signal transduction across the cytoplasm is dependent on the activation of catalytic regions associated to the receptors. Whereas receptor tyrosine kinases contain a catalytic activity in their cytoplasmic domains members of the cytokine receptor superfamily depend on intracellular catalytic molecules. Tyrosine phosphorylation of the cytokine receptors and cellular substrates is thought to be achieved by contact with and activation of intracellular tyrosine kinases after ligand binding and receptor dimerization. Cytoplasmically localized protein tyrosine kinases which play a major role in cytokine signaling include the Janus family kinases (JAK kinases) and src family-related kinases (Ziemiecki et al, 1994).

Although cytokine receptors activate similar intracellular signal transduction pathways, different receptors support the proliferation and differentiation in distinct haematopoietic lineages. Corresponding to cytokine studies, targeted mutagenesis in cytokine receptors has provided insight into the physiological roles of individual cytokine receptor subunits *in vivo*. Several cytokine receptors have been disrupted by targeted mutagenesis e.g. EpoR, IL-2R alpha, ß, IL-3R (ß-subunit) (Watowich et al, 1994).

Combined analysis of animal models for cytokine receptors and animal models for cytokines revealed understanding of the *in vivo* function of ligand/receptor pairs. A classic example is the SCF/c-kit ligand/receptor pair. Mutations at the steel (Sl) locus, encoding SCF, and the white spotting (W) locus, encoding the receptor tyrosine kinase c-kit, arose spontaneously and study of these mutations provided important information about interactions between haematopoietic cells and their stromal microenvironment (Broudy, 1997). An example for a gene targeted ligand/receptor pair is Epo and EpoR. The study of Epo-/- and EpoR-/- mice observed similar phenotypes in both models, exhibiting severe anaemia and embryonic lethality, thus indicating that the Epo function *in vivo* cannot be replaced by other cytokines or receptors (Wu et al, 1995).

1.2.15 Transcription factors in haematopoiesis

Cytokines mediate their effects through signal-transducing pathways which results in the activation of gene expression by specific transcription factors. The activation of cell specific transcription factors mediates the variety of proliferation and differentiation signals to which cells are exposed into coordinated cell specific gene expression. The production of functional blood cells from HSCs is a highly regulated process with haematopoietic transcription factors involved as important regulators of the correct temporal and spatial gene expression patterns.

Thus, an understanding of transcription factor function is essential to the study of haematopoietic development and commitment, differentiation and proliferation of haematopoietic cells. Similar to the arrangement of haematopoietic cell development and cytokines into a hierarchical system, with multi- or pluripotent progenitors and broadly acting cytokines at the top and lineage-specific progenitors and lineage specific cytokines at the bottom, transcription factors can be arranged along the haematopoietic hierarchy. A hierarchical relationship among haematopoietic transcription factors has been proposed (Orkin, 1995). Some transcription factors appear to function at an early stage of specification of HSCs and mutation of their genes affects a broad range of blood cells e.g. SCL, Rbtn2. Other transcription factors are limited in function to a later and more specific role e.g. GATA-1, Ikaros, PU.1 (Orkin, 1995).

Alike to functional cytokine studies, the advent of targeted mutagenesis has greatly advanced the study of haematopoietic transcription factors. The function of transcription factors within the haematopoietic system is implied from the range of cells that fail to develop in a mouse with a homozygous knockout mutation. Although speculative to some degree, the analysis of transcription factors on the basis of knockout phenotypes has provided much insight in their functional roles. Examples of some haematopoietic transcription factors and their main characteristics are described below.

The transcription factor SCL (for stem cell leukemia), also known as TAL1 (for T-cell acute leukemia-1), was originally identified as the product of a gene at the site of chromosomal translocations associated with acute T-cell lymphoblastic leukemia (Begley et al, 1989). SCL is a transcription factor of the basic helix-loop-helix (bHLH) DNA-binding domain family. During embryonic development, SCL is selectively expressed in extraembryonic and embryonic mesoderm (Kallianpur et al, 1994) and during normal haematopoiesis in erythroid cells, mast cells and megakaryocytes (Green et al, 1991). Mice lacking a functional SCL gene die around

embryonic day 9.5 due to a complete block in haematopoiesis (Robb et al, 1995; Porcher et al, 1996). Both, embryonic and definitive haematopoiesis are affected and it was found that SCL is essential for the generation of all haematopoietic lineages (Shivdasani et al, 1995; Robb et al, 1996). The analysis of Scl-/- mice and Scl-/- ES cells indicate a very early role for SCL, developmentally in the specification of haematopoietic cells from mesoderm and during normal haematopoiesis in the specification of HSCs with an additional role in regulating differentiation of erythroid, megakaryocytic and mast cells (Robb et al, 1996; Porcher et al, 1996; Elefanty et al, 1997).

Transcription factors influencing the proliferation and survival of haematopoietic multipotential progenitors are GATA-2, c-myb, and PU.1. The transcription factor GATA-2 is a zinc-finger protein and a member of the GATA-family. GATA-2 is thought to be important for the expansion and/or maintenance of the haematopoietic progenitor/stem cell population. Null mutation of GATA-2 does not eliminate the generation of haematopoietic progenitors but significantly impairs their expansion and/or maintenance, resulting in severe fetal liver anaemia and embryonic death at around 10.5 dpc (Tsai et al, 1994).

C-myb, a proto-oncogene encoded transcription factor is expressed at the highest level in immature haematopoietic progenitor cells (Weston, 1990). Mice lacking c-myb initially develop normally, but die of severe anaemia at the fetal liver stage of haematopoiesis (~15 dpc) (Mucenski et al, 1991). *In vitro* studies indicate that c-myb regulates the expression of proliferation-promoting genes or inhibits the transcription of differentiation-promoting genes (Badiani et al, 1994; Gonda et al, 1989). Primitive yolk sac haematopoiesis in c-myb -/- mice appears normal, but the number of myeloid and erythroid precursors for definitive haematopoiesis in fetal liver is greatly reduced, whereas megakaryocyte differentiation is not affected. These findings in mutant phenotype have been interpreted that c-myb has a function in controlling proliferation

of multipotential progenitors. The selective effect on definitive haematopoiesis is thought to be suggestive that the genetics of primitive and definitive stem cells may be different.

The transcription factor PU.1 (the product of the Spi-1 proto-oncogene) is a haematopoietic-specific member of the *ets* family. PU.1 is widely expressed in the haematopoietic system, principally in monocytes/macrophages, and B lymphocytes, but also in erythroid cells and granulocytes (Scott et al, 1994). Mutation of the PU.1 gene causes a defect in generation of B lymphocytes, monocytes and granulocytes, as might be predicted from the expression pattern. However, development of T lymphocytes is also defective, whereas erythrocyte and megakaryocyte progenitors are normal (Baribault et al, 1996; Scott et al, 1994). The absence of functional PU.1 results in death around embryonic day 18 due to abolished myeloid and lymphoid development (Scott et al, 1994). It is thought PU.1 might function either by regulating the development of a multipotent lymphoid/myeloid progenitor cell or through independent function in distinct lineages (Scott et al, 1994; 1997).
Transcription factors that appear restricted in their function to regulating the development of one or a few lineages are GATA-1 and Ikaros. These transcription factors probably function downstream within the haematopoietic hierarchy regulating decisions within the erythroid or lymphoid progenitor population, GATA-1 or Ikaros respectively.
Expression of the zinc finger protein GATA-1 within haematopoietic cells is restricted to the erythroid, megakaryocyte, eosinophil and mast cell lineages and multipotential progenitors (Shivdasani and Orkin, 1996). An essential role for GATA-1 in erythroid cell differentiation was demonstrated by disruption of the single X-linked gene in mouse ES cells and analysis in cell culture and chimaeric mice (Pevny et al, 1991; Simon et al, 1992). Lack of a functional GATA-1 results in the inability to complete the differentiation program of the erythroid lineage, due to arrest in maturation.

This is observed in both erythroid lineages, primitive and definitive (Pevny et al, 1995). GATA-1$^-$ embryos are embryonic lethal at the yolk sac stage (Fujiwara et al, 1996). These studies of targeted GATA-1 disruption provided strong evidence that GATA-1 is a key regulator of erythroid differentiation with survival and terminal maturation of erythroid precursors critically dependent on functional GATA-1.
The transcription factor Ikaros is thought to play a role analogous to that of GATA-1 within the lymphoid lineage. The Ikaros gene encodes lymphoid-restricted zinc finger transcription factors, which can be produced in six isoforms owing to differential splicing. Ikaros is expressed exclusively within lymphocytes and early haematopoietic progenitors and is thought to regulate the expression of a number of lineage-specific genes (Georgopoulos et al, 1992). Gene targeting in the Ikaros locus resulted in a complete absence of mature T and B lymphocytes and natural killer cells and a lack of early lymphoid progenitors (Georgopoulos et al, 1994; Wang et al, 1996; Winandy et al, 1995). In addition, lymph nodes, Peyer's patches, lymphocyte follicles and a mature thymus were absent in mutant mice. Results from these studies implicate Ikaros as a pivotal mediator of cell differentiation operating at the earliest stages of maturation of all lymphoid lineages including natural killer cells.
Although targeted gene disruption studies in mice provide considerable insight in haematopoietic transcription factor function and have led to the identification of critical transcription factors in the biology of HSCs, they have been less gainful for the identification and isolation of the HSC. Progress in order to understand haematopoiesis as a complete system in its entire complexity depends in part on complementary experiments using several different experimental strategies and methodologies.

1.3 Model systems for studying haematopoiesis

A variety of *in vitro* systems has been established by using haematopoietic precursors derived from various explanted adult or fetal tissues to complement *in vivo* studies of haematopoietic development and haematopoiesis (Medvinsky and Dzierzak, 1996; Yu et al, 1993).

Various cell types e.g. long-term bone marrow cell cultures, leukaemic cell lines, cell lines derived from different embryonic tissues such as fetal liver or embryonic yolk sac and cell lines possessing a stable pluripotent embryonic phenotype, such as embryonic teratocarcinomas, primordial germlines and embryonic stem cells, have been successfully used for studying cellular and molecular events of haematopoiesis *in vitro*.

Although very useful and amenable to various methodological techniques, most models are limited to certain sections and/or developmental stages of haematopoiesis and cannot recapitulate the process of haematopoiesis as a whole. Early developmental issues such as the regulation of mesoderm commitment to the haematopoietic lineages and the establishment of the haematopoietic system during embryogenesis are difficult to address due to the inaccessibility of mammalian embryos and difficulties to develop *in vitro* models that recapitulate the *in vivo* circumstances. Thus, most knowledge of the cellular and molecular processes during haematopoiesis is obtained from studies of the established haematopoietic system.

In addition to these limitations, it is not sufficient to merely identify genes or factors that are expressed in a manner consistent with a role in haematopoiesis, systems in which normal and/or abnormal function can be addressed are essential for an in-depth understanding of haematopoietic development and haematopoiesis. A model system that allows to recapitulate early haematopoietic development, that allows to identify and study the function of genes involved in haematopoiesis *in vivo* and *in vitro* and offers a method for genomic manipulation has become available with the use of embryonic stem cells for studying haematopoiesis.

1.3.1 Embryonic stem cells, *in vitro* model for haematopoiesis

The derivation of embryonic stem (ES) cells and their use for studying haematopoiesis has greatly advanced the understanding of haematopoietic development. ES cells are relatively accessible, easy to manipulate and allow circumvention of many of the limitations encountered from other cell types used for haematopoietic studies. They provide an alternative strategy for the identification and functional analysis of molecular control mechanisms in haematopoiesis and for studying developmental processes, both *in vitro* and *in vivo*.

ES cell lines are totipotent cell lines, derived directly from the inner cell mass (ICM) of preimplantation 3.5 dpc mouse blastocysts, and represent primary cultures with a high differentiation ability *in vitro* and *in vivo* (Evans and Kaufman, 1981; Martin, 1981). During normal mouse development, the ICM of a blastocyst develops into the embryo proper and the trophectoderm of the blastocyst gives rise to the extraembryonic membranes. When grown *in vitro*, ICM cells can be kept in an undifferentiated state as ES cells. The establishment of ES cells from ICM involves attachment of isolated blastocysts to tissue culture dishes, mimicking implantation and causing the trophectoderm to spread into a monolayer exposing the ICM. The proliferating ICM forms a cell clump which is disaggregated and the totipotent cells are cultured on fibroblast feeder cell layers to avoid differentiation (Robertson 1986, 1987). ES cells can be maintained *in vitro* in their undifferentiated state for extended periods of time by co-culturing on fibroblast feeder cell layers or in a feeder-free culture system in the presence of leukaemia inhibitory factor (LIF), also known as differentiation inhibitory activity (DIA) (Williams et al, 1988; Smith et al, 1988).

LIF is a glycoprotein, identified as the essential cytokine enabling ES cells to maintain in an undifferentiated state and to retain totipotent differentiation potential. ES cells closely resemble their normal *in vivo* counterparts and have a high differentiation potential *in vivo* and *in vitro* (Bradley et al, 1984).

When reintroduced into mouse blastocysts the differentiation potential of ES cells

can be seen in the high efficiency of ES cell chimera production and the contribution of ES cells to all three germ layers in resulting chimaeric mouse embryos, suggesting a high potential of ES cells to develop along all lineages of the embryo proper (Robertson, 1986). The ability of ES cells to contribute to the germline of a chimera allows to propagate the genome of ES cells through germline transmission. ES cell chimeras can be produced by injection of ES cells into host blastocysts (Hogan et al, 1994) or by aggregation of ES cells with morula cells (Nagy et al, 1990). The blastocyst injection method involves the injection of ES cells into pre-implantation embryos whereas the morula aggregation method involves the aggregation of 8-cell stage embryos with ES cells.

Embryos that are entirely derived from ES cells can be produced when two tetraploid 4-cell stage embryos are used for the aggregation with ES cells (Nagy et al, 1990). This results from observations that ES cells and tetraploid blastomers appear to have opposite developmental capabilities that can complement each other in aggregation chimeras, resulting in the formation of polarized chimeras in which the fetuses are ES cell derived and most of the extraembryonic tissues are provided by tetraploid components (Nagy et al, 1990, 1993). Mice entirely ES cell derived often die postnatally, with the viability depending on mouse strain and passage number of the ES cells used (Ueda et al, 1995; Carmeliet et al, 1996; Nagy et al, 1993). Although a limitation, this has not excluded further analysis of the haematopoietic development using entirely ES cell-derived embryos. Repopulation of the haematopoietic system of lethally irradiated recipients by transplantation of HSCs from the fetal liver of ES cell-derived embryos has been reported (Forrester et al, 1991). Embryos entirely derived from ES cells demonstrated, ES cells can maintain the potential to develop into embryos without positional or developmental cues from viable endogenous embryonic cells after *in vitro* culturing. Furthermore, it suggested that differentiation *in vitro* might provide a useful tool for examination of developmental control mechanisms.

In vitro differentiation of ES cells results in the formation of embryoid bodies (EBs) with endodermal, mesodermal and ectodermal layers (Doetschman et al, 1985). When LIF is withdrawn, ES cells will differentiate spontaneously into many different embryonic cell lineages including haematopoietic, endothelial, muscle and neuron lineages (Evans and Kaufman, 1981; Martin, 1981; Bradley et al., 1984; Doetschman et al, 1985).

The spontaneous differentiation pattern of ES cells can be influenced by the addition of exogenous factors or chemical inducers such as retinoic acid (RA) and dimethyl sulphoxide (DMSO) or by interaction of ES cells with supportive stromal cells.

RA has been found to induce in a concentration dependent manner neural and skeletal muscle differentiation (Bain et al, 1995; Wobus et al, 1994) but has also been found to inhibit haematopoietic differentiation (personal observation; Doostdar, 1997).

DMSO has been found to induce haematopoietic differentiation of ES cells (personal observation; Doostdar, 1997).

The capacity of undifferentiated ES cells to differentiate *in vitro* into terminally differentiated cell lineages makes them a valuable tool for studying early developmental processes. The appearance of terminally differentiated haematopoietic cells further indicates the intermediate presence of HSCs during ES cell differentiation. This suggests that some ES cells must commit to the haematopoietic system and become HSCs or cells with haematopoietic stem cell activity at some point between the undifferentiated state of being an ES cell with no direct haematopoietic potential and differentiating into an EB containing a range of mature haematopoietic lineages.

Several laboratories have taken advantage of the ES cell *in vitro* model system to study the early development of haematopoietic cells and various methods have been developed to achieve ES cell differentiation into haematopoietic cells (Burkert et al, 1991; Schmitt et al, 1991; McClanahan et al, 1993; Müller and Dzierzak, 1993; Keller et al, 1993; Hole et al, 1996).

Differentiating ES cells can give rise to both lineages of the haematopoietic system,

the lymphoid and the myeloid lineages, with all their mature cell types. Haematopoietic differentiation of ES cells *in vitro* is highly reproducible and provides access to populations of early precursors that are difficult, if not impossible, to access *in vivo*. Culture systems used to induce haematopoietic differentiation of ES cells include suspension cultures or cultures containing methyl cellulose medium, the formation of ES cell aggregates in hanging drop cultures prior to differentiation in suspension culture, and co-culturing of ES cells in direct contact with stromal supportive cells (Keller et al, 1993; Wiles and Keller, 1991; Hole et al, 1996; Nakano et al, 1994). These methods involve the removal of ES cells from contact with feeder cells or presence of LIF and provide culture conditions were ES cells generate aggregates of differentiating ES cells, referred to as embryoid bodies (EBs).

The appearance of haematopoietic cells during *in vitro* differentiation of ES cells in suspension culture is routinely observed as yolk sac like blood islands of haemoglobinised erythroid cells within developing EBs (Doetschman et al, 1985) and occurs spontaneously in the absence of exogenous growth factors, except those present in serum. The erythropoietic activity observed in EBs resembles the blood islands of embryonic yolk sac and contain erythroid cells and macrophages (Doetschman et al, 1985). However, EBs also contain progenitor cells capable of giving rise to myeloid and lymphoid cells (Burkert et al, 1991; Schmitt et al, 1991; McClanahan et al, 1993; Keller et al, 1993; Hole and Smith, 1994; Wiles and Keller, 1991; Gutierrez-Ramos and Palacios, 1992).

Cellular interactions within developing EBs resemble *in vivo* events between haematopoietic cells and their microenvironment, with the three dimensional structure of EBs providing a supportive microenvironment for *in vitro* haematopoiesis. Similar to haematopoietic tissues, EBs are composed of a range of heterogeneous cell types. Haematopoietic differentiation of ES cells has also been reported from ES cells induced to differentiate as single cells, cultured directly on a stromal cell layer.

ES cells differentiating on a cell layer of the stromal cell line OP9, which is derived from M-CSF deficient op/op mice (Yoshida et al, 1990), are able to develop into erythroid, myeloid and B cell lineages (Nakano et al, 1994).

Although *in vitro* differentiation of ES cells has the potential to allow the rapid analysis of cytokine effects, singly and in combination, most ES cell culture systems are supplemented with serum which complicates analysis. Nevertheless, addition of cytokines has been found to increase haematopoietic differentiation of ES cells. For instance, culture medium supplemented with Epo increases the number of erythroid cells in EBs. Addition of IL-3 has been found to increase erythrocytes, macrophages, mast cells and neutrophils (Wiles and Keller, 1991; Bigas et al, 1995). However, some factors lead to contradictory results. It has been argued that discrepancy between studies may, at least to some part, be due to the presence of serum in culture medium since serum contains variable amounts of factors and many other ill-defined substances. To overcome these problems a serum-free chemically defined medium for an ES cell culture system has been developed. ES cell differentiation to mesoderm in chemically defined medium was reported to be responsive to exogenously added factors (Johansson and Wiles, 1995). Although a promising approach, prolonged support of ES cell growth in an undifferentiated state was not achieved and spontaneous differentiation was observed. Although ES cell culture systems for the analysis of cytokine effects have been improved, it is important to note that EBs themselves produce cytokines and effects of added exogenous factor may result from direct and/or indirect action (Schmitt et al, 1991; McClanahan et al, 1993; Keller et al, 1993).

Haematopoietic differentiation within EBs, as defined by the onset of expression of haematopoietic specific genes and the appearance of specific haematopoietic precursor populations, has been found to follow an ordered sequence of events similar to those observed in the developing embryo (Schmitt et al, 1991; Burkert et al, 1991; Keller et al, 1993; McClanahan et al, 1993). Evidence for haematopoietic gene expression has been revealed in undifferentiated ES cells and during differentiation into EBs. In undifferentiated ES cells expression of Thy-1, c-kit, SCF can be detected, however, after induction of ES cell differentiation temporal expression of genes involved in early haematopoietic development in the embryo can be detected in EBs. Genes involved in erythroid differentiation such as adult and fetal type ß-globin and GATA-1 are among the first lineage specific genes expressed. Expression of cytokines such as IL-4, IL-6, G-CSF, M-CSF and their cognate receptors is detectable at later time points, with cytokine receptor transcripts in general expressed before cytokine transcripts (Schmitt et al, 1991; Keller et al, 1993; McClanahan et al, 1993; Hole et al, 1996). These gene expression studies are further supported by the kinetics of development of haematopoietic cell lineages within EBs.
The primitive erythroid lineage (nucleated erythrocytes) appears as the earliest haematopoietic population followed by the development of the definitive erythroid (enucleated erythrocytes) and myeloid lineages. This pattern of haematopoietic development within EBs strongly suggests that the molecular mechanisms involved in the establishment of the haematopoietic system *in vivo* also function within EBs *in vitro* (Schmitt et al, 1991; Keller et al, 1993; McClanahan et al, 1993).
Precursors with lymphoid potential defined by the expression of lymphocyte specific surface molecules, e.g. B220 and Thy-1 and by the ability to rearrange immunoglobulin (Ig) and T-cell receptor (TCR) genes have been observed later in EB differentiation, indicating that the lymphoid developmental programme is established following the development of the erythroid and myeloid lineages (Nakano et al, 1994; Potocnik et al, 1994).

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Analysis of the haematopoietic characteristics of ES cells by colony forming assays demonstrated the ability of ES cells to give rise to primitive haematopoietic progenitors as observed by the formation of haematopoietic colonies (Bigas et al, 1995; Hole et al, 1996). Although ES cell-derived haematopoietic progenitor and mature blood cells and the temporal sequence of their appearance suggest the presence of ES cell-derived HSCs within developing EBs, efforts to identify and isolate such primitive cells have not been successful to date.

Reconstitution of immunodeficient SCID or RAG-$2^{-/-}$ mice or lethally irradiated mice with ES cell derived haematopoietic cells has been reported from a number of studies (Palacios et al, 1995; Nisitani et al, 1994; Müller and Dzierzak, 1993; Hole et al, 1996). However, observations of the lymphoid potential and the longterm repopulating potential of ES cell derived cells in recipient mice varied. When transplanted into RAG-$2^{-/-}$ recipients, ES cell-derived cells have been shown to generate both B lymphocytes expressing surface immunoglobulin and $CD3^{+}$ T lymphocytes (Nisitani et al, 1994). Multilineage repopulation of SCID recipients with haematopoietic cells of ES cell origin has been reported on the basis of MHC class I antigen expression (Palacios et al, 1995). Limited lymphoid reconstitution potential of ES cells was observed after transplantation into lethally irradiated recipients and analysis by CFU-S assays (Müller and Dzierzak, 1993). Generation of primitive longterm repopulating and transiently engrafting stem cells in an ES cell culture system was demonstrated by the longterm survival and multilineage reconstitution of lethally irradiated mice by ES cell derived haematopoietic cells (Hole et al, 1996). Despite some discrepancy in observations between studies it is apparent that extensive haematopoietic differentiation can be achieved in ES cell culture systems which provides a unique model for studying haematopoietic development and haematopoiesis.

While identification and isolation of primitive haematopoietic stem cells/progenitor cells within differentiating EBs on morphological characteristics is not currently

possible, separation of these cells on a temporal basis has been achieved (Hole et al, 1996). The ability to clearly identify the time point of emergence of very primitive haematopoietic stem cells within an ES cell system allows to concentrate efforts and studies on EB cells containing HSCs and haematopoiesis supporting cell types. The use of such an exceptional model of haematopoiesis for molecular and mutagenic analysis is at present one of the most promising approaches for understanding haematopoiesis.

1.3.2 Insertional mutagenesis using ES cells

One strategy toward understanding haematopoietic development and haematopoiesis is to identify and functionally analyze genes required for these processes. *In vitro* mutagenesis in ES cells is a powerful alternative approach to classical *in vivo* mutagenesis, which enables the characterization of mutations in cell culture prior to the generation of mutant mice for studying gene function *in vivo*. Before the availability of ES cells for mutagenesis most mutations in mice arose spontaneously or were induced by radiation or chemical mutagens. Analysis of these 'anonymous' mutations was largely dependent on phenotype-driven screening strategies which require a large investment of manpower and resources. ES cells are applicable to a large number of mutagenic strategies, including analysis of spontaneous mutations, chemical mutagenesis and insertional mutagenesis (reviewed by Hill and Wurst, 1993). However, it is insertional mutagenesis, employing gene targeting or gene trapping methodology, which has proved to be the most advantageous strategy for analyzing developmental processes. The ability to efficiently mutate the mouse genome in a directed and subtle manner by combining insertional mutagenesis and ES cell systems has revolutionised transgenic analysis of mammalian development. Gene targeting in ES cells by homologous recombination enables the introduction of specific mutations into the mouse germ line in virtually any gene, whereas the gene trapping strategy randomly targets genes providing the means

for identifying novel genes. Both strategies produce defined tagged mutations and allow screening for the genetic alteration and characterization *in vitro* before the gene expression and gene function is studied by mutational analysis *in vivo*. Genetically altered ES cells are able to retain their differentiation potential and normality *in vivo* when reintroduced into the mouse blastocyst and establish themselves in the mouse germ line (Robertson et al, 1986; Robertson 1991).

1.3.2.1 Gene targeting using ES cells

Gene targeting by homologous recombination provides the highest possible level of control over producing mutations in cloned genes and has become a standard tool in the molecular dissection of function in developmental processes (Capecchi, 1989; Ramirez-Solis, 1993). In principle, gene targeting by homologous recombination involves the introduction of exogenous DNA with sequence homology to the gene of interest and a selectable marker gene. Depending on the design of the targeting vector, the homologous recombination event can yield a deletion, replacement, or insertion in the targeted genomic locus.

The vast majority of initial gene targeting strategies were designed to inactivate genes and produce null (knock-out) mutants for testing where and when in development a gene is required. Although the generation of knock-out animals has been very useful in the dissection of the function of some genes and has confirmed previous studies e.g. for the control of haematopoiesis by transcription factors such as PU.1, GATA-1, SCL, assessing the phenotypes of other gene knock-out animals has not been so straightforward as was assumed (Olson et al, 1995; Pevny et al, 1995; Shivdasani et al, 1995). For instance, in some cases the disruption of a gene can result in no discernible phenotype for the organ system analysed e.g. the cytokine GM-CSF (Dranhoff and Mulligan, 1994). Many developmental or regulatory processes are controlled by overlapping often highly plastic interactions, which makes it unlikely for

single gene disruptions to reveal their full involvement and function in development and regulation. A strategy to address these observations is the production of mice with multiple gene deletions. The other extreme of phenotypes are gene knock-out models which result in early embryonic lethality, e.g. receptor tyrosine kinase Flk-1 (Shalaby et al, 1995). Embryonic death of knock-out models hinders any attempt to study the role of these genes in downstream events or the role in other developmental processes. Strategies to overcome restrictions for analysis caused by embryonic lethality include molecular approaches by the generation of more subtle mutations and/or tissue-specific gene disruptions and *in vitro* differentiation and chimera production with knock-out ES cells.

In general, four complementary approaches are used to analyse the consequences of targeted mutations in genes suspected of functioning in haematopoiesis: (1) analysis of homozygous and/or heterozygous knock-out embryos and mice; (2) analysis of cell lineage contribution and distribution of homozygous and/or heterozygous knock-out ES cells in chimeric embryos and mice (3) analysis of haematopoietic chimeras generated by transplantation of homozygous and/or heterozygous knock-out ES cells into immunodeficient SCID or RAG-$2^{-/-}$ or lethally irradiated recipients and (4) *in vitro* differentiation of homozygous and/or heterozygous knock-out ES cells.

These complementary approaches have identified several genes that function in various aspects of haematopoietic development. For instance gene targeted animal models have been produced for haematopoietic transcription factors, for cytokines and cytokine receptors, and for haematopoietic adhesion molecules. Some examples have been described in this chapter.

Gene targeting strategies have greatly advanced the analysis of gene function in haematopoietic development and haematopoiesis, however, the gene targeting methodology cannot easily be adapted to a large scale approach. Each targeting vector has to be individually constructed, is dependent on the availability of genomic DNA sequence information of the target gene to be cloned and/or sequenced, and each

targeting event must be individually verified, with each of these steps requiring comprehensive efforts to be achieved. In contrast, random insertional mutagenesis of ES cells is relatively easy to achieve and could provide the basis for rapid and functional analysis of the murine genome.

1.3.3.2 Gene trapping using ES cells

Gene trapping is an alternative approach for the identification and functional characterization of genes that randomly targets genes but can be used to detect genes potentially important in a developmental process of interest by specific screening strategies. Gene trapping does not depend on the availability of genomic DNA sequence information, thus can be used in large-scale screens of the genome in order to detect novel genes involved in developmental processes. This strategy applies the rationale that developmentally regulated genes may code for developmentally important molecules. Evidence for the success of applying random mutagenesis to dissecting developmental pathways is ample demonstrated by studies of *Drosophila* or *C. elegans* development (Cooley et al, 1988; Hope, 1991).
Unlike random mutagenesis by chemical mutagens or X-rays, the gene trapping strategy produces random mutations by the insertion of a gene trap construct into the genome and allows insertions into transcribed loci to be detected from among a large group of random insertions. Gene trap constructs are designed so that the only possibility of activating a reporter gene is if the construct integrates into the proper position to be transcribed under the transcriptional control of a genomic locus. This causes a tagged mutation and allows the regulation of the locus to be studied by assaying the activity of the reporter gene.
A number of vector constructs for gene trapping have been described (Friedrich and Soriano, 1993; Hill and Wurst, 1993). Basically two types of gene trapping vectors that differ in their requirements for reporter gene activation have been developed, the

promoter trap vectors and the gene trap vectors. A schematic description of the two basic gene trapping vectors is represented in Figure 1.2.
Promoter trap vectors simply consist of a promoterless reporter gene the expression of which requires insertion of the vector into an exon of a gene to generate a fusion transcript between the trapped endogenous gene and the reporter gene. Gene trap vectors contain a splice acceptor sequence upstream of a promoterless reporter gene, thereby forcing the reporter construct to be processed as a separate exon. Integration of this type of vector into an intron results in the generation of a fusion transcript between the endogenous gene and the reporter gene through the use of the splice acceptor. Since introns rather than exons are the targets of a gene trap vector, the number of target sites in the genome is much greater than for a promoter trap vector. Both vector types are likely to act as an insertional mutagen by disrupting the endogenous function of the trapped gene. Promoter trap vectors by being an additional sequence fragment to an endogenous exon and gene trap vectors by acting as an 'artificial' additional exon. Although the vector constructs randomly target different integration sites, exons for promoter trap vectors and introns for gene trap vectors, both must be trapped into a transcriptional active gene in order for a clone to survive in the presence of a drug selecting for positive clones.
Conventional gene trap constructs commonly utilize the neomycin resistance gene (neo^R) as the selectable marker and a promoterless lacZ gene (ß-galactosidase or ß-gal) as the reporter which is easily assayed for monitoring the transcriptional activity of the endogenous gene. Since ß-gal activity is not a selectable marker the addition of a selectable marker in a gene trap construct is essential. However, the addition of a selectable marker gene with separate promoter elements produces a background of total random insertions without ß-gal activity. Thus, screening for ß-gal activity is performed on numerous random integrants without a trapping event. To circumvent this limitation, a selectable reporter gene, ß-geo, which is a translational fusion between ß-gal and neo and encodes a protein with both activities has been developed

(Friedrich and Soriano, 1991). The use of the ß-geo reporter gene thus allows direct selection of gene trapping events while maintaining the ability to detect patterns of endogenous gene expression by monitoring ß-gal activity. However, it must be noted that the use of a selectable reporter with combined selection and reporter activity results in a gene trap clone population for genes expressed in undifferentiated ES cells and possible later during development. Genes not expressed in undifferentiated ES cells but later in development are not included in this population since survival of the clones is dependent on the expression of the gene for drug resistance which is under the transcriptional control of the trapped gene.

Gene trap constructs are routinely inserted into ES cells by electroporation, which is a simple means to deliver DNA, or by retroviral infection. ES cell clones that contain gene trap events are selected by drug resistance and expanded as undifferentiated clones *in vitro*. The *in vitro* and *in vivo* expression of the trapped gene can easily be assayed by staining ES cells or ES cell derived embryos for ß-gal activity. Using a chromogenic substrate (X-Gal), the spatial and temporal activity of the trapped locus in ES cells or tissues expressing ß-gal can be monitored by a blue precipitate.

Generating numerous ES cell lines containing gene trap constructs is relatively simple and requires only basic tissue culture and molecular biological skills. However, it is the subsequent screening strategy of gene trap clones that determines the efficiency and success of an experimental approach employing the gene trapping methodology. Initial screening procedures of gene trap clones for mutations affecting early embryogenesis concentrated on the establishment and phenotypic screening of mutant mouse lines or as a prescreening strategy, on the production of ES cell chimera embryos (Friedrich and Soriano, 1991; Gossler et al, 1989). *In vivo* screening of gene trap clones thus involves the production of a large number of chimeric embryos which requires considerable technical expertise, as well as being very demanding on time and resources. The rate limiting step for a large scale gene trap screen is therefore the

in vivo analysis and not the isolation of ES cell lines containing gene trap integrations (Friedrich and Soriano, 1991; Wurst et al, 1995; Joyner, 1991).

Recently, several modified trapping strategies have been introduced that allow specific selection for integrations in particular subsets of genes (Skarnes et al, 1995; Forrester et al, 1996, Baker, 1997). General strategies that can be used to achieve specificity of a gene trapping approach for the field of interest include (1) the design of gene trap constructs that rely on the integration in certain sequences of endogenous genes for reporter gene expression or (2) the use of 'conventional' gene trap constructs combined with a very specific and stringent prescreening strategy prior to analysis in chimeric or transgenic animals.

The use of a 'secretory trap' vector which relies on capturing the N-terminal signal sequence of an endogenous gene for reporter gene expression was reported as a prescreen strategy for trans-membrane or secreted proteins in ES cells (Skarnes et al, 1995). Others developed an induction gene trap screen that preselected gene trap clones *in vitro* for integrations into genes that lie downstream of receptor/ligand-mediated signalling pathways. By monitoring the reporter gene expression of gene trap clones after exposure to retinoic acid, gene trap integrations induced, repressed or non-responsive to retinoic acid were identified (Forrester et al, 1996).

A preselection for developmentally regulated genes that are not constitutively expressed but expressed in selected cell types was based on *in vitro* differentiation of ES cell gene trap clones into EBs in conditions supporting differentiation into neuronal cell lineages. The differentiated ES cell gene trap clones were than assayed for co-expression of ß-gal and antigens present within neuronal cell lineages.

Four gene trap clones with single copy integrations were chosen for generating chimeric embryos and found to be expressed in the developing tissues as indicated from *in vitro* analysis (Baker et al, 1997). In addition to screening based on ß-gal expression the generation of fusion transcripts facilitates to directly clone the transcribed region upstream of the insertion site using the 5' RACE protocol (5' rapid

amplification of cDNA ends) (Skarnes et al, 1992). The cDNA sequence information allows identification of homologies to known sequences and novel sequences and can be used as probes to examine the expression pattern of their cognate genes.

In vitro prescreening strategies for gene trapping events have proven to be powerful alternative approaches to enhance the utility of this technology. Identification of genes that might be important in early developmental processes is clearly indicated with much effort directed toward improving prescreen strategies and refining *in vitro* differentiation of ES cells. Combining gene trapping and *in vitro* haematopoietic development in ES cells may prove to be one of the most promising avenues in identifying HSCs and molecular mechanisms of haematopoietic development.

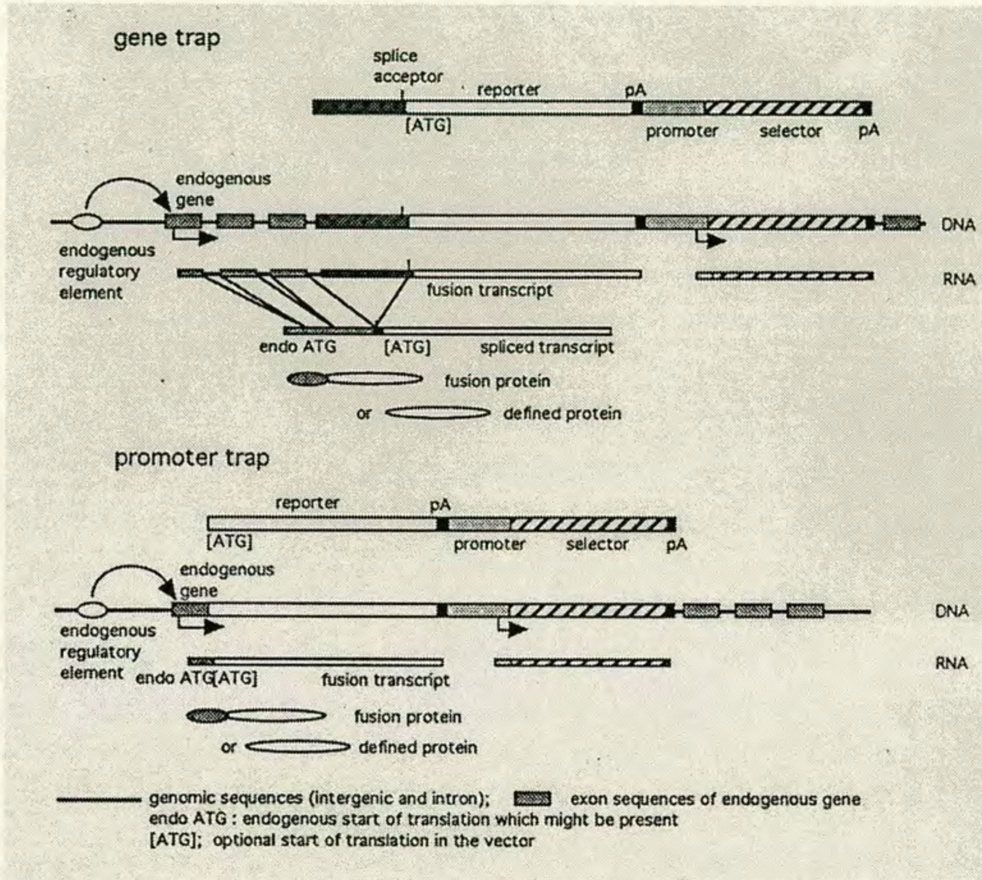


Figure 1.2: A schematic description of the general strategy of gene trapping.
(Top) Gene trap: A splice acceptor site is linked to the reporter gene. Insertions into an intron of an endogenous gene generates fusion transcripts through the use of the splice acceptor.
(Bottom) Promoter trap: Insertions into an exon of an endogenous gene is required for the generation of fusion transcripts (from Gossler and Zachgo, 1993).

2. *AIMS AND OBJECTIVES*

The *in vitro* differentiation properties of ES cells are widely utilized for the study of developmental processes. The ES cell system (EFC-1), on which this project is based has previously been used to investigate the cellular and molecular control of early haematopoietic differentiation and to establish the culture conditions required for the development of transplantable *in vitro* HSCs. A highly reproducible ES cell culture system has been developed in which the temporal pattern for the presence of multilineage long-term repopulating HSCs has been defined (Hole et al, 1996). These features of the ES cell system make it a valuable foundation for the present project, (1) as a basis and standard for culture conditions and haematopoietic differentiation of gene trap cell lines, and (2) as a source for the study of HSCs in relation to their microenvironment.

The aim of the present project has been to characterize the molecular events which accompany the haematopoietic differentiation process by the means of the identification and characterization of novel genes and by the refined analysis of expression patterns of known haematopoietic genes.

Various strategies can be applied for the identification of novel genes involved in developmental processes, one of which is the gene trapping approach in ES cells. In order to ensure effective and efficient use of resources, *in vitro* pre-screening and characterization of gene trap clones used for analysis *in vivo* by chimeric embryo production would be advantageous. Therefore part of this project seeks to design an *in vitro* pre-screening strategy specific for the enrichment of gene trap cell clones with trapped haematopoietic genes from a gene trap clone library.

The ability of ES cells to form complex, three-dimensional developmental aggregates, EBs, during *in vitro* differentiation allows the study of haematopoietic cells in relation to their haematopoietic microenvironment. The temporal pattern of the presence of

transplantable HSCs in the ES cell system used in this study provides a powerful model system for the identification and isolation of HSCs. Although EBs develop morphological features, they do not display a morphological organization as identified in mouse embryos, thus making it difficult to identify specific areas within the EB. The detection of specific haematopoietic transcripts within intact EBs can give crucial clues about the morphological organization and localization of haematopoietic cells. The identification of haematopoietic areas within EBs will be the starting point for the analysis of the spatial relationship of haematopoietic cells and their microenvironment. It is also likely to provide information to devise a strategy for the isolation of the HSCs present during the *in vitro* differentiation of ES cells. The temporal gene expression pattern during *in vitro* differentiation of ES cells can be studied by standard methodologies such as RT-PCR analysis, however, the analysis of spatial gene expression is more complicated. Means must be used to maintain the morphological structure of the EBs but also for the analysis of gene expression within an EB.

This project seeks to develop conditions suitable for the spatial analysis of gene expression in EBs, based on non-isotopic *in situ* hybridization methodology, in order to identify and analyse the co-ordinate gene expression during haematopoietic differentiation *in vitro*.

3. *MATERIAL AND METHODS*

3.1. Embryonic Stem Cell Culture Procedures

3.1.1 Solutions and Media for Embryonic Stem Cell System

All solutions and media used for the embryonic stem cell system were prepared with autoclaved tissue culture grade water.

3.1.2 General Solutions for Embryonic Stem Cell System

Phosphate Buffered Saline (PBS)

One litre of PBS solution in tissue culture grade water contained 10 PBS tablets. This solution was autoclaved.

Trypsin Solution (TVP)

TVP solution consisted of 250 mg trypsin, 372 mg EDTA disodium dissolved in 1 litre PBS supplemented with 10 ml chicken serum. This solution was filter sterilized and stored in aliquots at -20°C.

Gelatine Solution

A 1% (w/v) gelatine stock solution was prepared with tissue culture grade water, autoclaved and stored at 4°C. Stock solution was diluted with PBS to obtain 0.1% (w/v) gelatine solution.

3.1.3 Preparation of Media for Embryonic Stem Cell System

Freshly prepared media were tested for sterilisation by adding 5 ml of medium to 5 ml tryptose phosphate broth and incubation at 37°C for 24-48 hours.

Culture medium (FCS-Medium)

FCS-medium consisted of 1x Glasgow's Modified Eagle's Medium (GMEM), 0.2% Sodium bicarbonate, 1% non-essential amino acids, 2% L-Glutamine/Pyruvate (stock solution 1:1, L-Glutamine (200 mM) : Pyruvate (100 mM)), 0.1 mM 2-mercaptoethanol supplemented with 10% fetal calf serum (culture medium).

Differentiation medium (DIF-Medium)

DIF-medium consisted of 1x Glasgow's Modified Eagle's Medium (GMEM), 0.2% Sodium bicarbonate, 1% non-essential amino acids, 2% L-Glutamine/Pyruvate (stock solution 1:1, L-Glutamine (200 mM) : Pyruvate (100 mM)), 0.1 mM 2-mercapto-ethanol supplemented with 10% fetal calf serum (differentiation medium).

3.1.4 Embryonic Stem Cell Lines and 'Gene Trap' Cell Lines

The embryonic stem (ES) cell lines used were EFC-1 (Nichols et al, 1990), R1 (Nagy et al, 1993), and CGR8 (Mountford et al, 1994). ES cell lines containing a gene trap integration are referred to as 'gene trap' lines in this study. Gene trap cell lines I114 and R68, both derived from the ES cell line R1, contain conventional gene trap vectors with a splice acceptor sequence fused to the reporter gene *lacZ* and the bacterial neomycin-resistance gene driven by the phosphoglycerate kinase-1 (PGK1) promoter. *In vivo* expression of I114 was found to be restricted to yolk sac (YS) and fetal liver (FL), R68 was found to be expressed in foetal heart and various other areas (Forrester et al, 1996). Gene trap cell line ST598, derived from the ES cell line CGR8, contains a 'secretory trap' vector with the reporter gene ß-geo, which is a *lacZ*-neomycin phosphotransferase fusion gene linked to a splice acceptor sequence, a signal sequence

and a transmembrane domain, which relies on capturing the N-terminal signal sequence of an endogenous gene to generate an active ß-gal fusion protein. *In vivo* expression of this gene trap cell line has been found to be restricted to YS and FL (Skarnes et al, 1995; Skarnes W, personal communication). Gene trap cell line Zin40 contains a gene trap construct with a splice acceptor sequence linked to the ß-geo reporter gene and is ubiquitous expressed *in vivo* (Smith A, personal communication). Gene trap cell line Zin40 was derived from the ES cell line CGR8.

3.1.5 Embryonic Stem (ES) Cell Maintenance

Unless stated otherwise, the centrifuge used was a MSE Mistral 1000 containing a 61080-147 swing out rotor. ES cell lines and 'gene trap' lines were routinely passaged and maintained in an undifferentiated state under feeder-free culture conditions in the presence of leukaemia inhibitory factor/differentiation inhibiting activity (LIF/DIA), as described (Smith, 1991; Hole and Smith, 1994). All experiments described used cell lines of less than 30 passages. Unless specified, the term ES cells used for describing culture procedures covers ES cell lines without and with gene trap integration. Undifferentiated ES cells were maintained on gelatinized tissue-culture flasks in FCS medium supplemented with LIF at a concentration of 100 U/ml (Smith et al, 1988). LIF was obtained from the supernatant of Cos-7 cells transfected with a LIF expression plasmid. All of the solutions used were prewarmed to 37°C in a waterbath. 25 cm^2 tissue-culture flasks were coated with gelatine by adding 5 ml of 0.1% (w/v) gelatine solution and incubation at room temperature for at least 15 min before thorough aspiration. The ES cells were passaged every 2 days by trypsinization, up to a maximum number of 30 passages. The spent culture medium was aspirated and the confluent ES cells were washed gently with 5 ml PBS. 2 ml of TVP was added to cover the cell layer and incubated at 37°C for 2 min. To release the cells, the flask was knocked several times. The resulting cell suspension was collected into a 15 ml plastic centrifuge tube and 8 ml FCS medium added before the cells were pelleted by

centrifugation for 5 min at 1 000 rpm. After the supernatant was aspirated, the cells were resuspended in 10 ml FCS medium. The number of cells was determined using a haemocytometer. From a confluent 25 cm^2 flask the typical total cell number was ~5 to 8x 10^6. 1x 10^6 ES cells were added to each of two 25 cm^2 gelatine-coated flasks in a volume of 10 ml FCS medium. LIF was added to a final concentration of 100 U/ml. One of these flasks was used for the next passage after inspection for contamination and appropriate cell density using a microscope. The ES cell culture was incubated at 37°C in a 5% CO_2 atmosphere.

3.1.6 Formation of Embryoid Bodies (EBs)

3.1.6.1 Formation of Undifferentiated ES Cell Aggregates

Three different methods were used to form undifferentiated ES cell aggregates prior to differentiation into embryoid bodies (EBs). For all procedures using ES cell aggregates and EBs wide-bore plastic pipettes were used.

3.1.6.2 Non-gelatinized Method

The non-gelatinized method was used to obtain rapidly a large number of ES cell aggregates for preliminary *in situ* hybridization experiments and ß-gal analysis of gene trap cell lines. ES cells form aggregates that are only loosely attached to the substrate when cultured at high-density on non-gelatinized tissue culture flasks. ES cells were generated as a single cell suspension, as described above for ES cell maintenance. 2x 10^6 ES cells in 10 ml of FCS medium were seeded into a 25 cm^2 non-gelatinized tissue culture flask and LIF added to a final concentration of 100 U/ml.
After two days incubation at 37°C in a 5% CO_2 atmosphere, the ES cell aggregates were harvested. The spent medium was carefully aspirated and the ES cell aggregates were gently washed off the substrate with 10 ml FCS medium by using a wide-bore plastic pipette. The wash medium was transferred to a 20 ml plastic universal tube

and the ES cell aggregates were allowed to settle. The medium was aspirated and the ES cell aggregates were resuspended in 10 ml of DIF medium.

3.1.6.3 Cellform-coated Plate Method

Formation of ES cell aggregates on cellform-coated plates was performed using a modified protocol as described (Folkman and Moscona, 1978; B. Rosen, personal communication). The cellform-coated plate method was applied to assess the practicability of the rapid and simultaneous production of uniform ES cell aggregates from a large number of different ES cell and gene trap cell lines for large-scale screening. Different plastic ware and variations in cell numbers plated were tested to obtain information for optimal conditions for ES cell aggregate formation. Tissue culture grade plastic plates and bacteriological plastic plates were coated with a diluted solution of cellform poly (2-hydroxyethyl methacrylate) (poly(HEMA)). 3 g of cellform poly(HEMA) powder was dissolved overnight in 25 ml 95% EtOH. The viscous solution was centrifuged for 5 min at 2 500 rpm to remove particulate matter. This stock was then diluted 1/10 with 95% EtOH and approximately 100 μl per 1 cm plate diameter was pipetted into the plates. The plates were allowed to dry for 48 hours with the lids in place at 37°C. Uncoated plates were used as controls. Single cell suspensions of ES cells (see above 3.1.5; ES cell maintenance) of various concentrations (~3-10 x 10^4 cells/ml) were plated into the dishes and LIF added to a final concentration of 100 U/ml. After 2 days incubation at 37°C in a 5% CO_2 atmosphere, the ES cell aggregates were harvested into a 20 ml plastic universal tube and were collected by centrifugation at 800 rpm for 3 min. The supernatant was aspirated and the ES cell aggregates were resuspended in DIF medium.

3.1.6.4 Hanging Drop Method

The hanging drop method was used for time-course experiments. This method is time consuming but provides pre-differentiated ES cell aggregates of uniform size. Figure 3.1 illustrates this method. A single cell suspension of ES cells was generated as described above. 6x 10^5 ES cells in 20 ml FCS medium (~3 x 10^4 cells/ml) were placed into a 20 ml plastic universal tube and a final concentration of 100 U/ml of LIF added. Eight square plastic plates (10 cm diameter) were taken and 8 ml distilled water added to the base of each. Using a Biohit multi-channel pipette, 10 µl aliquots of cell suspension were pipetted onto the upturned underside of a square plate lid. Approximately 200-250 discrete 10 µl aliquots were placed on each lid, ensuring a reasonable distance between drops to avoid coalescence. The lid was replaced on to the base with a smooth, swift action to avoid confluence of the drops. After the cell suspension was dispensed on the square plate, they were placed in a humidified 5% CO_2 atmosphere at 37°C and the hanging drops were cultured for two days. Each 10 µl aliquot contains ~300 ES cells which settle at the bottom of the hanging drop and multiply to form a smooth, spherical body of uniform size. After two days the drops from all of the plates were removed with a plugged pasteur pipette and placed in a 20 ml plastic universal tube. The ES cell aggregates (~10^3 aggregates/10 ml medium) were collected by centrifugation at 800 rpm for 3 min. The supernatant was aspirated and the ES cell aggregates resuspended in 10 ml of DIF medium.

3.1.7 Differentiation of ES Cell Aggregates in Suspension Culture

10 ml of DIF medium was placed into a petri dish and 200 µl of a mixture of the antibiotics penicillin/streptomycin, each at 200 U/ml, were added. The ES cell aggregates, either formed using the non-gelatinized method, the cellform-coated plate method or the hanging drop method and resuspended in 10 ml DIF medium were transferred to the petri dish containing DIF medium and penicillin/streptomycin. The suspension was cultured at 37°C in a 5% CO_2 atmosphere. The day on which

the ES aggregates were harvested and transferred to DIF medium was assigned as day 0 of differentiation. The medium was changed every two days by allowing the embryoid bodies (EBs) to settle in a 20 ml plastic universal tube and aspirating the supernatant. EBs which had adhered to the substrate were gently washed off using a wide-bore plastic pipette. 10 ml of DIF medium were placed into a new petri dish and a further 10 ml of DIF medium was used to resuspend and to transfer the EBs to the petri dish. The suspension culture was then incubated as above. In time course experiments, operations were carried out as shown in Figure 3.2 over a period up to 8 days. On the final day of a time course, EBs which had undergone various days of differentiation were obtained.

3.1.8 Exposure of Undifferentiated ES Cell Aggregates to Morphogen

ES cell aggregates formed by the hanging drop method (see above) were exposed to 1.0% DMSO or 10^{-8} M of all-trans RA for the first 48 hours of differentiation and allowed to differentiate for a further period before being assayed (Doostdar, 1997; personal observations). The appropriate volume of DMSO was directly added to the petri dish containing DIF medium and penicillin/streptomycin. Retinoic acid was dissolved in 100% ethanol at a stock concentration of 10^{-2} M and stored in the dark at -70°C. The retinoic stock solution was serially diluted in DIF medium to 10^{-6} M and the appropriate volume of this dilution was added to the DIF medium and antibiotics containing petri dish. The EBs were harvested after the exposure to 1.0% DMSO or 10^{-8} M retinoic acid as described and washed through 15 ml of PBS before transferring into suspension culture.

3.1.9 Harvesting and Counting of Embryoid Bodies

EBs were harvested by allowing them to settle in a 20 ml plastic universal tube as described above. The supernatant was removed and the EBs resuspended in 2 ml PBS. This was placed on a petri dish of 3 cm diameter which was placed on a grid. The EBs within a 1 cm^2 area were counted and this multiplied by 7 to obtain the total number of EBs.

3.1.10 CFU-A Assay

A CFU-A (colony-forming unit-A) assay is an *in vitro* assay that detects primitive haematopoietic progenitors (CFU-A). The *in vitro* CFU-A assay was set up as described previously (Pragnell et al, 1988; Lorimore et al, 1990). Briefly, a feeder layer consisting of 0.6% agar in alpha MEM with conditioned medium from two cell line (AF1-19T, a source of GM-CSF and L929, a source of CSF-1) was poured in 3 cm diameter tissue culture grade dishes (1 ml per layer). EBs were added to 0.3% agar in alpha MEM and formed the upper layer. All EB samples were assayed in triplicates. Approximately 50 EBs were plated into a dish. To estimate the volume needed per EB sample three 10 μl aliquots were placed onto a microscope slide and the EBs counted. Means per EB sample were used to calculate the required volume for the CFU-A assay per sample. The dishes were incubated for 11 days at 37°C in a humidified atmosphere with 5% O_2/10% CO_2. Quantification of positive CFU-A colonies was performed visually using a microscope.

3.1.11 ß-Galactosidase Expression Patterns in Embryoid Bodies

ß-Galactosidase activity can be assayed by *in situ* staining since the action of ß-gal on the exogenously added substrate X-gal produces an insoluble blue cleavage product which precipitates at the site of enzyme activity. The ß-gal staining assay was performed essentially as described previously (Beddington et al, 1989). In brief, EBs from ES cell lines containing gene trap integrations allowed to differentiate for various periods of time were transferred into separate wells of a 24-well plate and fixed for 10 min at 4°C in 1 ml fixation solution (0.2% glutaraldehyde in PBS, pH 7.3, containing 2 mM $MgCl_2$, 5 mM EDTA). After fixation, the EBs were washed twice in wash solution (PBS pH 7.3 containing 2 mM $MgCl_2$, 0.1% DOC, 0.02% Tween-20, 0.005% bovine serum albumin) for 10 minutes each. The wash solution was replaced by staining solution and the EBs were incubated at 37°C overnight (12-16 hours). 25 ml of staining solution consisted of 25 ml PBS, pH 7.3, containing 0.25 mg/ml spermidine, 41 mg $K_3Fe(CN)_6$, 52.5 mg $K_4Fe(CN)_6$, 0.34 mg NaCl and 1 mg/ml X-gal colour substrate (Boehringer Mannheim). The staining reaction was stopped by 3 washes in PBS, pH 7.3, and the ß-gal expression patterns were assessed by microscopy.

3.1.12 Quantitative ß-Galactosidase Activity Assay

The detection of ß-gal activity in EBs from ES cell lines containing gene trap integrations was performed using a colorimetric assay as described (Eustice et al, 1991). Briefly, EBs were lysed by three freeze/thaw cycles and the protein concentration of cell lysates was determined measuring the optical density at 280 nm. ß-Gal activity assays were performed with equivalent amounts of protein (40 µg) using o-nitrophenyl-ß-D-galactopyranoside (ONPG) as substrate. Samples were incubated at 37°C over night and the absorbance at 405 nm was determined using an Elisa reader.

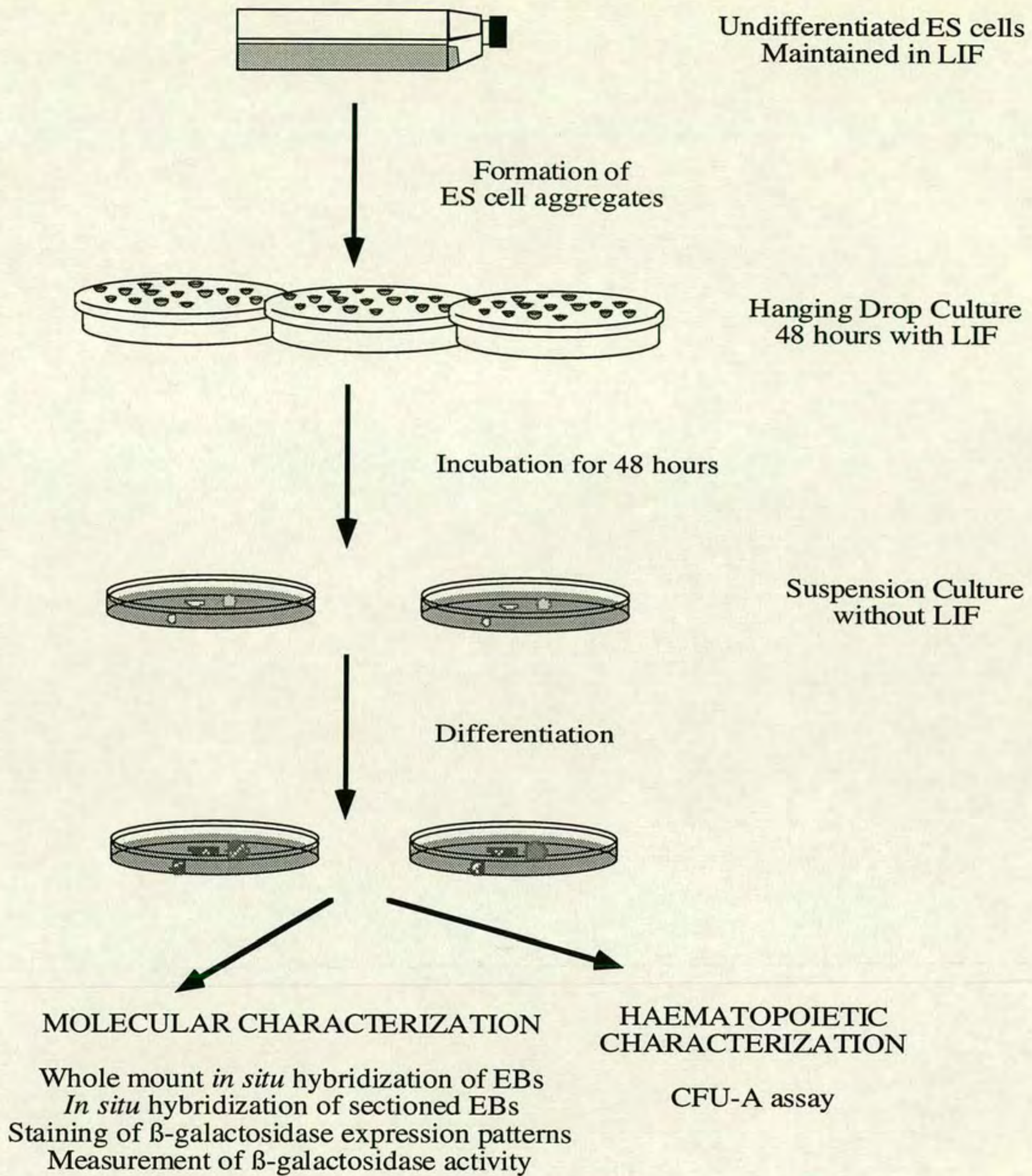


Figure 3.1: Summary of *in vitro* differentiation procedures and molecular characterization. Aliquots of a single cell suspension of ES cells in FCS medium supplemented with LIF were pipetted onto bacterial dish lids. The hanging drops were cultured for two days. ES cell aggregates were harvested into a petri dish containing DIF culture medium without LIF and allowed to differentiate. DIF medium was replaced every two days. EBs were characterized by molecular biological methods and haematopoietic differentiation was assessed by the CFU-A assay.

EXPERIMENT DAY 0

EXPERIMENT DAY 2

EXPERIMENT DAY 4

passage

48 hr

passage

48 hr

passage

Undifferentiated ES cells
Maintained in LIF

Formation of
ES cell aggregates

Formation of
ES cell aggregates

Differentiation

EB day 0 differentiation

Formation of
ES cell aggregates

Differentiation

Differentiation

Hanging Drop Culture
48 hr with LIF

EB day 0 differentiation
Suspension Culture without LIF

EB day 2 differentiation

Figure 3.2: Schematic description of the performance of an ES cell differentiation time course. The ES cell line was maintained in an undifferentiated state in medium supplemented with LIF. ES cells were passaged and ES cell aggregates were formed using the hanging drop method every two days. ES cell aggregates were harvested into petri dishes containing culture medium lacking LIF and allowed to differentiated into EBs for varying periods of time.

3.2 *In Situ* Hybridization of Embryoid Bodies

All procedures for *in situ* hybridizations on EBs, whole mount and on sections, were performed at standards for RNA work. All solutions used were treated for possible contaminating RNase activity with 0.1% DEPC overnight (~16 hours) at 37°C and autoclaved or DEPC treated H_2O was used for preparations. Glass-ware was baked at 180°C overnight (~16 hours) before use and sterile plastic-ware from previously unopened bags was used. All chemicals used were kept separate from routine laboratory work.

3.2.1 RNA Probe Synthesis

Single stranded riboprobes were synthesized as run-off transcripts from linearized plasmid templates using bacteriophage RNA polymerases (T3, T7, SP6) under standard conditions essentially as described by the manufacturer (Boehringer Mannheim).

Plasmid DNA was prepared by the standard alkali lysis method followed by phenol/chloroform purification and subsequent EtOH precipitation (see Sections 3.3.2 and 3.3.8). Plasmid DNA was linearized by restriction endonuclease digestion as described in Section 3.3.4. However, the incubation time for the linearization digest was prolonged since initial riboprobe synthesis did not lead to the expected yield of riboprobe. To further ensure pure transcript and to reduce possible contamination of uncut plasmid DNA electrophoretic separation of the linearization mixture was performed. The appropriate band with linearized plasmid was cut from the gel and then phenol/chloroform extracted.

All reagents for the transcription reactions were supplied by Boehringer Mannheim. Transcription was carried out in sterile 1.5 ml eppendorf tubes which were kept on ice during the preparation set up. The reagents were mixed in the following order as described below. Transcription mixtures consisted of ~1 µg linearized and purified template DNA in DEPC-treated water, 2 µl of 10x NTP labelling mixture,

2 μl 10x supplied transcription buffer, 20 units RNase inhibitor and DEPC-treated water to give a total volume of 20 μl when 40 units of appropriate bacteriophage RNA polymerase was added. The reagents were mixed gently and incubation was carried out at 37°C for 2 hours. 2 μl of RNase free DNase (10 units/μl) were added to the reaction mixture to digest the plasmid DNA template. The template digest reaction was incubated at 37°C for 15 min and then stopped by adding 2 μl of 200 mM EDTA, pH 8.0. Ethanol precipitation of labelled RNA transcripts in the presence of LiCl was carried out by adding 2.5 μl of 4 M LiCl and 75 μl of pre-chilled 100% ethanol. This mixture was placed at -70°C for at least 2 hours or overnight. The precipitated RNA probes were collected by centrifugation for 15 min at 13,000 rpm in an Eppendorf microfuge at 4°C and rinsed with 50 μl ice-cold 75% ethanol (made with DEPC-treated water). The RNA pellet was dried briefly under vacuum and resuspended in 25 μl of DEPC-treated water by heating for 10 min at 65°C. Labelled riboprobes were stored at -70°C until used in hybridization experiments.

The RNA probe synthesis reaction was checked on an formaldehyde agarose gel followed by northern transfer and colorimetric staining of bands (Sections 3.3.11; 3.3.12; 3.3.14) and by dot detection of spotted RNA on a nylon membrane for the estimation of the yield of labelled RNA (see below).

3.2.2 Estimation of Labelled RNA Probe Concentration

Estimation of riboprobe yield was performed in a side by side comparison of DIG-labelled sample riboprobe with a DIG-labelled control RNA sample provided by Boehringer Mannheim. The procedure was essentially performed as described in the manufacturer's application manual 'The DIG-System User's Guide for Filter Hybridization', Boehringer Mannheim.

Briefly, prediluted DIG-labelled control RNA (20 ng/μl) was serially diluted in RNA dilution buffer (DEPC-treated H_2O, 20x SSC and formaldehyde mixed in a volume ratio at 5+3+2) to obtain samples at 1 ng/μl, 100 pg/μl, 10 pg/μl and 1 pg/μl.

A ten-fold serial dilution of each newly synthesized DIG-labelled or Fluorescein-labelled riboprobe sample was made with RNA dilution buffer. 1 μl of each concentration of the diluted control RNA was spotted in a row on a nylon membrane (Boehringer Mannheim). In subsequent rows, 1 μl of the corresponding dilutions of experimental riboprobes were spotted (Figure 3.3). The membrane was left to dry for 5 min before the RNA samples were fixed to the membrane by cross-linking with UV-light for 30 sec.

The solutions used for signal detection varied depending in which hybridization procedure the riboprobes were applied. Therefore general terms for the solutions are used to describe the signal detection procedure. Details of solution components are listed in Table 3.1.

The membrane was placed into a sterile petri dish plate, washed briefly (~1 min) in 5 ml washing buffer before incubated in 10 ml blocking solution for 30 min at room temperature. Anti-DIG-AP-conjugated antibodies were diluted at 1:50 000 and Anti-Fluorescein-AP-conjugated antibodies were diluted at 1:20 000 in blocking solution. The membrane was incubated in 5 ml of diluted antibody solution for 1 hour at room temperature. The membrane was then washed twice in 10 ml wash buffer for 15 min each before equilibrated for 2 min in detection buffer. The colour substrate solution for signal development was prepared freshly immediately prior use. The detection buffer was removed and 5 ml of colour substrate solution was added to the membrane. The colour development was allowed to occur at dark for 3 to 16 hours. After spots appeared in sufficient intensity, the reaction was stopped by washing the membrane three times in sterile H_2O for 5 min each. The spot intensity of the control and experimental dilutions were compared to estimate the concentration of the experimental riboprobes.

Figure 3.3: Estimating the yield of DIG-labelled or fluorescein-labelled riboprobe. Dilutions of labelled control RNA and the newly labelled experimental RNA were spotted on, fixed and directly detected on a nylon membrane with colorimetric detection.

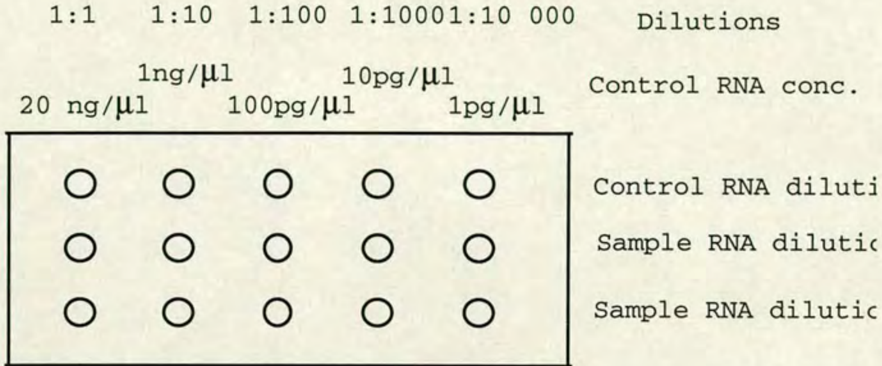


Table 3.1: Solutions for colorimetric signal detection of labelled RNA spotted on nylon membrane

Solution	Hybridization protocol established for mouse embryos	Hybridization protocol established for chicken embryos
Wash buffer	0.1 M TrisHCl pH 7.5, 0.15 M NaCl	0.1 M maleic acid, 0.15 M NaCl, pH 4.5 with NaOH
Blocking solution	3% (w/v) bovine serum albumin in wash buffer	1% (w/v) BBR in wash buffer
Detection buffer	0.2 M TrisHCl pH 9.5, 0.01 M $MgCl_2$	0.1 M TrisHCl pH 9.5, 0.1 M NaCl
Colour substrate solution	2.3 µl/ml BCIP, 3 µl/ml NBT in detection buffer	3.5 µl/ml BCIP, 4.5 µl/ml NBT in detection buffer

3.2.3 Whole Mount *In Situ* Hybridization of Embryoid Bodies

Initial *in situ* hybridization experiments on EBs were performed following procedures as described for non-isotopic *in situ* hybridization on mouse embryos (Rosen and Beddington, 1993). Because of limitations in the suitability of this protocol for *in situ* hybridization on EBs, in particular in combination with confocal microscopy, a protocol established for chicken embryos was used (Graham A; personal communication). Ultimately procedures combining both protocols were found to be most suitable for the EBs. The main features of these protocols are summarized in Table 3.2.

The following sections describe the protocols followed for *in situ* whole mount hybridization of EBs and modifications introduced, control experiments for optimization of conditions and procedures and the establishment of *in situ* hybridization procedures most suitable for EBs. Guidelines for modifications were obtained following manufacturer's recommendations for the use of reagents and the Boehringer Mannheim manual 'Non radioactive *in situ* hybridization application manual', second edition.

Unless stated otherwise, the procedures were carried out according to the respective protocol. Pretreatments were performed in batches in sterile 5O ml centrifuge tubes. Washes were carried out by allowing EBs to sink to the bottom of the container and carefully removing the liquid with a sterile glass pasteur pipette connected to a vacuum aspirator. Great care was taken to always leave a small volume of liquid on the EBs to prevent drying. Prehybridization and hybridization reactions were performed in sterile 5 ml universal tubes. The tubes were closed with the lid during incubations to avoid evaporation of the solution. Incubation of samples was performed using a waterbath at the appropriate temperature. Tween-20 was used as a substitute for Nonidet-P-40.

3.2.3.1 *In situ* Hybridization of Whole Mount EBs following Procedures Originally Established for Mouse Embryos

Fixation of Embryoid Bodies

EBs were harvested by allowing them to settle in a 50 ml centrifuge tube as described in Section 3.2.9 and washed twice with 10 ml cold PBS. The EBs were resuspended in 10 ml freshly prepared 4% paraformaldehyde in PBS and stored overnight (16-18 hours) at 4°C. The fixed EBs were washed twice with 10 ml cold PBT before being dehydrated on ice by washing once with each of 25%, 50%, and 75% methanol-PBS and twice with 100% methanol. 10 ml of solution was used for each wash. One wash took 5-10 min. The EBs were resuspended in 15 ml of 100% methanol and stored at -20°C.

Pretreatments: Permeabilization and Postfixation

The EBs were rehydrated on ice by washing once with each of 75%, 50%, and 25% methanol-PBS, as described above for dehydration. The following wash and incubation steps were carried out at room temperature. The EBs were washed three times with 10 ml PBT for 5-10 min before treated in three changes of 10 ml RIPA for 30 min each. Post-fixation of the EBs was performed by resuspension in 10 ml PG-PBT and incubation for 20 min. The EBs were then washed three times with 10 ml RIPA for 5 min and three times with 10 ml PBT for 5 min.

Prehybridization and Hybridization

The EBs, resuspended in 3 ml of PBT, were divided into equal volume samples by transferring them to sterile 5 ml universal tubes. The number of samples was dependent on the number of different probes used for hybridization. The EBs were split into the same number as that of the different probes used, plus two controls.

These controls were, one of EBs incubated only with antibody conjugate without probe and one sample incubated neither with antibody conjugate nor with probe. The samples of EBs were washed with 2 ml of 1:1 hybridization buffer to PBT at room temperature and allowed to sink. They were then washed with 2 ml of hybridization buffer for 5-10 min at room temperature before prehybridization for 1-3 hours at 70°C in 1 ml hybridization buffer containing 100 μg/ml tRNA and 100 μg/ml sheared, denatured herring sperm DNA (ssDNA). Both tRNA and ssDNA were phenol/chloroform extracted before used as blocking reagents in prehybridization and hybridization reactions. The prehybridization solution was removed and 0.5 ml of hybridization buffer containing 100 μg/ml tRNA, 100 μg/ml ssDNA and a 1:100 dilution of denatured digoxigenin labelled RNA probe was added (see Section 3.2.1 for RNA probe synthesis).

The RNA probe was denatured by heating to 80°C for 5 min and placing on ice for 5 min. The EBs in this hybridization mixture were incubated overnight (16-18 hours) at 70°C. After hybridization, the samples were washed once with 3 ml hybridization buffer for 10 min at 70°C, once with 2x SSC-FT for 5 min at 65°C and twice with 3 ml 2x SSC-FT for 30 min at 65°C. The EBs were allowed to cool to room temperature, before being washed twice with 3 ml 1x TBST for 10-15 min.

Antibody Binding

The EBs were blocked by incubation for 1 hour at room temperature in 2 ml 10% heat-inactivated sheep serum in 1x TBST. The blocking solution was then replaced with 1 ml 1% heat-inactivated sheep serum in 1x TBST containing 375 mU/ml (1:2000 dilution) anti-digoxigenin Fab-alkaline phosphatase conjugate (Boehringer Mannheim) and incubated overnight (16-18 hours) at 4°C.

Heat-Inactivation of Sheep Serum

A two-fold dilution of sheep serum in DEPC-treated water was heated to 70°C for 30 min and placed on ice for 5 min. The denatured protein was pelleted by centrifugation for 2 min at 1 000 rpm (microfuge) and the supernatant was used in reaction solutions.

Washing and Colour Development

The antibody conjugate solution was removed and the EBs were washed four times with 1x TBST at room temperature. Three wash steps with 3 ml 1x TBST for 5 min were followed by one with 5 ml 1x TBST for 30 min. The EBs were then washed twice with freshly prepared APB, each with 3 ml for 10 min. They were then transferred to alternate wells of a sterile 24 well titre plate. 1 ml of freshly prepared staining solution containing 3.375 µl nitro blue tetrazolium chloride (NBT) (stock 100 mg/ml in dimethyl formamide (DMF), 70% (v/v)) and 2.33 µl/ml 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) (stock 50 mg/ml in DMF) (Boehringer Mannheim) in APB was added and the dishes were placed in dark at room temperature. The progress of the reaction was observed for brief intervals under a binocular magnifier (300x magnification). Signals corresponding to the RNA species used were visible within 15 min. Incubations were continued for 1 to 24 hours. The staining reactions were stopped by adding 2 ml PBT containing 1 mM EDTA and rinsing the EBs five times in 2 ml PBT containing 1 mM EDTA. The EBs were transferred to alternate wells of a new 24 well titre plate and stored in the dark at 4°C, before photographed for documentation.

In some *in situ* hybridization experiments, diamino-benzidine (DAB) stain was used for colour development. For this all procedures were performed as described for NBT/BCIP or Fast Red colour substrate with the exception that peroxidase (POD) conjugated anti-DIG antibodies were used for the detection of hybridized riboprobe. For the subsequent colour development DAB Fast-tablets (Boehringer Mannheim) were used according to the manufacturer's instruction.

3.2.3.2 Two-Colour Whole Mount *In situ* Hybridization of EBs

Two-colour *in situ* hybridization of whole EBs was performed using riboprobes labelled with DIG or fluorescein simultaneously in hybridization conditions as described above (Section 3.2.1).Visualization of hybridization events was carried out sequentially using two different enzymatic detection systems.

After posthybridization washes in TBST, the EBs were incubated in blocking-buffer and then with AP conjugated anti-fluorescein antibodies (1:2000) as described in Section 3.2.3.1. The signal was developed by using Fast Red tablets (Boehringer Mannheim) as colour substrate according to the manufacturer's instructions. The staining reaction was stopped by three washes in TBST, 5 min each, before the EBs were incubated overnight with peroxidase-conjugated anti-DIG-antibodies. The signal was developed using DAB Fast-tablets (Boehringer Mannheim) according to the manufacturer's instruction. The staining reaction was stopped by three washes with PBT, 10 min each, before evaluation of signal patterns using a microscope.

When two alkaline phosphatase conjugated antibodies were used for the detection of hybridized riboprobes, AP-conjugated anti-fluorescein antibodies and AP-conjugated anti-DIG antibodies, Fast Red and NBT/BCIP (both Boehringer Mannheim) were used as colour substrate. Signal for DIG-labelled riboprobes was detected following exactly the procedure for single probe hybridization as described in Section 3.2.3.1. The colour reaction was stopped with 2 ml PBT +1 mM EDTA, which effects the inactivation of any remaining AP activity from AP-conjugates from the first signal detection step. The EBs were then washed 3x in TBST before incubated with AP-conjugated anti-fluorescein antibodies at 4°C overnight. Colour development of signals from fluorescein labelled riboprobes the colour substrate Fast Red was used following the manufacturer's instructions. The colour reaction was stopped with 3x washes in PBT, 5 min each, and the EBs were then evaluated using a microscope.

Solutions for whole mount *in situ* hybridizations

PBS	Phosphate buffered saline
PBT	Phosphate buffered saline, 0.1% Tween-20
RIPA	Detergent mix: 150 mM NaCl, 1% Tween-20, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris pH 8.0
PG-PBT	4% paraformaldehyde, 0.2% EM grade glutaraldehyde in PBT

Hybridization buffer:

50% ultrapure formamide, 5x SSC pH 4.5 (from 20x SSC stock solution, acidified with citric acid), 50 $\mu g\ ml^{-1}$ heparin, 0.1% Tween-20

SSC-FT	2x SSC pH 4.5, 50% formamide, 0.1% Tween-20
TBST	Diluted from a 10x stock solution: 100 ml of stock solution consisted of 8 g NaCl, 0.2 g KCl, 25 ml 1 M TrisHCl pH 7.5, 10 ml Tween-20
APB	Alkaline phosphate buffer: 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20, 100 mM TrisHCl pH 9.5

3.2.3.3 *In situ* Hybridization of Whole Mount EBs Following Procedures Originally Established for Chicken Embryos

Fixation of Embryoid Bodies

EBs were harvested as described in Section 3.2.9 and washed twice with 10 ml cold PBS containing 2 mM EGTA. The EBs were resuspended in 10 ml freshly prepared 4% formaldehyde in PBS + 2 mM EGTA, pH 7.5 adjusted with NaOH, and stored for fixation overnight (16-18 hours) at 4°C. The fixed EBs were washed twice in 10 ml PBT before being dehydrated on ice by washing with 50% methanol-PBT and twice with 100% methanol for 10 min each. The EBs were resuspended in 15 ml of 100% methanol and stored at -20°C.

Pretreatments: Permeabilization and Postfixation

The EBs were rehydrated on ice by washing once with each of 75%, 50%, and 25% methanol-PBS and washed twice with 10 ml PBT for 5-10 min at room temperature before being treated with 2 ml proteinase K (10 μg/ml) in PBT at 37°C for 10 min. The proteinase K solution was prewarmed at 37°C for 30-60 min prior to use. Proteinase K solution was removed and the EBs were rinsed briefly with PBT before post-fixation in 5 ml freshly prepared 4% formaldehyde + 0.1% glutaraldehyde in PBT for 20 min at room temperature. The EBs were then washed twice with 10 ml PBT for 5 min.

Prehybridization and Hybridization

The EBs, resuspended in PBT, were divided into equal volume samples by transferring them into sterile 5 ml universal tubes. The samples of EBs were rinsed once with 2 ml of 1:1 hybridization buffer to PBT at room temperature and the EBs were allowed to sink. They were then washed with 2 ml of hybridization buffer for

5-10 min at room temperature before prehybridization for 1-3 hours at 70°C in 1 ml hybridization buffer. After prehybridization, 0.5 ml of prewarmed hybridization mix containing denatured labelled riboprobe was added. The EBs in this mix were incubated overnight for 16-18 hours at 70°C. The following rinse and wash steps were carried out at 70°C using prewarmed solutions. After hybridization, the samples were rinsed twice with 2 ml hybridization buffer, twice washed for 30 min with 2 ml hybridization buffer and once washed with 2 ml 1:1 hybridization buffer to MABT for 20 min. The EBs were allowed to cool to room temperature, before being rinsed three times with 3 ml MABT and washed twice with MABT for 30 min.

Antibody Binding

The EBs were blocked by incubation for 1 hour at room temperature in 2 ml of MABT buffer containing 2% Boehringer Blocking Reagent (BBR) followed by incubation for 30 min in 2 ml 20% heat inactivated sheep serum in MABT + 2% BBR. The blocking solution was then replaced with 1 ml 20% heat-inactivated sheep serum in MABT + 2% BBR containing appropriate antibody conjugate (Boehringer Mannheim) and incubated overnight (16-18 hours) at 4°C.

Heat-Inactivation of Sheep Serum

Sheep serum was heat-treated at 60°C for 30 min and placed on ice for 5 min. The denatured protein was pelleted by centrifugation for 5 min at 1 000 rpm (microfuge) and the supernatant was used in reaction solutions.

Washing and Colour Development

The antibody conjugate solution was removed and the EBs were rinsed three times with MABT at room temperature and then washed three times for 1 hour each with MABT. The EBs were then washed twice with 3 ml NTMT before being transferred

to alternate wells of a sterile 24 well titre plate. 1 ml of freshly prepared staining solution in NTMT was added and the dishes were placed in dark at room temperature. The progress of the reaction was observed for brief intervals under a binocular magnifier (300x magnification). Signals corresponding to the RNA species used were visible within 15 min. Incubations were continued for 1 to 24 hours. The staining reactions were stopped by adding 2 ml PBT containing 10 mM EDTA and rinsing the EBs three times in 2 ml PBT containing 10 mM EDTA. The EBs were stored in the dark at 4°C, before being photographed for documentation.

Solutions for whole mount *in situ* hybridizations

PBS Phosphate buffered saline

PBT Phosphate buffered saline, 0.1% Tween-20

Hybridization buffer:

50% ultrapure formamide, 1.3x SSC pH 4.5 (from 20x SSC stock solution, acidified with citric acid), 5 mM EDTA pH 8.0, 50 μg ml^{-1} yeast RNA, 0.2% Tween-20, 0.5% CHAPS, 100 μg ml^{-1} heparin

MABT Maleic acid buffer:

100 mM maleic acid, 150 mM NaCl, 0.1% Tween-20, pH 7.5

BBR Boehringer blocking reagent

Blocking buffer 2% (w/v) BBR in MABT

NTMT 100 mM NaCl, 100 mM TrisHCl pH 9.5, 1% Tween-20

Staining solution: 4.5 μl/ml NBT and 3.5μl/ml BCIP (Boehringer Mannheim) in NTMT buffer

3.2.3.4 *In Situ* Hybridization of Whole Mount EBs Combining Procedures of Protocols Established for Mouse Embryos or Chicken Embryos

Following considerable effort to optimize the *in situ* hybridization procedures on EBs a combination between the hybridization described above was used.

Fixation of embryoid bodies

EBs were harvested by allowing them to settle in a 50 ml centrifuge tube as described in Section 3.2.9 and washed twice on ice with 10 ml cold PBS containing 2 mM EGTA. The EBs were resuspended in 10 ml freshly prepared 4% paraformaldehyde in PBS + 2 mM EGTA, pH 7.5 adjusted with NaOH, and stored for fixation overnight (16-18 hours) at 4°C. The fixed EBs were washed twice on ice with 10 ml cold PBT before being dehydrated on ice by washing once with each of 25%, 50%, and 75% methanol-PBS mixtures and twice with 100% methanol. All solutions were used cold and one wash took 5-10 min. The EBs were resuspended in 15 ml of cold 100% methanol and stored at -20°C.

Pretreatments: Permeabilization and Postfixation

The EBs were rehydrated on ice by washing once with each of 75%, 50%, and 25% methanol-PBS and washed twice with 10 ml PBT for 5-10 min at room temperature before being treated with 2 ml proteinase K (10 μg/ml) in PBT at 37°C for 10 min. The proteinase K solution was prewarmed at 37°C for 30-60 min prior use. Proteinase K solution was removed and the EBs were rinsed briefly with PBT before postfixation in 5 ml freshly prepared 4% paraformaldehyde in PBT for 20 min at room temperature. The EBs were then washed twice with 10 ml PBT for 5 min. Subsequent procedures, prehybridization and hybridization, the detection of hybridized riboprobe by antibody binding and colour development was performed as described above in hybridization procedures established for chicken embryos (Section 3.2.3.3).

Table 3.2: Summary of main features of the whole mount *in situ* hybridization protocols used for hybridization of EBs

	Hybridization protocol established for mouse embryos	Hybridization protocol established for chicken embryos
Fixation	4% paraformaldehyde	4% formaldehyde
Pretreatments		
Permeabilization	'cocktail' of ionic and non-ionic detergents	Proteinase K treatment
Postfixation	4% paraformaldehyde + 0.2% glutaraldehyde	4% formaldehyde + 0.2% glutaraldehyde
Hybridization buffer	50% ultrapure formamide, 5x SSC pH 4.5, 50 μg ml^{-1} heparin, 100 μg/ml tRNA, 100 μg/ml ssDNA, 0.1% Tween-20	50% ultrapure formamide, 1.3x SSC pH 4.5, 5 mM EDTA pH 8.0, 100 μg ml^{-1} heparin, 50 μg ml^{-1} yeast RNA, 0.5% CHAPS, 0.2% Tween-20,
Hybridization temperature	70°C	70°C
Riboprobe concentration	~ 1μg/ml	~ 1μg/ml
Posthybridization washes	TBST	MABT
Blocking reagent	bovine serum albumin	Boehringer blocking reagents

3.2.4 Signal Detection by Confocal Fluorescence Microscopy

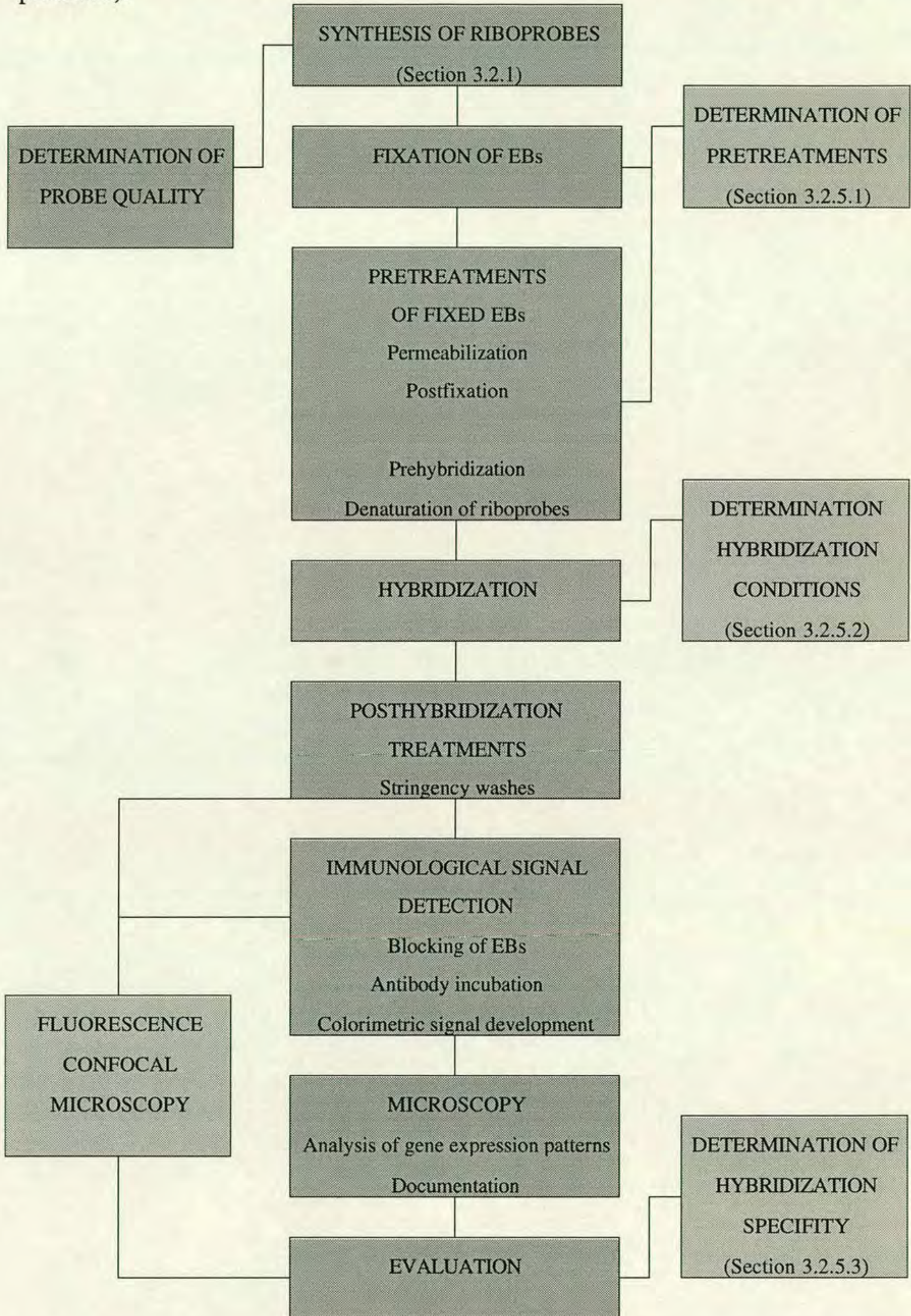
Fluorescence labelled EBs were analyzed using a Zeiss LSM 410 invert laser scanning confocal microscope according to the manufacturer's instructions. EBs were labelled with fluorescence, either by hybridization with fluorescein labelled riboprobes or by hybridization with DIG-labelled riboprobes detected by anti-DIG-antibodies conjugated with fluorescein or rhodamine. When direct fluorescein labelled riboprobes were used, the hybridization procedures were followed up to the posthybridization washes, according to one of the hybridization protocols described above.

When fluorescence labelled antibodies were used, the procedures were followed as described up to antibody staining and washes, with the difference that the sample EBs were kept dark from the use of antibodies onwards. Prior to analysis by confocal microscopy three additional wash steps with the appropriate wash buffer were performed.

The samples were mounted using an anti fading mounting medium (Sigma).

The laser scanning microscope was equipped with a laser lamp for excitation wavelengths of 488 nm and 543 nm, which were used to detect signal from fluorescein labels or rhodamine labels, respectively. Confocal images were stored as graphic files using the manufacturer's proprietary LSM software based on Microsoft Windows. Images of optical sections of whole-mount *in situ* hybridized EBs obtained by confocal microscopy analysis are shown in Section 3.2.5.4 (Determination of hybridization specification) in Figures 3.6.3 and 3.6.4.

Figure 3.4: Flow diagram of procedures for whole mount in situ hybridization of EBs (see Sections 3.2.3.1, 3.2.3.2 and 3.2.3.4 for whole-mount *in situ* hybridization protocols).



3.2.5 Determination of Whole Mount *In situ* Hybridization Procedures suitable for EBs

The procedures used for *in situ* hybridization can be broadly divided into four sections: 1) synthesis of labelled probes; 2) sample preparation - fixation and pretreatments; 3) hybridization with labelled probes; and 4) signal detection of hybridized probes (see Figure 3.4: Flow diagram for whole mount *in situ* hybridization). Although different whole mount *in situ* hybridization protocols follow a common concept many alternatives exist for each process required for this experimental methodology. Many criteria that can influence the quality of the result may need to be individually optimized for each experimental system. Thus, to find a workable balance, if any, between the resolution and sensitivity of RNA detection in samples and the retention of sample morphology, various conditions may have to be tested. The measures taken to obtain applicable conditions for the detection of specific mRNA in EBs by whole mount *in situ* hybridization combined with colorimetric or fluorescence signal detection are described below.

3.2.5.1 Determination of Pretreatment Conditions

Effects of postfixation treatment

Difficulties in signal detection associated with fixation procedures and the type of fixative used in *in situ* hybridization procedures have been reported (Guiot and Rahier, 1995; Carmo-Fonseca et al, 1991). To assess the influence of fixation procedures on the morphology of EBs and the sensitivity of signal detection, various fixatives were used for postfixation treatment and their effects on EBs compared. The fixatives tested have been reported to be adequate for *in situ* hybridization of a variety of tissue and cell types and were tested at concentrations and incubation times as recommended by manufacturers for *in situ* hybridization consumables (application manuals: '*In situ* hybridization: A guide to radioactive and non-radioactive *in situ* hybridization systems', Amersham Life Science, 1994; 'Nonradioactive *in situ* hybridization: Application manual, second edition', Boehringer Mannheim, 1996). Crosslinking fixatives, 4% paraformaldehyde in PBS, 4% paraformaldehyde and 0.1% glutaraldehyde in PBS, or 2% glutaraldehyde in PBS, and precipitating fixation mixtures (v/v), ethanol/acetic acid (95/5) or methanol/acetone (50/50) were used for postfixation of EBs after proteinase K treatment. Day 6 EBs were prepared for hybridization as described in Section 3.2.3.4 and split into aliquots for postfixation in different fixatives. Postfixation was carried out as described except that substitute solutions and varying incubation times were used (see Table 3.3 for incubation times). The effects on autofluorescence levels and morphology of EBs of each fixative was examined by confocal microscopy (see Table 3.3 for summary).

In general, autofluorescence levels measured at the excitation wavelength 488 nm compared to 543 nm were higher in all EB samples, with the exception of glutaraldehyde treated EBs which showed high autofluorescence at both wavelengths. Precipitating fixatives influenced autofluorescence levels in EBs less compared to crosslinking fixatives. However, morphology preservation of EBs was higher after incubation in crosslinking fixatives (data not shown).

Effects of fixation and postfixation on EBs after incubation in hybridization solution

Spectra of autofluorescent molecules are very broad compared to the relatively narrow spectra of fluorescence probes. This makes it difficult to avoid autofluorescence by simply choosing a fluorescence probe out of the range of the autofluorescence occurring in an experimental system (Van de Lest et al, 1995). However, tests using various fixatives for postfixation to investigate the cause of autofluorescence in EBs (see above) indicated that the use of the fluorescence label rhodamine may result in improved conditions for the signal detection of hybridized riboprobes. The maximum emission of rhodamine label is at 555 nm and autofluorescence in EBs at the 543 nm laser wavelength was relatively moderate compared to autofluorescence levels observed at 488 nm.

The standard procedures for fixation of EBs included overnight incubation in 4% paraformaldehyde and dehydration in methanol to allow the storage of EB samples at -20°C. This meant that EBs were exposed to fixative solutions for prolonged periods of time. The duration of fixation has been reported to have an important impact on the sensitivity of *in situ* hybridization conditions (Guiot and Rahier, 1994). Furthermore, enhanced autofluorescence by preincubation in formaldehyde has been observed in sectioned specimens (Van de Lest, 1995).

To assess the influence of this prolonged fixation on the sensitivity of the *in situ* hybridization method combined with confocal microscopy, the first fixation time of EB samples was varied. Day 6 EBs fixed as described (Section 3.2.3.4) and EBs harvested on the day of use and fixed for 30 min in freshly prepared 4% paraformaldehyde were examined for the effects of fixation treatments on autofluorescence levels and morphology. The fixed EBs were incubated in proteinase K and each sample was split into 4 aliquots for various postfixation conditions. Fixatives used for postfixation were 4% paraformaldehyde, 0.4% paraformaldehyde, EtOH/acetic acid (95/5) or methanol/acetone (50/50).

These pretreated EBs were then subjected to a mock hybridization without added riboprobes in order to examine the effect of all procedures required for whole mount *in situ* hybridization of EBs. The procedures were carried out as described (see Section 3.2.3.4) except that postfixation solutions were substituted as described above. Samples of EBs were taken after each treatment step and autofluorescence levels and morphology examined. A summary of the influences from preparative treatments is listed in Table 3.4.

After exposure to hybridization conditions all EBs showed very high autofluorescence levels at both wavelengths, 488 nm or 543 nm, irrespectively of the fixation conditions used. This observation indicated that the prolonged incubation in organic conditions (hybridization mix contains 50% formamide), required for hybridization is the limiting step for signal detection by fluorescence confocal microscopy.

Morphology preservation of EBs fixed in paraformaldehyde was higher compared to EBs fixed in EtOH/acetic acid or methanol/acetone. EBs postfixed in precipitating fixatives were disrupted into sticky cell clumps (data not shown).

Table 3.3: Influence of postfixation solutions on autofluorescence levels and morphology of EBs. Day 6 EBs were fixed with 4% paraformaldehyde and pretreated with proteinase K. For postfixation crosslinking and precipitating fixatives were compared.

	Autofluorescence 488 nm	Autofluorescence 543 nm	Morphology
Before proteinase K treatment	+	+	++
After proteinase K treatment without postfixation	+	+	---
Postfixation in crosslinking fixatives (20 min at RT)			
4 % paraformaldehyde	++	+	+
4 % paraformaldehyde and 0.1% glutaraldehyde	++	+	+
2% glutaraldehyde	++	++	+
Postfixation with precipitating fixatives			
EtOH/acetic acid (15 min at RT)	+	-	--
Methanol/acetone (4 min at -20˚C)	+	-	--

Morphology: ++ well-preserved structure; + fairly well-preserved structure; --- complete loss of strutcture; -- severe loss of structure; - some loss of sturcuture; Autofluorescence: ++ very high level of autofluorescence; + high level of autofluoresence; - low level of autofluorescence;

Table 3.4: Influence of fixation and postfixation solutions on autofluorescence levels and morphology of EBs. Day 6 EBs were fixed with 4% paraformaldehyde overnight or for 30 min and treated with proteinase K. For postfixation crosslinking and precipitating fixatives were compared. These EBs were then subjected to a mock hybridization without added riboprobes.

	Autofluorescence		Morphology
	488 nm	543 nm	
Before proteinase K treatment after fixation			
4% paraformaldehyde overnight	nr	nr	+++
4% paraformaldehyde for 30 min	nr	nr	+++
without fixation	nr	nr	+++
After proteinase K treatment before postfixation			
4% paraformaldehyde overnight	nr	nr	-
4% paraformaldehyde for 30 min	nr	nr	--
without fixation	*	*	--- *
Postfixation in 4% paraformaldehyde (20min at RT)			
4% paraformaldehyde overnight	++	+	+
4% paraformaldehyde for 30 min	++	+	+
Postfixation in 0.4% paraformaldehyde (20 min at RT)			
4% paraformaldehyde overnight	++	+	-
4% paraformaldehyde for 30 min	++	+	-
Postfixation in EtOH/acetic acid (15 min at RT)			
4% paraformaldehyde overnight	+	-	--
4% paraformaldehyde for 30 min	+	-	--
Postfixation in methanol/acetone (4 min at -20°C)			
4% paraformaldehyde overnight	+	-	--
4% paraformaldehyde for 30 min	+	-	--

Morphology: ++ well-preserved structure; + fairly well-preserved structure;
--- complete loss of strutcture; -- severe loss of structure; - some loss of sturcuture;
* EBs were disrupted after treatment and could not be recovered
Autofluorescence: ++ very high level of autofluorescence; + high level of autofluoresence;
- low level of autofluorescence; nr not relevant;

3.2.5.2 Determination of Hybridization Conditions

Effects of riboprobe concentration during hybridization

To determine whether the riboprobe concentration influences the sensitivity of hybridization reactions and/or is the cause for background staining hybridization of EBs with different probe concentrations were performed. The probe concentration has been reported to influence the sensitivity of signal detection, though the influence can reach a plateau with further increased probe concentrations having no effect or resulting in background staining. However, the optimal concentration for probes also depends on the size of the probe and on the sensitivity of the detection system (Guiot and Rahier, 1995).

Day 6 EBs were hybridized with anti-sense probes specific for actin mRNA or globin mRNA following the protocol described in Section 3.2.3.1. Riboprobe concentrations used were either at the recommended concentration (1:100 dilution) or at a 8 fold increased concentration. Subsequent colorimetric signal detection produced from both probe concentrations the expected specific staining patterns for globin or actin. No obvious difference between low or high probe concentration was observable thus confirming a high sensitivity and a high stringency of the hybridization conditions used.

Comparison of whole mount *in situ* hybridization protocols

Initial whole mount *in situ* hybridizations on EBs were performed following a protocol established for mouse embryos (see Section 3.2.3.1; Rosen and Beddington, 1993). This protocol utilizes alternative pretreatment conditions for the permeabilization of EBs prior to hybridization, which have been described as mild but efficient. EBs are cell clusters with very fragile structures and are prone to disruption when handled under rough conditions. Thus, the *in situ* hybridization protocol mentioned above was the protocol of choice. Although suitable in combination with colorimetric signal detection of hybridized probe, in combination with fluorescence confocal microscopy the hybridization conditions appeared to be the cause for limited sensitivity for signal detection. Therefore, the suitability of a more conventional whole mount *in situ* hybridization method was tested (see Section 3.2.3.3; Graham A, personal communication). The main difference between these hybridization protocols are the pretreatment conditions for the permeabilization of EBs prior to hybridizations (Table 3.2). The former protocol uses a 'cocktail' of ionic and non-ionic detergents, the latter uses proteinase K treatment.

Two samples of EBs, day 6 and day 4, were hybridized with fluorescein labelled antisense probes specific for actin or globin according to the respective procedures described above (Section 3.2.3.1 and 3.2.3.3), and used for signal detection by confocal microscopy. The detection of specific hybridization events by confocal microscopy was hampered in all samples by autofluorescence background (Figures 3.6.3 and 3.6.4). However, combined with colorimetric signal detection the protocol utilizing proteinase K appeared to provide higher sensitivity. It was mentioned in the original protocol, that the use of a detergent mix for pretreatments may be intrinsically less sensitive than protocols that use proteinase K (Rosen and Beddington, 1993).

3.2.5.3 Determination of Signal Detection Conditions

Direct and indirect signal detection

Confocal laser scanning fluorescence microscopy for detection of specific hybridization events was attempted for its reported high sensitivity in signal detection, three-dimensional analysis by non-invasive sectioning, and for the possible quantitation of fluorescence signal (Shotton, 1989). Initial efforts to combine whole mount *in situ* hybridization of EBs with confocal microscopy used anti sense riboprobes directly labelled with fluorescein. The use of probe with fluorochromes directly conjugated to the ribonucleotide sequence, could allow visualization of probe-target hybrids by fluorescence microscopy immediately after the hybridization reaction. However, when direct labelled riboprobes were used for the hybridization of EBs difficulties in signal detection were encountered due to autofluoresence background.

In a protocol for *Drosophila* embryos using directly labelled riboprobes, it was reported that the fluorescein in the RNA probe itself did not appear to contribute to the signal. However, the use of an anti-fluorescein antibody to detect the fluorescein-tagged RNA probe and a secondary antibody conjugated with fluorescein isothiocyanate (FITC) was reported to be successful (Biotechniques, 20, 748-750). Indirect signal detection in EBs employing confocal microscopy was attempted by hybridization of EBs with DIG labelled riboprobes and subsequent incubation with anti-DIG antibodies conjugated with a fluorochrome label, fluorescein or rhodamine. A schematic description of this approach is shown in Figure 3.5. Colorimetric signal detection was performed as a control for the hybridization reactions. The hybridization procedures were carried out as described in Section 3.2.3.4.
Although, the distinct expression patterns for actin and globin could be detected in control samples by colorimetric signal development, signals for actin or globin expression from confocal microscopy analysis did not produce reliable images due

to interfering autofluorescence background (Figures 3.6.3 and Figures 3.6.4). Problems in the use of fluorescence detection of non-radioactive probes, caused by background autofluorescence in cells and tissues has also been acknowledged by a manufacturer of products for application in hybridization experiments (application manual: '*In situ* hybridization: A guide to radioactive and non-radioactive *in situ* hybridization systems', Amersham Life Science, 1994).

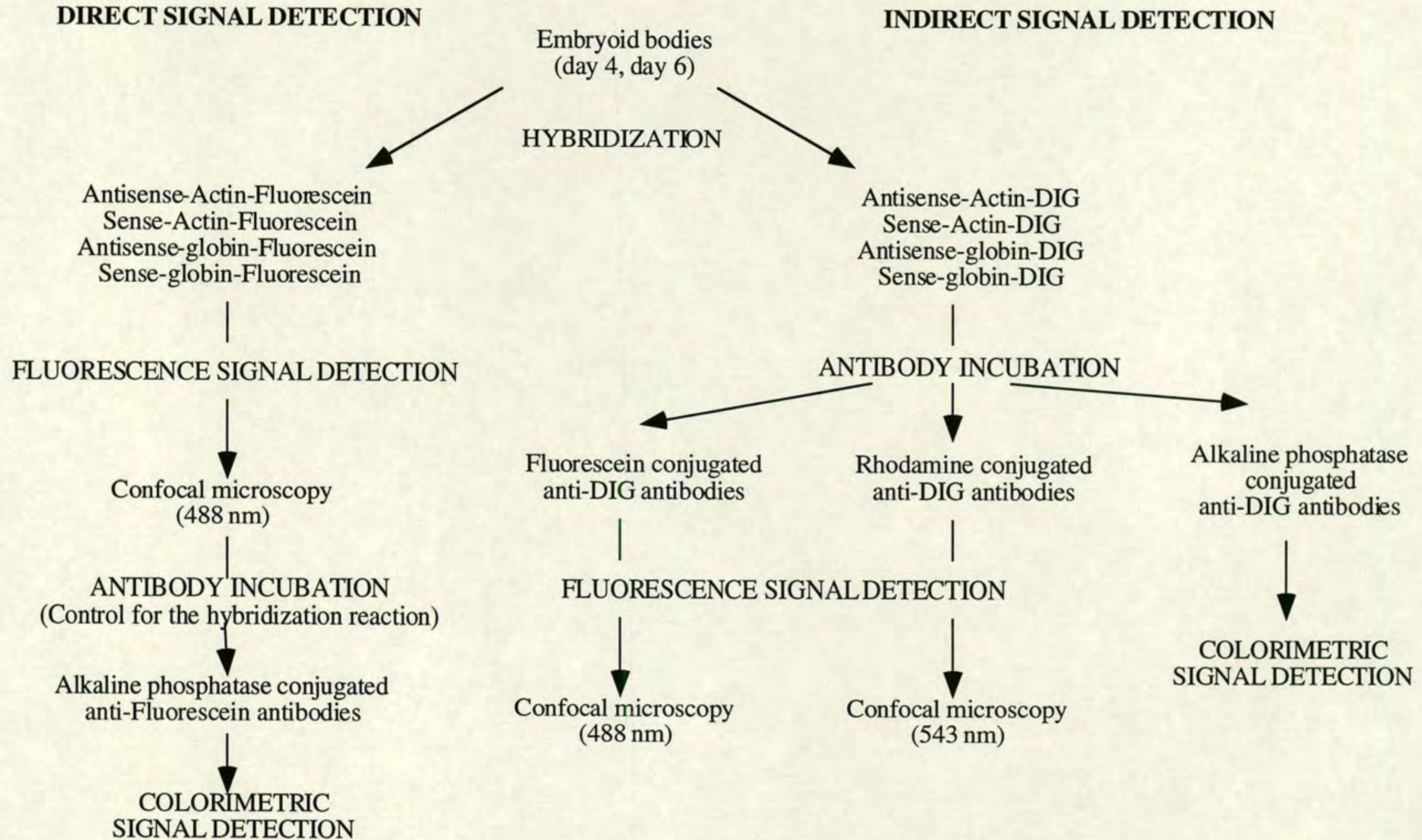
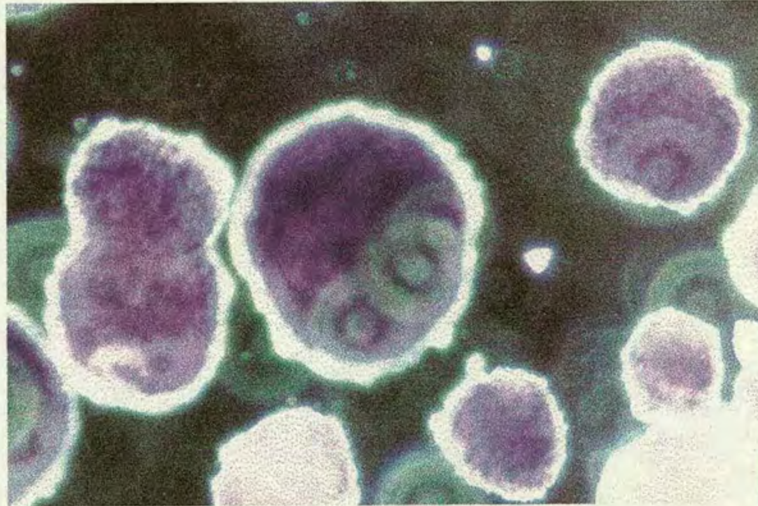


Figure 3.5: Schematic description of the comparison of direct and indirect signal detection systems after whole-mount in situ hybridization of EBs.

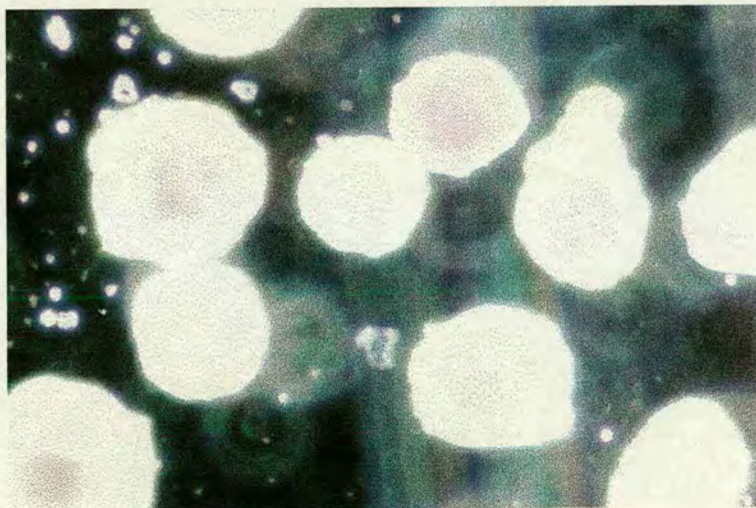
3.2.5.4 Determination of Hybridization Specificity

Control reactions for hybridization reactions

Controls for the specificity of the hybridization procedures were performed using: (1) anti-sense riboprobes for actin mRNA for hybridization which served as positive control; (2) antibody conjugate without probe, as control for unspecific antibody binding; (3) neither probe nor antibody added, as control for endogenous alkaline phosphatase activity; (4) corresponding sense-sequences of anti-sense probes as specific controls for particular riboprobes, addressing non-specific binding of probes. These control reactions were performed for each hybridization protocol used. Figures 3.6.1-3.6.2 shows images of these 'general' controls. Specific controls using corresponding sense-sequences to particular anti-sense riboprobes are shown with their respective anti-sense image in Results Section 4.3.

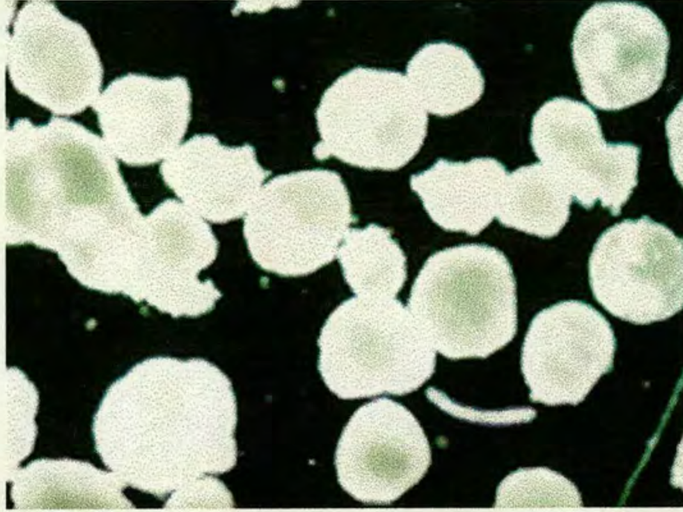


Positive signal for actin expression in day 6 EBs.

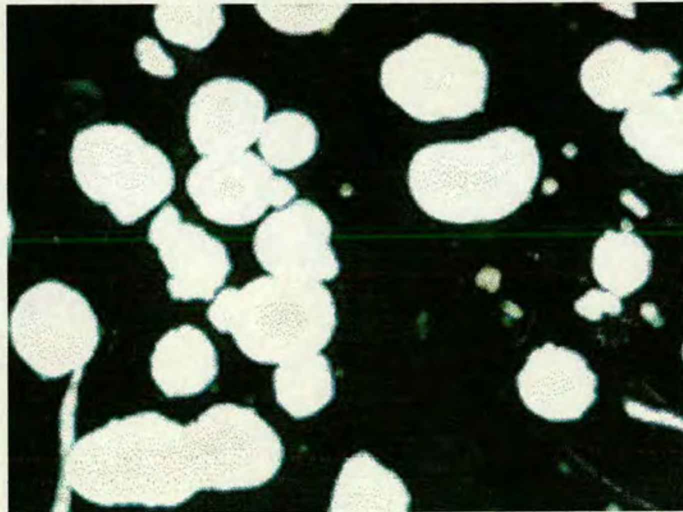


Background level for actin expression by exposure of day 6 EBs with sense probes for actin.

Figure 3.6.1: Positive control hybridizations for whole-mount *in situ* hybridization experiments. Day 6 EBs were hybridized with DIG-labelled antisense riboprobes specific for actin expression (top) or exposed to DIG-labelled riboprobes with corresponding sense sequences to actin sequence(bottom). After hybridization, riboprobe-target RNA hybrids were detected by immunoenzymatic colorimetric signal detection (Magnification 300x).

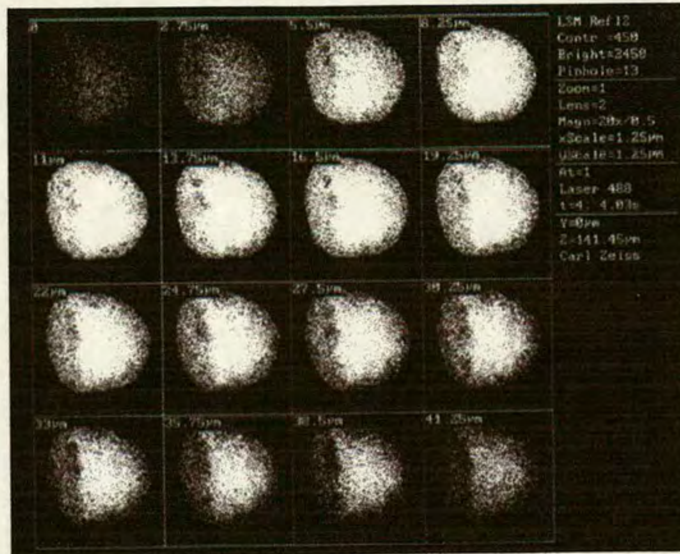


Background level from non-spcecific antibody binding during immunoenzymatic colorimetric signal detection in whole-mount *in situ* hybridization reactions.

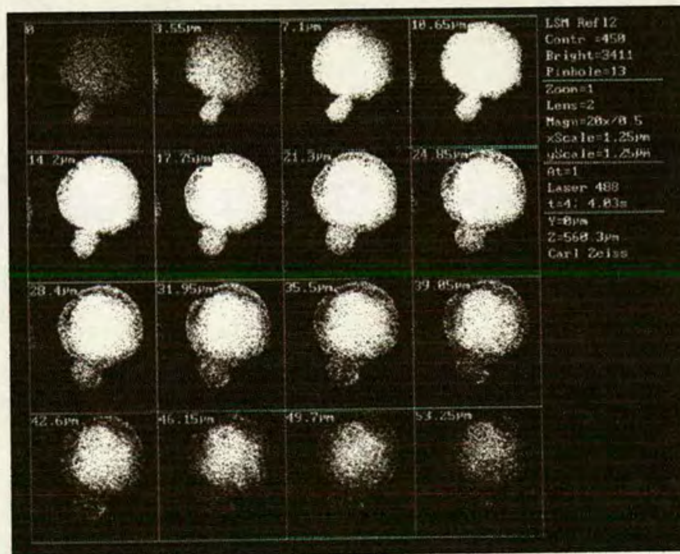


Background level from non-specific alkaline phosphatase activity during immunoenzymatic colorimetric signal detection in whole-mount *in situ* hybridization reactions.

Figure 3.6.2: Negative control reactions for whole-mount *in situ* hybridization experiments. Day 6 EBs were incubated in hybridization solutions without riboprobe but exposed to alkaline-phosphatase conjugated anti-DIG antibodies and incubated in colorimetric signal detection solutions (top). Day 6 EBs were incubated in hybridization solutions without riboprobe and in antibody incubation buffer without antibody added incubated in colorimetric signal detection solutions (bottom) (Magnification 300x).

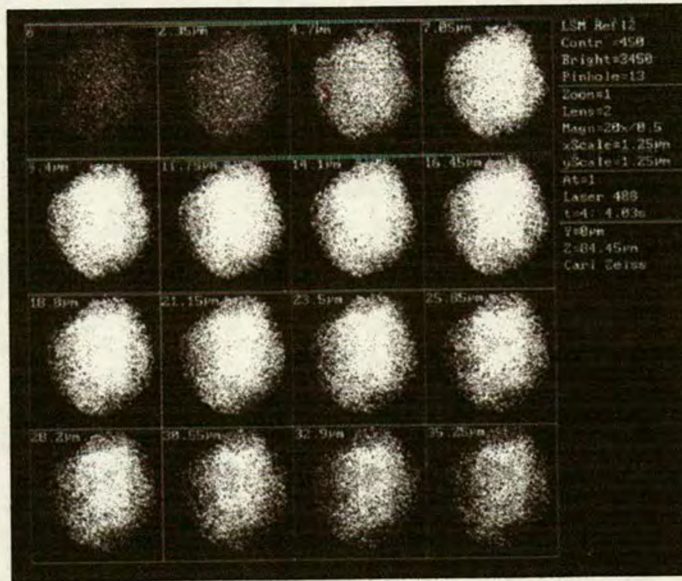


Sequence of optical sections through an EB hybridized with fluorescein-labelled antisense riboprobes specific for *actin* expression.

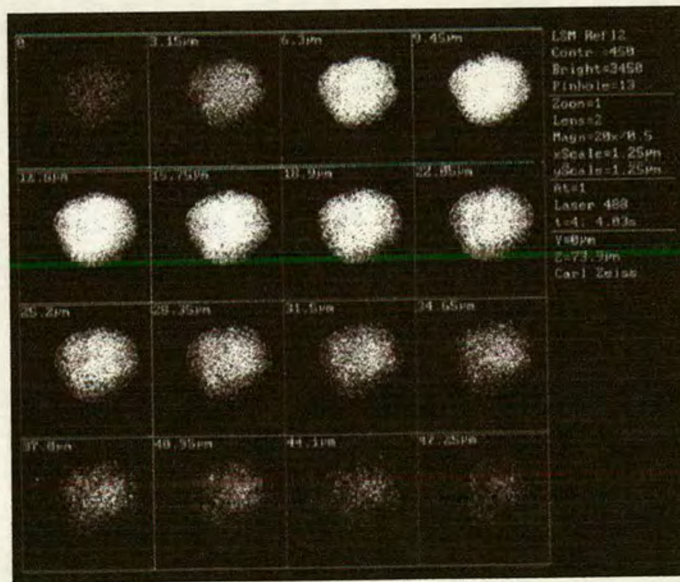


Sequence of optical sections through an EB incubated in hybridization solutions without riboprobe.

Figure 3.6.3: Whole-mount *in situ* hybridization of EBs combined with confocal microscopy for signal detection. Day 6 EBs were hybridized with fluorescein-labelled antisense riboprobes specific for *actin* expression (top) or incubated in hybridization solution without added riboprobe (bottom) as control sample for autofluorescence levels. The high autofluorescence level in control EBs demonstrates the difficulties in combining *in situ* hybridization with confocal microscopy.



Sequence of optical sections through an EB hybridized with fluorescein-labelled antisense riboprobes specific for *globin* expression.



Sequence of optical sections through an EB incubated with fluorescein-labelled sense riboprobes corresponding to *globin* sequence.

Figure 3.6.4: Whole-mount *in situ* hybridization of EBs combined with confocal microscopy for signal detection. Day 6 EBs were hybridized with fluorescein-labelled antisense riboprobes specific for globin expression (top) or incubated with fluorescein-labelled sense riboprobes corresponding to globin sequence and confocal microscopy was used to assess signal. High autofluorescence levels hampered the assessment of specific expression patterns.

3.2.6 *In situ* Hybridization of Sections of EBs

Procedures for *in situ* hybridization of EB sections were derived from hybridization protocols for mouse embryo sections (Wilkinson and Green, 1990; Nichols et al, 1996), from whole mount *in situ* hybridization protocols used for zebrafish (Broadbent, J; Graham, A, personal communication) and from whole mount *in situ* hybridization procedures on EBs as described in this study (see Section 3.2.3). The following sections are a detailed description of the procedures used to perform *in situ* hybridization on sectioned EBs. Contents of solutions that are specific for this hybridization protocol are listed below. A schematic description of the procedures required for *in situ* hybridization of sectioned EBs is illustrated in Figure 3.8.

Preparation of sectioned embryoid bodies

Fixation of embryoid bodies

EBs were harvested by allowing them to settle in a 50 ml centrifuge tube as described in Section 3.2.9 and washed twice with 10 ml cold PBS. The EBs were resuspended in 10 ml freshly prepared 4% paraformaldehyde in PBS and fixed overnight (16-18 hours) at 4°C. The fixed EBs were washed twice with 10 ml cold saline (0.83% NaCl) before being dehydrated on ice by washing with ethanol-saline mixtures: once with 50%, twice with 70%, once each of with 85%, 95% ethanol-saline and twice with absolute ethanol. 10 ml of solution was used for each wash. One wash took 15-30 min. The EBs were resuspended in 15 ml of absolute ethanol and stored at -20°C.

Embedding of EBs for wax sectioning

Embedding was performed in a chemical resistant 50 ml plastic centrifuge tube (Corning). Fixed EBs, stored at -20°C in EtOH were used for embedding. The ethanol solution was replaced with 10 ml histoclear, three times for 30-45 min each at room

temperature. The EBs were then incubated for 30 min in a 1:1 histoclear : paraffin wax mix at 60°C, followed by three changes of paraffin wax, each for 30 min at 60°C. The EBs were transferred to a prewarmed mould placed on a heater block at ~60°C using a prewarmed 5 ml plastic pipette. The mould was placed on an even surface after the EBs had settled to the bottom and allowed to set overnight (~16 hours) at room temperature. These wax blocks with embedded EBs were stored at 4°C until used for sectioning and *in situ* hybridization. Moulds were formed by wrapping aluminium foil around a small object to obtain a mould with relative small diameter (~1 cm) and flat bottom area. Since EBs are relatively small in size and samples consisted of a population of EBs it was important to use moulds with a relative small diameter that allowed to embed a large number of EBs in a small area. However, the size of the wax block had to have a large enough surface for sectioning. EBs do not display an obvious anterior and posterior structure, therefore it was not required to orientate the EBs during embedding in wax.

Subbing of slides (TESPA-slides)

Subbing of slides, to enable cell adherence and maintenance during *in situ* hybridization procedures, was essentially performed as previously described (Wilkinson and Green, 1990). Standard sized glass slides (BDH) were placed in a metal rack and immersed for 10 sec each in 10% HCl/70% ethanol, followed by distilled water and 95% ethanol. The slides were dried in an oven at 150°C for 5 min and then allowed to cool to room temperature. Subsequently, the slides were dipped in 2% TESPA (3-aminopropyl-triethoxysilane) in acetone for 10 sec, washed twice with acetone and then distilled water for 10 sec each before being dried at 42°C. TESPA coated slides were wrapped in plastic foil and stored desiccated at 4°C.

Sectioning of wax embedded EBs

The wax block with embedded EBs was mounted on an 'empty' wax block in a standard cassette used for automatic embedding. This was required to be able to use a Jung Multicut 2045 Leica microtome.

The mounted wax block was trimmed into a rectangle shape and 7 μm sections were cut using disposable blades. Sections were placed on slides flooded with sterile DEPC-treated H_2O at ~40°C and allowed to expand. Subsequent sections were placed on subsequent slides and sets of 5 slides with 3-4 single sections in following order were prepared (Figure 3.7). Excess water was carefully removed using a sterile pasteur pipette and the sections were dried overnight at 37°C.

The slides with sections were stored desiccated at 4°C until required for *in situ* hybridization with riboprobes.

Figure 3.7: Schematic diagram of serial sections of EBs
Slides were labelled with date and sample information and records of each section were taken. The sections were preferably placed at the lower half of the slide to ensure complete immersion during handling in coplin jars.

Slide 1	Slide 2	Slide 3	Slide 4	Slide 5
sections	sections	sections	sections	sections
1	2	3	4	5
6	7	8	9	10
11	12	13	14	15

Pre-treatment of sections prior to hybridization

The appropriate number of slides with adherent wax sections of EBs were placed into glass coplin jars, except for acetic acid treatment and the hybridization reaction, and handled in sets for all procedures required for *in situ* hybridization of sections. The maximum number of slides handled in a set was 10 slides/coplin jar. Solution volume for incubations in coplin jars was 25-30 ml. Slides were dewaxed in 30 ml Histoclear, twice for 10 min and than washed with 100% EtOH for 2 min. Subsequently, the slides were washed quickly through 30 ml of 100 % ethanol, 95%, 85%, 70%, 50% and then 30% ethanol : saline solutions, followed by one wash with saline for 5 min and then three washes with PBS for 5 min each. Proteinase K treatment was performed by incubation with 20 μg/ml proteinase K in TE buffer, pH 8.0 for 5 min at 37°C. The proteinase K solution was freshly diluted from a frozen stock solution (10 mg/ml) and prewarmed for at least 30 min prior to use. After proteinase K treatment, the slides were washed twice with PBS for 5 min before fixed with freshly prepared 4% paraformaldehyde in PBS for 20 min. Postfixation washes were twice with PBS for 5 min each. For acetic anhydride treatment, 250 ml of sterile 0.1 M tri-ethanolamine HCl, pH 8.0, solution were added to a histology glass container and a glass rack to hold the slides was placed into the solution. This was set up with a rapidly rotating stir bar and the slides were then transferred into the glass rack.

0.63 ml acetic anhydride was added and the slides were incubated for 10 min with constant stirring. The slides were then transferred back into a Coplin jar and washed twice in PBS for 5 min each. Subsequent dehydration of the slides was performed by washing them quickly through ethanol/PBS solutions with increasing ethanol concentrations. Solutions of 30%, 50%, 70%, 85%, 95% ethanol-PBS and 100% ethanol were used. The slides were drained after dehydration, transferred to a glass rack and placed at a dustfree area to air-dry for approximately 10 min at room temperature before being hybridized with riboprobes.

Prehybridization of sectioned EBs

Prehybridization was performed by placing the slides in a coplin jar and adding 25 ml of hybridization mixture without probe and subsequent incubation at 65°C for 2-3 hours.

Hybridization of sectioned EBs

For hybridization ~2.5 µl hybridization mix containing riboprobe was applied per square centimetre of coverslip. The appropriate volume of riboprobe (5-7.5 µl), depending on probe concentration, was pipetted into a 0.5 ml eppendorf tube and a complementing volume of prewarmed hybridization mix was added to make up the total volume. The mixture was carefully applied to cover the sections on a slide. Care was taken that the sections did not dry whilst applying the hybridization mixture. To spread the hybridization mix, a coverslip was lowered carefully on top of the sections to avoid trapping of air bubbles and sealed to the slide with nail varnish. The slides were then placed horizontally in a plastic container containing tissue soaked with 50% formamide, 5x SSC and sealed by wrapping with cling film and foil and then incubated overnight (16-20 hours) at 65°C for hybridization.

Posthybridization treatment

For posthybridization washes the slides were transferred into a coplin jar with 25 ml wash solution I. One slide after the other was carefully removed from the 65°C incubator and using a pair of tweezers the solid nail varnish was carefully peeled off to release the coverslips. After the coverslips were removed, the slides were quickly placed into a coplin jar containing 25 ml of prewarmed wash solution I to avoid drying of the sections. The slides were washed twice in wash solution I at 65°C for 30 min each. Followed by two washes in wash solution II at 65°C for 30 min each. Ribonuclease treatment as recommended by other protocols was omitted since no background from non-specific riboprobe binding was encountered.

Colorimetric detection of labelled riboprobe hybridized to target mRNA

After the posthybridization washes at 65°C, the slides were washed twice in 1x TBST buffer for 10 min each. Residual remains of the nail varnish or hybridization solutions were carefully removed using tweezers and the slides were then transferred into a new coplin jar containing MABT washing buffer. The slides were equilibrated for about 2 min in MABT before being incubated in blocking buffer for 1-2 hours. This was performed at room temperature.

Incubation with alkaline phosphatase conjugated antibodies (Anti-DIG-AP at 1:5000 or Anti-Fluorescein at 1:2000) occurred at 4°C overnight (16-20 hours) in antibody solution containing antibodies specific for the riboprobe label. The antibody incubation was followed by washes at room temperature. Three washes with MABT for 15 min each and three washes with NTMT solution for 5 min each. The NTMT solution was always prepared freshly from stock solutions on the day of use. Colour development of bound antibodies for hybridized riboprobe was performed by incubation overnight (16-20 hours) in NTMT buffer containing 4.5 µl NBT/ml buffer and 3.5 µl BCIP/ml buffer. The slides were kept dark at room temperature.

Depending on the specificity of the riboprobe signal for hybridization events started to appear after 5-6 hours and was completed after overnight incubation. Prolonged incubation in colour development solution did not cause background staining. The colour reaction was stopped by 5-7 washes in PBT, 5 min each.

For some staining reactions signal amplification was performed by using alkaline phosphatase conjugated anti-alkaline phosphatase antibodies (APAAP-complex, Boehringer Mannheim). For this, normal colour development and washes was followed by incubation with APAAP-complex (250 mU/ml) in antibody solution at 4°C overnight (~16 hours) and subsequent colorimetric signal development as described above.

The slides can be kept for several weeks in PBT at 4°C without changes or loss in staining patterns. To increase staining intensity and to remove sedimented stain

from the slides, the slides were passed through increasing methanol/PBS solutions (50%, 75%, 100%) prior to mounting. The developed slides were mounted with Aqua mount (BDH) mounting medium and the staining patterns were assessed using a microscope.

3.2.7 Two-Colour *In Situ* Hybridization of Sections

Two-colour *in situ* hybridization of sectioned EBs was performed to investigate co-expression patterns of genes. Riboprobes labelled with DIG or fluorescein were hybridized simultaneously to sectioned EBs as described in Section 3.2.6; 'Hybridization of sectioned EBs'. The labels of hybridized probes were detected by using alkaline phosphatase conjugates (Anti-DIG-AP and Anti-Fluorescein-AP) and two different colour substrates (NBT/BCIP and Fast Red). The detection reactions were carried out consecutively. After hybridization and posthybridization washes, the DIG-labelled probe was detected as described above in Section 3.2.6; 'Colorimetric detection of labelled riboprobes hybridized to target mRNA'. After development of signal for DIG-labelled probe, the colour reaction was stopped by 5-7 washes with PBT, 5 min each. To inactivate any remaining AP-activity of bound antibodies four washes, 5 min each, in 0.1 M glycine pH 2.2 (acidified with HCl), 0.1% (v/v) Tween-20 were performed. This inactivation step was followed by three washes in MABT for 5 min each. The sections were then blocked for 1-2 hours in blocking buffer before incubation with Anti-Fluorescein-AP (1:2000) overnight at 4°C. The antibody incubation was followed by three washes in MABT, 15 min each, and equilibration in 0.1 M TrisHCl, pH 8.3, 0.1% Tween-20 for 5 min. This solution was replaced by Fast Red colour substrate solution (1 Fast Red tablet dissolved in 2 ml 0.1 M TrisHCl pH 8.3) and the sections were kept dark for colour development overnight. The colour reaction was stopped with 3-5 washes in PBT, 5 min each. The slides were then passed through increasing methanol/PBS solutions (50%, 75%, 100%), mounted with Aqua mount solution before evaluated using a microscope.

Solutions for *in situ* hybridization of sectioned EBs

PBS Phosphate buffered saline

TE buffer 10 mM TrisHCl pH 8.0, 1 mM EDTA pH 8.0

Hybridization Mix:

50% formamide, 5x SSC, pH 4.5 (acidified with citric acid),

50 μg/ml tRNA, 50 μg/ml heparin, 1% SDS (sodium dodecyl sulphate)

Solution I:

50% formamide, 5x SSC, pH 4.5 (acidified with citric acid), 1% SDS

Solution II:

50% formamide, 2x SSC, pH 4.5 (acidified with citric acid)

TBST Diluted from a 10x stock solution:

100 ml of stock solution consisted of 8 g NaCl, 0.2 g KCl,

25 ml 1 M TrisHCl pH 7.5, 10 ml Tween-20

MABT Maleic acid buffer:

100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.1% Tween-20

BBR Boehringer blocking reagents

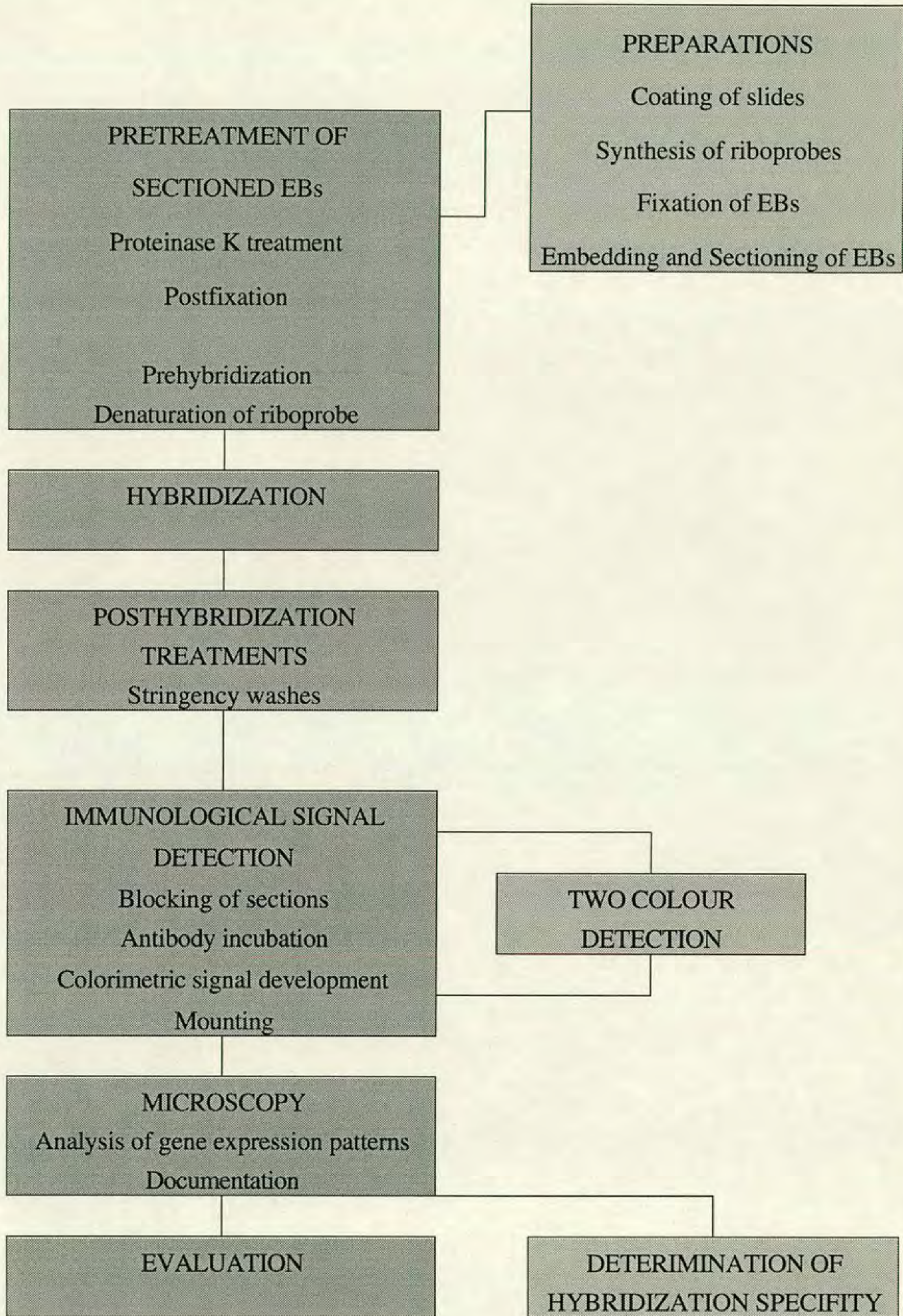
Blocking buffer MAB buffer with 2% BBR

Antibody solution:

MAB buffer with 2% BBR and 1% heat-inactivated sheep serum

NTMT 100 mM NaCl, 100 mM TrisHCl pH 9.5, 1% Tween-20

Figure 3.8: Flow diagram of prodcedures for *in situ* hybridization on sectioned EBs.



3.3 Molecular Biological Methods

Procedures Involving Molecular Biology Techniques

Unless stated otherwise, all molecular biological procedures were standard techniques and carried out essentially as described by Sambrook et al, (1989); Ausubel et al, (1994); and Davis et al, (1986).

3.3.1 Solutions and Media for Molecular Biological Procedures

All solutions and media used for molecular biological procedures were prepared according to sterile routine standards. Distilled water was used for the preparation of solutions and media.

General Solutions for Molecular Biology

a) PBS

One litre of PBS solution, in distilled water contained 10 PBS tablets.

This solution was autoclaved.

b) TBE Buffer

TBE buffer was made up at 10x concentration. One litre of this buffer, in distilled water contained 108 g Tris base, 55 g boric acid and 7.4 g EDTA.

Preparation of Media for Bacterial Strains

a) LB Medium for *E. coli*

One litre of LB medium, in distilled water contained 10 g tryptone, 5 g yeast extract and 5 g NaCl.

b) LB Plates for *E. coli*

One litre of medium consisted of LB medium and 1.5% (w/v) nutrient agar and the antibiotic Ampicillin (100 g/ml).

3.3.2 Plasmid Extraction

3.3.2.1 Plasmid Mini-preparation

The plasmid mini preparation was performed with cultures grown at 37°C in 3 ml LB medium containing the selective compound ampicillin, for 6-18 hours using the alkaline lysis method as described (Sambrook et al, 1989).

3.3.2.2 Plasmid Preparation Using Magic Maxipreps DNA Purification System (Promega)

Large-scale plasmid preparation was performed with cultures possessing the recombinant plasmids which were used for RNA probe synthesis. The procedure was carried out according to the manufacturer's instructions. See Table 3.5 for recombinant plasmids used for *in situ* hybridization of EBs.

Table 3.5: Recombinant plasmids used for *in situ* hybridization of EBs.

Plasmid/Vector	Source
actin in pSP64	Gift of Dr Barry Rosen, Edinburgh, UK
BMP-4 in pSP72	Gift of Dr Brigid Hogan, Nashville, USA
brachyury in pBluescript	Gift of Dr Nicholas Hole, Durham, UK
CD34 in pBluescript	Gift of Dr John Brown, London, UK
c-kit in pPECE	Gift of Dr Lesley Forrester, Edinburgh, UK
FLK-1 in pGEM7	Gift of Dr Janet Rossant, Toronto, Canada
alpha-globin in pUC18	Gift of Dr Nicholas Hole, Durham, UK
SCL in pGEM3	Gift of Dr Anthony Green, Cambridge, UK
SCF in pBluescript	Gift of Dr Nicholas Hole, Durham, UK
TGF in pBluescribe	Gift of Dr Dickson, Glasgow, UK
vav in M13Mp19	Gift of Prof. Adams, Melbourne, Australia
goosecoid in pBluescript	Gift of Dr Nicholas Hole, Durham, UK
pBluescript SK(+/-)	Stratagene, UK

3.3.3 Preparation of DNA for Subcloning

Manipulations of DNA were carried out as described (Sambrook et al, 1989). Subcloning was performed to obtain a recombinant plasmid consisting of a cDNA insert and a vector with two promoters. This was carried out with full length cDNA encoding the gene product actin, c-kit, globin, vav, and the cloning vector, pBluescript SK +/-.

3.3.4 Restriction Endonuclease Digestion

Digestion of DNA by restriction endonucleases was performed, to obtain appropriate DNA fragments for DNA subcloning or for the synthesis of radiolabelled DNA probes, and for the linearization of DNA templates for riboprobe synthesis. Restriction endonuclease reactions typically consisted of 1-2 μg plasmid DNA, 2 units restriction enzyme(s) (Boehringer) and 1x appropriate reaction buffer in a total volume of 20-50 μl, made up with sterile DEPC treated H_2O. The reaction mix was incubated at 37°C for DNA digestion for at least 1 hour up to 16 hours. The restriction endonuclease digestion products were then resolved by agarose electrophoresis (see 3.3.6).

3.3.5 Transformation of Competent Cells *(E. coli)*

Competent cells were prepared using the $CaCl_2$ method as described (Davis et al, 1986). Transformation was used to obtain bacteria possessing the vector plasmid or the recombinant plasmids which were used for subcloning, RNA probe synthesis and [alpha-^{32}P] dCTP labelling of DNA probe.

Competent cells, stored in aliquots of 1 ml in eppendorf tubes at -70°C were thawed at 4°C. 100 μl of cells were transferred to a sterile eppendorf tube and a solution of DNA (5 μl) containing 50-100 ng DNA was added. This transformation mix was gently mixed and placed on ice for 30 min followed by 5 min at 37°C. 1 ml LB medium was added and the solution was transferred to a 15 ml sterile plastic

universal tube. Following incubation in a 37°C shaker for 1 hour, the cells were collected by centrifugation for 5 min at 3 000 rpm (MSE Mistral 6L centrifuge containing a 59563 swing-out rotor). Most of the supernatant was removed. The supernatant left in the tube (~100 μl) was used to resuspend the pellet and the solution was used for plating out onto plates with the selective compound ampicillin. The cells were grown at 37°C for at least 16 hours before clones were picked and used for plasmid mini preparations.

3.3.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to check plasmid DNA preparations and to separate restriction endonuclease digest fragments. This was performed essentially as described (Sambrook et al, 1989). The gels consisted of 1.0% (w/v) agarose in 0.5x TBE, containing 0.5 μg ethidium bromide per ml. Running buffer was TBE buffer (0.5x). The DNA sample volume loaded on a gel varied between 3 μl and 25 μl. Before loading onto a gel, the DNA sample was mixed with 2 μl loading buffer (50% (v/v) glycerol, 1 mM EDTA (pH 8.0), 0.25% (w/v) bromophenol blue in water). DNA was detected by illumination with UV light after electrophoresis for 1 to 4 hours at 80 mV. The gels were then photographed for documentation.

3.3.7 Recovery of DNA Fragments from Agarose Gel

Linearized plasmid DNA or restriction endonuclease digest fragments, separated by agarose gel electrophoresis, were recovered for riboprobe synthesis or labelling with ^{32}P dCTP, respectively. After electrophoresis, the appropriate DNA fragment was cut from the gel using a clean scalpel blade and placed into a 0.5 ml eppendorf which had been pierced with a 26G needle and plugged with siliconized glasswool. The small eppendorf tube was placed into a 1.5 ml Eppendorf tube and then centrifuged for 10 min at 10 000 rpm. Centrifugation effected the separation of

DNA fragments from agarose and allowed the collection of DNA into the 1.5 ml Eppendorf tube. Transfer of the DNA was checked using a UV transilluminator. Recovered DNA samples were then phenol/chloroform extracted (see below) before used for riboprobe synthesis or radio-labelling by nick translation manipulation.

3.3.8 Phenol/Chloroform Extraction of a DNA Sample Followed by Ethanol Precipitation

Phenol/chloroform extraction was performed to purify DNA samples from protein or from agarose gel remains. Subsequent ethanol precipitation allowed concentration of the DNA sample. The phenol/chloroform extraction was carried out by adding about one sample volume of phenol plus one sample volume of chloroform : isoamyl alcohol (24:1) to the DNA sample and vortexing the mixture for 1 min. The organic and aqueous phases were separated by centrifugation for 5 min at 13 000 rpm in an Eppendorf microfuge. The aqueous solution of DNA (upper layer) was removed into a new eppendorf tube with a pipette. The extraction procedure was repeated with chloroform : isoamyl alcohol (24:1). The aqueous solution of DNA was then ethanol precipitated with 2 volumes 100% ethanol and 1/10 volume 3M NaAc. This was carried out for at least 30 min at -70°C. The precipitated DNA was collected by centrifugation for 15 min at 13 000 rpm in an Eppendorf microfuge. After washing, by adding 1 ml of 70% ethanol, centrifugation at 13 000 rpm for 5 min and drying in a vacuum concentrator for at least 5 min, the DNA was dissolved in 20-100 μl DEPC-treated water.

3.3.9 Procedures Involving RNA

For manipulations involving RNA, sterile plasticware from previously unopened bags was used. Glassware was baked at 180°C for at least 16 hours before use. Solutions were maintained as free of RNase by DEPC treatment or by using (0.1%) DEPC-treated water. DEPC treatment of solutions consisted of the addition of 0.1% DEPC and incubation for 16 hours at 37°C before sterilization. Eppendorf tubes were centrifuged in a MSE Micro Centaur microfuge.

3.3.10 Total RNA Extraction

Extraction of total RNA from fetal liver, fetal brain, posterior region tissue from 129/OLA or CBA mouse embryos and spleen, bone marrow and thymus from CBA or 129/OLA mice was performed using the RNAzol B method (Biotecx). The RNA extraction was carried out according to the manufacturer's instructions, as follows. Isolated mouse tissue cells, were resuspended in 1 ml DEPC-treated PBS and transferred to a sterile 1.5 ml eppendorf tube. The solution was then centrifuged for 1 min at 10 000 rpm and the supernatant removed. The cell pellet was lysed in 0.8 ml RNAzol B and the lysate passed through a 23G needle. 80 μl of chloroform was added and samples were mixed thoroughly by agitation for 15 sec (but not vortexed). The samples were placed on ice for 5 min and centrifugation was performed for 15 min at 13 000 rpm. The aqueous phase (upper layer) was transferred to a new eppendorf tube. A volume of the aqueous phase was about 50 % of the initial volume of RNAzol B used. An equal volume of isopropanol was added and the samples incubated at 4°C for 5-16 hours to precipitate RNA. The tubes were centrifuged as described above and the supernatant removed. The RNA pellet was washed with 75% Ethanol (made with DEPC-treated water) and centrifuged at 4°C for 10 min at 13 000 rpm. After removal of the ethanol, the pellet was dried at room temperature for 5-10 min. It was resuspended in an appropriate volume (50-100 μl) of 1 mM EDTA by incubation at 65°C for 5 min. 1 μl was used to measure the

A_{260} and A_{280}, as described in Section 3.3.16.2. The RNA extraction procedure was repeated from the RNAzol addition steps for preparations which had an A_{260}/A_{280} ratio less than 1.5.

3.3.11 Formaldehyde/Agarose Gel Electrophoresis

Total RNA samples or riboprobe samples were resolved by denaturing formaldehyde/agarose gel electrophoresis as described (Ausubel et al, 1994). A 1% (w/v) formaldehyde/agarose gel was prepared by dissolving the appropriate amount of agarose in DEPC treated H_2O, cooling it to 60˚C, and adding 10x formaldehyde gel running buffer (0.4M MOPS pH 7.0, 0.1M sodium acetate, 0.01M EDTA) and formaldehyde to give final concentrations of 1x and 2.2M, respectively. The RNA samples were prepared by adding 0.5-10 μg RNA in DEPC treated H_2O, 1/10 of the total volume of 10x formaldehyde gel running buffer, 50% formamide and formaldehyde to give a final concentration of 2.2 M. The samples were then incubated at 65˚C for 15 min, chilled on ice for 3 min and 1/5 of the total sample volume of formaldehyde loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol; 1 mM EDTA pH 8.0; 50% glycerol in DEPC treated H_2O) was added and mixed. The samples were loaded and electrophoresed at 5 V/cm for 2-3 hours. The gels were then photographed and used for northern blotting.

3.3.12 Northern Blot

Capillary transfer of RNA fractionated by denaturing formaldehyde/agarose electrophoresis, onto a nylon membrane (Boehringer Mannheim) was performed according the manufacturer's instructions.

The formaldehyde/agarose gel was placed in a glass dish and rinsed 3 times for 10-15 min with DEPC-treated H_2O to remove formaldehyde. The capillary transfer apparatus was prepared as follows. A plastic dish was filled with DEPC-treated 20x SSC and a plate was placed over the dish. Three pieces of 3MM Whatman paper were cut, wet with 20x SSC and draped over the plate with overhanging ends hanging into buffer to act as wick. Possible air bubbles were smoothed out by rolling a sterile 10 ml plastic pipette over the paper. The top right-hand corner of the gel was cut off to assist orientation during succeeding operations and then placed upside down on the filter paper avoiding air bubbles. The gel was surrounded with strips of parafilm to avoid short-circuiting of the buffer. A piece of nylon membrane, which was handled with forceps, was cut the same size as the gel and wet by placing it into water for 3-5 min. The wetted membrane was then placed onto the gel avoiding any air bubbles between the membrane and the gel. The surface of the membrane was flooded with 20x SSC and three pieces of 3MM Whatman paper, cut to exactly the same size as the gel, were placed on top of the membrane. A stack of paper towels were placed on the 3MM paper and a weight of ~500 grams was placed on top. The transfer of RNA to membrane by capillary action was allowed to proceed overnight (16-18 hours). The paper towels and the 3MM papers above the gel were then removed and the positions of the gel slots were marked with a pencil on the membrane. The membrane was rinsed in 2x SSC for 3 min and the RNA UV-crosslinked before hybridized with non-isotopic riboprobes or alpha-^{32}P labelled DNA probes.

3.3.13 Non-Isotopic Filter Hybridization

Non-isotopic hybridization of filters with RNA or DNA was performed to check labelled riboprobes used for *in situ* hybridization of EBs. The procedure was essentially performed according to the manufacturer's instructions (Boehringer Mannheim, 'The DIG system user's guide for filter hybridization'). Hybridization was performed in sterile strong chemical resistant 50 ml plastic centrifuge tube (Corning). Each membrane was placed in such an universal tube and hybridization mix without riboprobes (5 ml/10 cm^2) was added. The tube was then placed in a standard hybridization roller tube (Hybaid) and prehybridized for 2-3 hours at 65˚C. Riboprobes used for hybridization were denatured by incubation at 65˚C for 10 min and placed on ice for 5 min. The prehybridization solution was replaced by hybridization mix (0.5 ml/10 cm^2) containing riboprobe (100 ng/ml). Great care was taken that the membrane did not dry during solution exchanges. Hybridization occurred at 65˚C under constant rotation for 12-18 hours. The membrane was washed at 65˚C, twice in 5 ml 2x wash solution for 5 min each and twice in 0.5x wash solution 15 min each. The hybridization reaction was followed by colorimetric signal detection of hybridized labelled riboprobe.

Solutions for non-isotopic hybridization of membranes

Hybridization Mix:

5x SSC, pH 7.0 (acidified with citric acid), 50% formamide,
0.1% sodium-lauroylsarcosine, 0.02% SDS (sodium deoxycholate),
2% Boehringer blocking reagents

2x Wash solution :

2x SSC, pH 7.0, 0.1% SDS

0.5% Wash solution:

0.5x SSC, pH 7.0, 0.1% SDS

3.3.14 Colorimetric Detection of Non-Isotopic Labelled Riboprobes on Membranes

Colorimetric signal detection was performed with membranes with RNA, hybridized with DIG-labelled and/or fluorescein-labelled riboprobes or membranes with newly synthesized riboprobes for size and quality check. The membrane was placed into a sterile petri dish plate, equilibrated briefly (~1 min) in 5 ml in MABT washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.3% Tween-20) before incubated in 10 ml blocking solution (1% Boehringer blocking reagents in MAB) for 1-2 hours at room temperature. Anti-DIG-AP-conjugated antibodies were diluted at 1:50 000 and Anti-Fluorescein-AP-conjugated antibodies were diluted at 1:20 000 in blocking solution. The membrane was incubated in 5 ml of diluted antibody solution overnight (12-16 hours) at 4°C. The membrane was then washed twice in 10 ml wash buffer for 15 min each before equilibrated for 2 min in detection buffer (0.1 M TrisHCl pH 9.5, 0.1 M NaCl) which was prepared from stock solutions on the day of use. The colour substrate solution for signal development was prepared freshly immediately prior to use. The detection buffer was removed and 5 ml of colour substrate solution (4.5 μl/ml NBT, 3.5 μl/ml BCIP in detection buffer) was added to the membrane. The colour development was carried out in the dark for 3 to 16 hours. After signal in sufficient intensity appeared, the reaction was stopped by washing the membrane three times in sterile H_2O for 5 min each. The spot intensity of the control and experimental dilutions were compared to estimate the concentration of the experimental riboprobes.

3.3.15 Radiolabelled Filter Hybridization

Filters with RNA were hybridized with radiolabelled DNA probes as controls for plasmids used for *in situ* hybridization of EBs.

DNA probes labelled with [alpha-^{32}P] dCTP (Amersham) were prepared using a NICK translation system (Promega) according to the manufacturer's instructions. The hybridization procedures were performed as described (Sambrook et al, 1989) using Church/Gilbert buffer as hybridization mix. After hybridization, the filters were exposed overnight to a phosphoimager screen for detection of specific hybridization events. The screen was then scanned using a Molecular Dynamics laser scanner and analysed using Molecular Dynamics software.

3.3.16 Southern Blot

Capillary transfer of DNA fragments separated by agarose electrophoresis was carried out as described for RNA with the exception that the gel was submerged in denaturation solution (0.5 M NaOH, 1.5 M NaCl), twice for 15 min, rinsed with H_2O and then neutralized in neutralization buffer (0.5 M TrisHCl, pH 7.5 with HCl, 3 M NaCl), twice for 15 min before placed on a capillary transfer apparatus as described above (see Section 3.3.12; 'Northern Blot'). These incubations were carried out on a shaker.

3.3.17 Optical Density Measurement

The concentration of DNA or RNA in a sample was determined using an Unicam Helios UV-Vis Spectrometry spectrophotometer.

3.3.17.1 Determination of DNA Concentration

The absorbance at 260 nm of a DNA solution is directly proportional to the amount of DNA in the sample. An absorbance at 260 nm of 1.0 corresponds to 50 μg of double-stranded DNA per ml. To obtain the DNA concentration of a sample, the following formula was applied:

$$\text{DNA (mg/}\mu\text{l)} = 0.05 \times A_{260} \times \text{dilution factor}$$

Sterile distilled water was used as a reference and also to dilute the DNA sample appropriately. The absorbance was measured in 1 ml solution using a quartz cuvette of 1 cm path length. To determine the purity of DNA in a sample the ratio of the absorbance at 260 nm and 280 nm (A_{260}/A_{280}) was calculated. Pure preparations of DNA have A_{260}/A_{280} values of ~1.8.

3.3.17.2 Determination of RNA Concentration

The absorbance at 260 nm of a RNA solution is directly proportional to the amount of RNA in the sample. An absorbance at 260 nm of 1.0 corresponds to 40 μg of RNA per ml. To obtain the RNA concentration of a sample the following formula was applied:

$$\text{RNA (mg/}\mu\text{l)} = 0.04 \times A_{260} \times \text{dilution factor}$$

Sterile distilled water was used as a reference and also to dilute the RNA sample appropriately. The absorbance was measured in 1 ml solution using a quartz cuvette of 1 cm path length. To determine the purity of RNA in a sample the ratio of the absorbance at 260 nm and 280 nm (A_{260}/A_{280}) was calculated. Pure preparations of RNA have a A_{260}/A_{280} values of ~2.0.

4. *RESULTS*

4.1 *In vitro* differentiation of ES cell lines containing gene trap construct insertions and their 'parental' ES cell lines

Haematopoietic *in vitro* differentiation of four ES cell lines containing gene trap construct insertions (thereafter referred to as 'gene trap' cell lines), selected on the basis of their spatial expression patterns in chimaeric embryos (I114, R68, ST598, Zin40), their respective parent ES cell lines (R1, CGR8) and a control ES cell line (EFC-1) was assessed. Three of these gene trap lines (I114, R68, ST598) had been shown to have a restricted expression pattern *in vivo*, one gene trap line (Zin40) had been found to be expressed ubiquitously *in vivo* (see Material and Methods Section 3.1.4; Forrester et al, 1996; Skarnes et al, 1995; Smith A, personal communication). The experiments were designed to assess the effect of gene trap construct insertions on haematopoietic lineage development and the correlation of *in vitro* and *in vivo* reporter gene expression as a pilot study for the potential use of *in vitro* differentiation as a large scale prescreen of a gene trap library. All experiments were performed in sets, i.e. ES cells with gene trap insertions, their parental cell lines, and the ES cell line EFC-1 as an internal control were assayed at the same time.

4.1.1 CFU-A analysis of gene trap cell lines and ES cell lines

The haematopoietic differentiation potential *in vitro* of the gene trap cell lines (ST598, Zin40, I114, R68), their parent ES cell line (CGR8, R1) and the control ES cell line (EFC-1) was assessed by CFU-A analysis. ES cell aggregates of each cell line were produced by the hanging drop method, and were allowed to differentiate *in vitro* over a time course of 8 days before CFU-A analysis. The emergence of CFU-A colonies in all cell lines tested, indicated the presence of primitive haematopoietic progenitors within these developing EBs (Figure 4.1).

CFU-A colonies are essentially composed of macrophages derived from a clonogenic primitive progenitor cell (termed CFU-A). The CFU-A shares properties in common with the CFU-S (colony forming unit, spleen), such as responsiveness to stem cell regulators and self renewing potential as observed by replating analysis. The CFU-A can be considered as an *in vitro* equivalent to the CFU-S (Lorimore et al, 1990; Hole et al, 1996). ES cells have the potential to differentiate into haematopoietic and other lineages and it has been reported that ES cell lines containing gene trap constructs can maintain their differentiation potential despite the integration of foreign DNA (Robertson, 1986; 1991). Comparison of temporal CFU-A profiles showed that in all cell lines analyzed the first appearance of CFU-A colonies was around day 4-6 of *in vitro* differentiation. The consistency of onset of the emergence of CFU-A colonies in different cell lines suggested a 'common' developmental programme for *in vitro* haematopoiesis, induced by the removal of conditions that prevent ES cell differentiation. Undifferentiated ES cell aggregates were unable to form haematopoietic colonies in the CFU-A assay. This and the absence of substantial numbers of CFU-A colonies prior to 4 days of *in vitro* differentiation indicated the need for differentiation within the EB to enable the development of primitive haematopoietic progenitors.
The cloning efficiency in the CFU-A assay is defined by the percentage of EBs plated that form haematopoietic CFU-A colonies. Each sample was assayed in triplicate and the number of CFU-A colonies were scored after 8-12 days incubation. The numbers expressed in graphs showing data from time course studies of haematopoietic differentiation represent the means of 3 assay plates for each time point. The error bars for each time point describe the standard deviation. Variations in the onset of emergence and cloning efficiency of CFU-A colonies between different experiments were within the consistency of this *in vitro* assay. Variations between experiments as observed for the ES cell line EFC-1 (Fig. 4.1 A and B) are most likely derived from batch differences of components (conditioned medium AF1-19T, a source of GM-CSF and L929, a source for CSF-1 was a gift from the Beatson Institute in Glasgow) used

to set up the assay, differences in passage numbers of the ES cells, and technical variation. For instance, the consistency of the upper agar layer, which contains the EBs, affects the formation of CFU-A colonies. If the agar layer is too soft the supportive culture conditions for the formation of CFU-A colonies is not provided. The number of EBs/plate should not be more then 50. Plating of > 50 EBs results in a decrease in CFU-A colonies due to depletion of factors in the medium. When used as a rapid screen for haematopoietic differentiation the size and structure of the CFU-A colonies are scored by eye. This may also be a source of variation. In a qualitative analysis of colonies derived from EBs in CFU-A assay conditions, the diameter of colonies produced was reported to be a measure of the multipotency of the clonogenic cell. Large CFU-A colonies (>2mm in diameter) were derived from more primitive haematopoietic progenitors (Hole et al, 1996). The insertion of gene trap constructs into ES cells may have mutagenic effects. Since the procedure for gene trap construct integration involves subcloning steps these mutagenic effects may be different between different clones. Comparisons of the temporal CFU-A profiles of gene trap cell lines to their respective parent ES cell lines revealed different differentiation characteristics. Each gene trap and ES cell line, displayed a characteristic temporal CFU-A pattern (Figure 4.1), which may have reflected different clonal mutagenic effects.

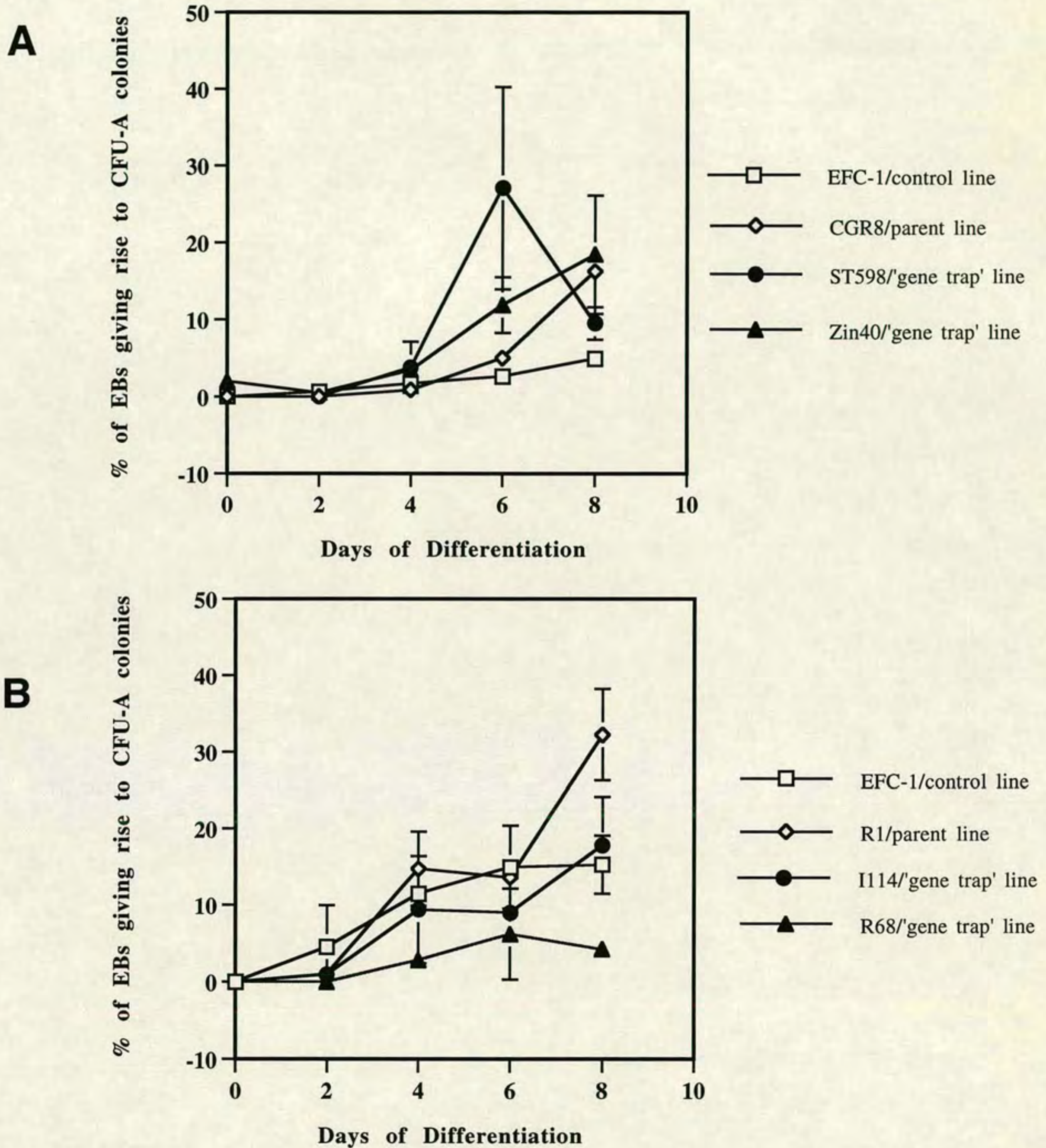


Figure 4.1: Time course analysis of the emergence of CFU-A colonies within differentiating EBs. The number of EBs forming CFU-A colonies is expressed as a percentage of plated EBs (clonal efficiency). Time is expressed as a number of days EBs were allowed to differentiate in suspension culture in the absence of LIF. Each time point represents the mean of three plates assayed. The error bars express ±standard deviation. (A) Comparison of the parental ES cell line CGR8 and derived 'gene trap' lines ST598 and Zin40 with the control ES cell line EFC-1. (B) Comparison of the parental ES cell line R1 and derived 'gene trap' lines I114 and R68 with the control ES cell line EFC-1.

4.1.2 Effects of morphogenic factors on haematopoietic differentiation of gene trap cell lines and ES cell lines

To further characterize the differentiation potential of the different gene trap and ES cell lines, the effects of exogenous factors on the haematopoietic differentiation of ES cells was analyzed. Previous studies using the ES cell line EFC-1 in the described culture conditions have shown that the haematopoietic commitment of this ES cell line can be influenced by the effects of morphogens. The morphogenic factors dimethyl sulphoxide (DMSO) and retinoic acid (RA) have been found to influence haematopoietic differentation of the ES cell line EFC-1 in a dose-dependent manner, as measured by CFU-A analysis. DMSO has been observed to increase the numbers of CFU-A colonies formed by differentiating EBs, whereas RA decreases haematopoietic differentiation (Doostdar L, 1997). The exposure of ES cell aggregates from the ES cell line EFC-1 to 1% DMSO for the first 48 hours of *in vitro* differentiation and further differentiation in suspension culture can result in a 2 fold increase in the percentage of CFU-A colonies. Contrary to this increase by DMSO, the exposure of ES cell aggregates to 10^{-6} M RA results in the inhibition of haematopoietic differentiation in EBs (Figure 4.2.1).

The opposing effects of these two morphogenic factors on haematopoietic commitment were investigated on the gene trap lines and their parent ES cell lines to assess whether the effects of DMSO and RA are cell line specific or act in a general manner on ES cell lines.

The possible utilization of these two morphogenic factors as a strategy for the design of a specific *in vitro* prescreen of gene trap insertions was examined. Identification of trapped genes potentially involved in early haematopoietic commitment may be likely, if the response to DMSO and RA could be used as a general indicator for haematopoietic development. These experiments would also allow insight in the correlation of the *in vivo* and *in vitro* expression patterns of the reporter gene.

The influence of DMSO and RA on gene trap lines and different ES cell lines was investigated by incubating undifferentiated ES cell aggregates of each cell line with either of the morphogenic factors for the first 48 hours of differentiation. After the exposure to the morphogenic factor, the developing EBs were allowed to differentiate for various periods of time in the absence of added factors. Time course studies over 6 days of differentiation comprised three samples of each cell line for each time point. DMSO induced ES cell aggregates, RA induced ES cell aggregates and control ES cell aggregates which were not exposed to any morphogenic factors but cultured under haematopoietic commitment supporting culture conditions. CFU-A analysis of the developing EBs revealed that DMSO and RA can influence the haematopoietic differentiation potential of gene trap cell lines and ES cell lines (Figures 4.2.1-4.2.4). Whereas the three ES cell lines (CGR8, R1, EFC-1) displayed a 'common' response to the morphogenic factors DMSO or RA, the gene trap lines showed an individual response to these morphogenic factors. An increase in CFU-A colony numbers after exposure to DMSO was observed in the original ES cell lines, with the increase starting at around day 4 of differentiation, the earliest day when CFU-A colonies emerge under standard conditions. Suppression of haematopoietic differentiation was observed in the three original ES cell lines after the exposure to RA (Figures 4.2.1 and 4.2.2). The resemblance of the temporal CFU-A profile between DMSO induced ES cell aggregates and their untreated counterpart ES cell aggregates suggested that DMSO may act predominantly on the haematopoietic differentiation potential of ES cells. The observed changes in haematopoietic commitment during ES cell differentiation after exposure to DMSO or RA can most likely be attributed solely to these morphogenic factors, since the ES cell culture system used was a feeder-free system, without cytokine supplementation or conditioned medium. This temporal and quantitative analysis of haematopoietic commitment in gene trap and ES cell lines by CFU-A formation indicated that the opposing effects of DMSO and RA are not

ES cell line dependent but a common response of some ES cell lines to these two morphogenic factors.

Each gene trap line showed an individual characteristic CFU-A profile compared to their parent ES cell line (Figures 4.2.3 and 4.2.4). The diverse responses to DMSO and RA on haematopoietic commitment of the gene trap lines are likely to be caused by clonal variation in subclones of ES cells and/or by the mutagenic effect of the inserted gene trap construct(s). However, recessive mutagenic effects of gene trap constructs may be compensated for by the second allele.

With the exception of the gene trap line Zin40 (Figure 4.2.3), the effects of DMSO or RA on haematopoietic commitment were not detectable directly after the exposure to these factors. This emphazises the necessity of *in vitro* differentiation within EBs as a prerequisite for the emergence of primitive haematopoietic progenitors. The haematopoietic differentiation potential of the gene trap line ST598 (Figure 4.2.3) was negatively affected by both morphogenic factors. DMSO reduced the number of CFU-A colonies and RA inhibited the formation of CFU-A colonies during *in vitro* differentiation of ST598. The gene trap line Zin40 showed an increase in the number of CFU-A colonies after DMSO exposure and reduction after RA treatment (Figure 4.2.3). The gene trap lines I114 and R68 showed similar responses to the morphogenic factors with the number of CFU-A colonies increasing after exposure to DMSO and inhibition of haematopoietic differentiation after exposure to RA (Figure 4.2.4).

The individual responses after exposure of ES cell aggregates to morphogenic factors may allow confident preselection of gene trap lines that could be of interest in terms of haematopoietic development. Combined with information of the corresponding ß-gal activity during *in vitro* differentiation of gene trap lines it may be a promising strategy for the identification of genes involved in early haematopoietic development.

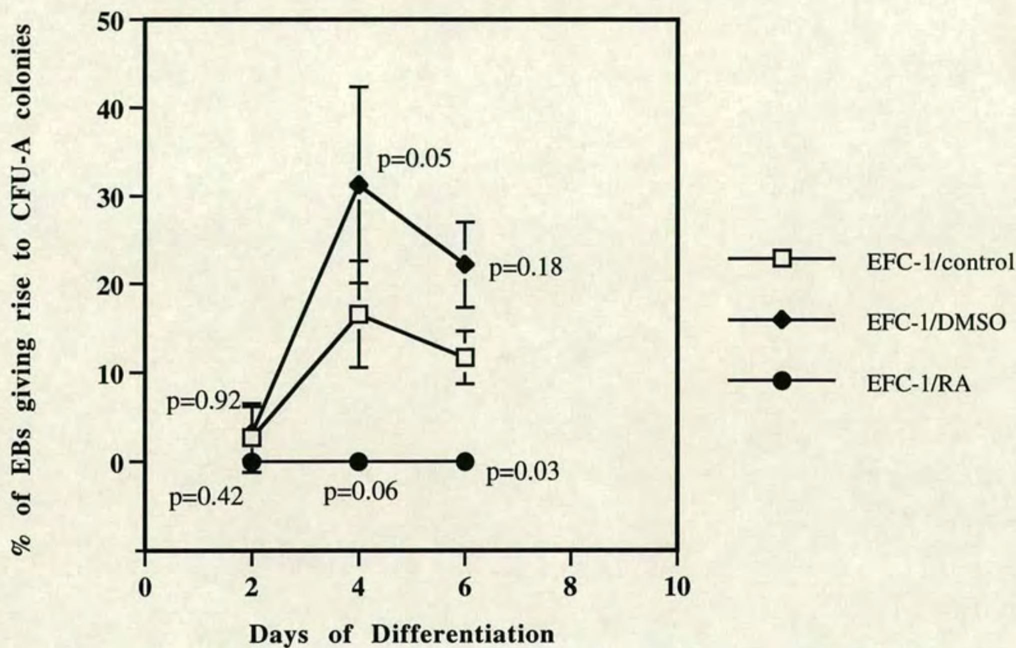
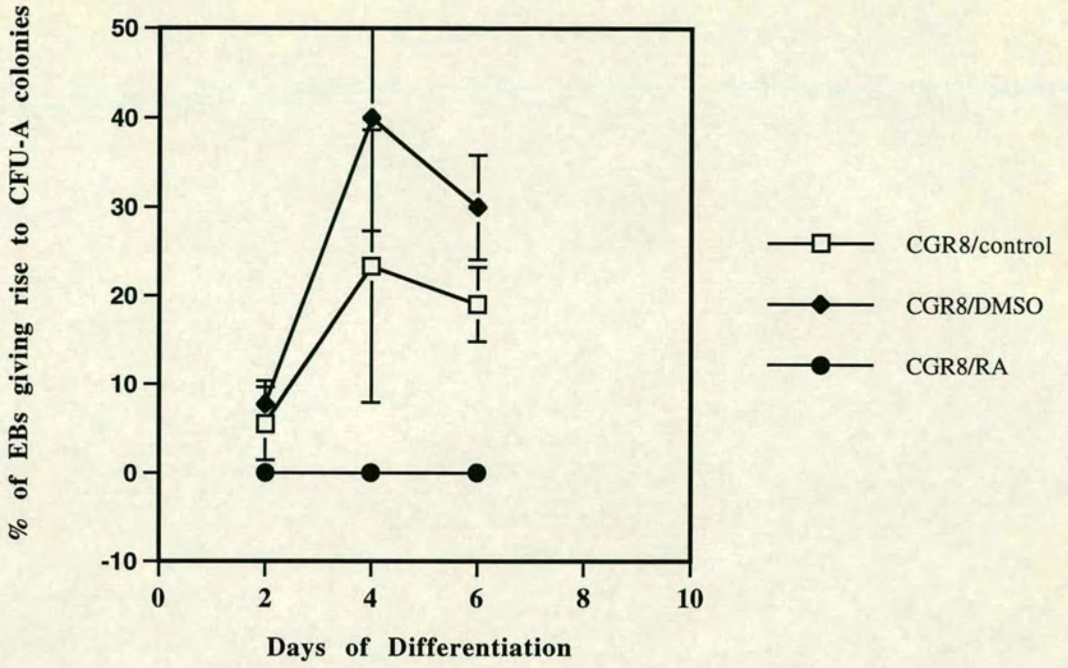


Figure 4.2.1: Time course analysis of the emergence of CFU-A colonies from the internal control cell line EFC-1 after induction with DMSO or RA. EScell aggregates were induced with DMSO and RA, respectively for the first 48 hours of differentiation and allowed to differentiate for up to 6 days before being assayed by CFU-A analysis. Each time point represents the mean of three plates assayed. Error bars express ± standard deviation. P-values are calculated from a paired 2-tail T-test comparing means at each time point between control EBs and induced EBs.



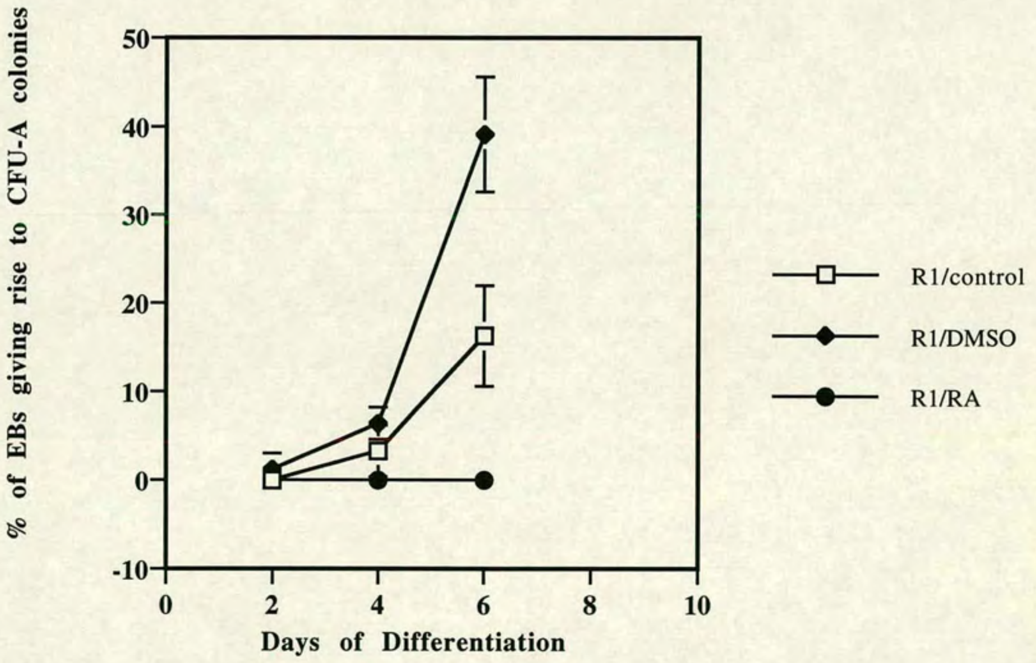
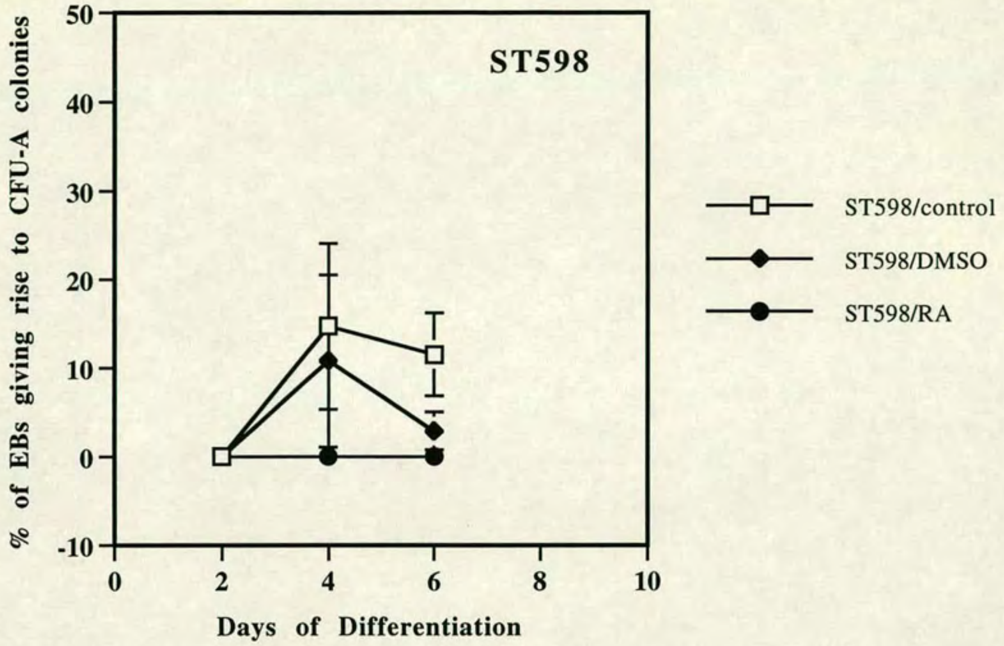


Figure 4.2.2: Time course analysis of the emergence of CFU-A colonies from the parental cell lines CGR8 and R1 after induction with DMSO or RA.



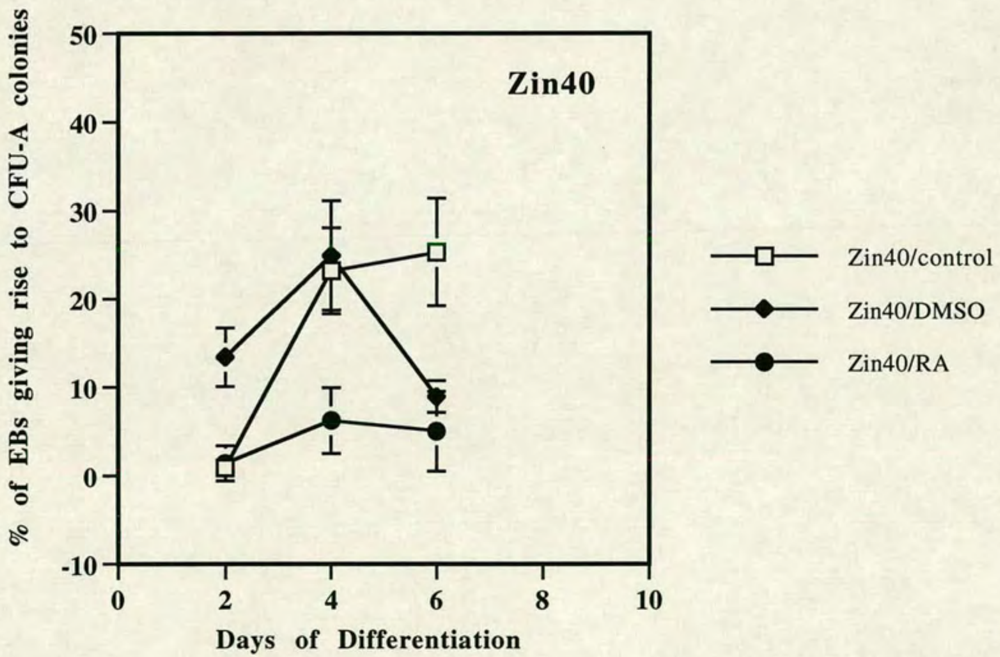
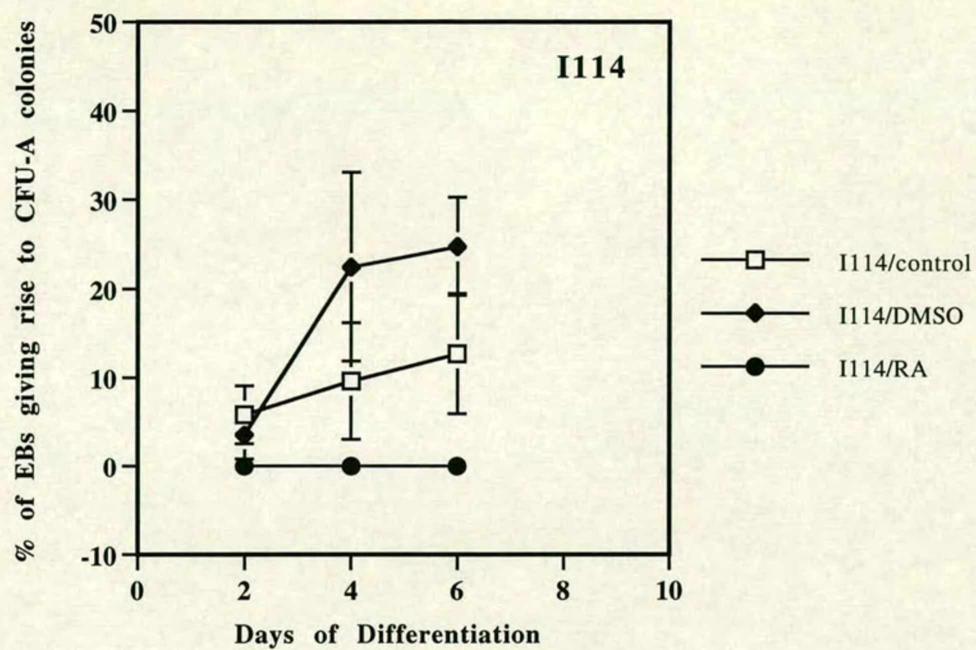


Figure 4.2.3: Time course analysis of the emergence of CFU-A colonies from the gene trap cell lines ST598 and Zin40 after induction with DMSO or RA (parental ES cell line CGR8).



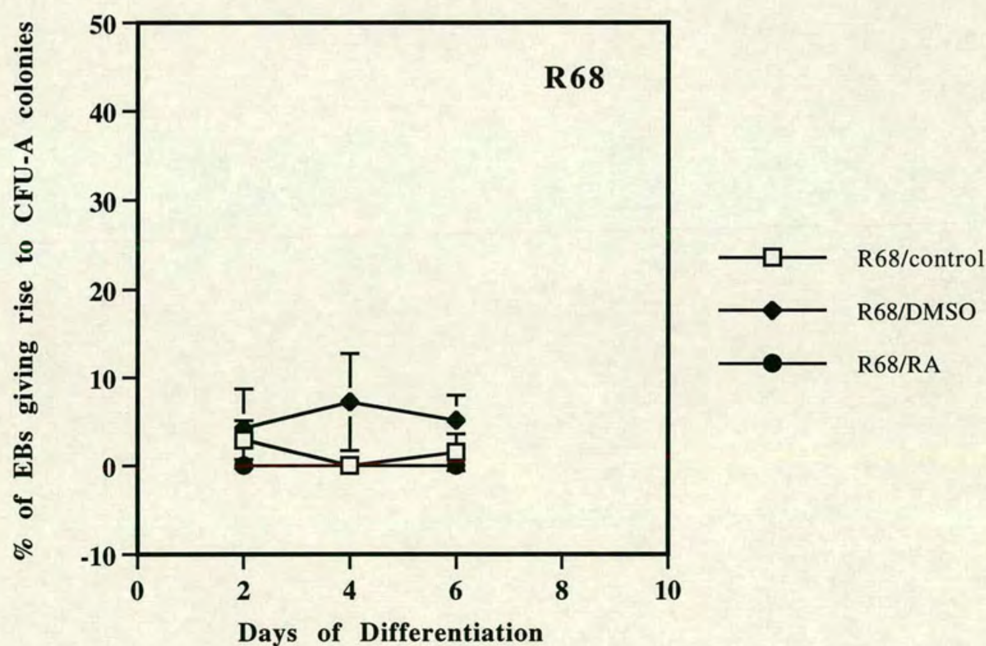


Figure 4.2.4: Time course analysis of the emergence of CFU-A colonies from the gene trap cell lines I114 and R68 after induction with DMSO or RA (parental ES cell line R1).

4.1.3 Quantitation of the ß-galactosidase activity in gene trap cell lines during *in vitro* differentiation

ß-Galactosidase (ß-gal) is a commonly used marker in gene trap constructs and its activity can easily be detected by utilizing a colorimetric assay (Eustice et al, 1991). The activity of functional ß-gal fusion protein reflects the expression pattern of the trapped endogenous gene and can be used to monitor changes in gene expression levels. Monitoring the ß-gal activity of EBs derived from gene trap lines during *in vitro* differentiation allows the assessment of temporal reporter gene expression. However, no details about spatial expression patterns, cell types and numbers of cells expressing active ß-gal fusion proteins can be obtained by this approach since pooled EB populations are used. For the analysis of spatial expression an X-gal staining assay can be used (Beddington et al, 1989).
Haematopoietic commitment of ES cell and gene trap lines can be influenced by exposure to morphogenic factors, suggesting that ß-gal activity at around day 4 of *in vitro* differentiation after exposure to DMSO or RA may allow *in vitro* pre-screening of gene trap events potentially interesting for early haematopoietic development. Increase in ß-gal activity of differentiating gene trap line derived EBs after exposure to DMSO, detected in a time period between day 3 and day 6 of differentiation, could be used as indication for the possible involvement of the trapped endogenous gene in early haematopoietic development. Together with suppression of ß-gal activity in the same time period, after exposure to RA, this strategy should allow the preselection of developmentally regulated gene trap clones.

Using the gene trap lines I114, R68, ST598, and Zin40 time course studies monitoring ß-gal activity during *in vitro* differentiation and after exposure to morphogenic factors were performed. Results presented show data from time course studies and induction studies with DMSO and RA which are representative data from an individual experiment (Figures 4.3, 4.4.1, 4.4.2, and Table 4.1).

All experiments were repeated 3 times, however, complete consistency between individual experiments was difficult to achieve since pooled EBs were used. Although routine culture procedures were employed throughout ES cell culture studies, slight variations in differentiation of EB populations have to be taken in consideration. In addition, possible differences in stability of lacZ fusion transcripts and ß-gal fusion protein can complicate a temporal and quantitative analysis of ß-gal activity. The quantitative assessment of temporal reporter gene expression in gene trap lines over an 8 day time course of *in vitro* differentiation revealed a characteristic ß-gal activity pattern for each gene trap line, reflecting the individuality of gene trap cell lines which emerges through the random integration of gene trap constructs into the ES cell genome (Figure 4.3). Parental ES cell lines (R1 and CGR8) and ES cell line EFC-1 were included as controls for the definition of background activity in this ß-gal activity assay. No significant background activity was observed in these controls.
Temporal changes in ß-gal activity during differentiation indicating developmentally regulated expression of the trapped genes were observed in gene trap lines R68 and ST598. Constitutive ß-gal activity was detected in gene trap lines Zin40 and I114. Corresponding to its constitutive and ubiquitous expression *in vivo*, ß-gal activity in gene trap line Zin40 was found to be at a high level. The gene trap line I114 displayed low level of ß-gal activity above background levels.

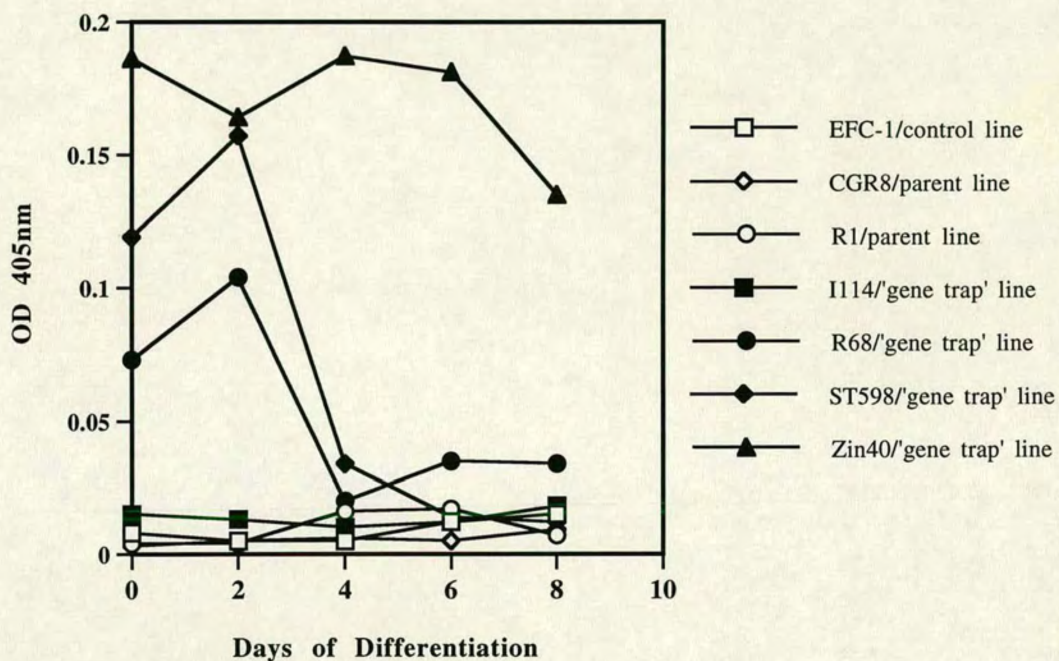


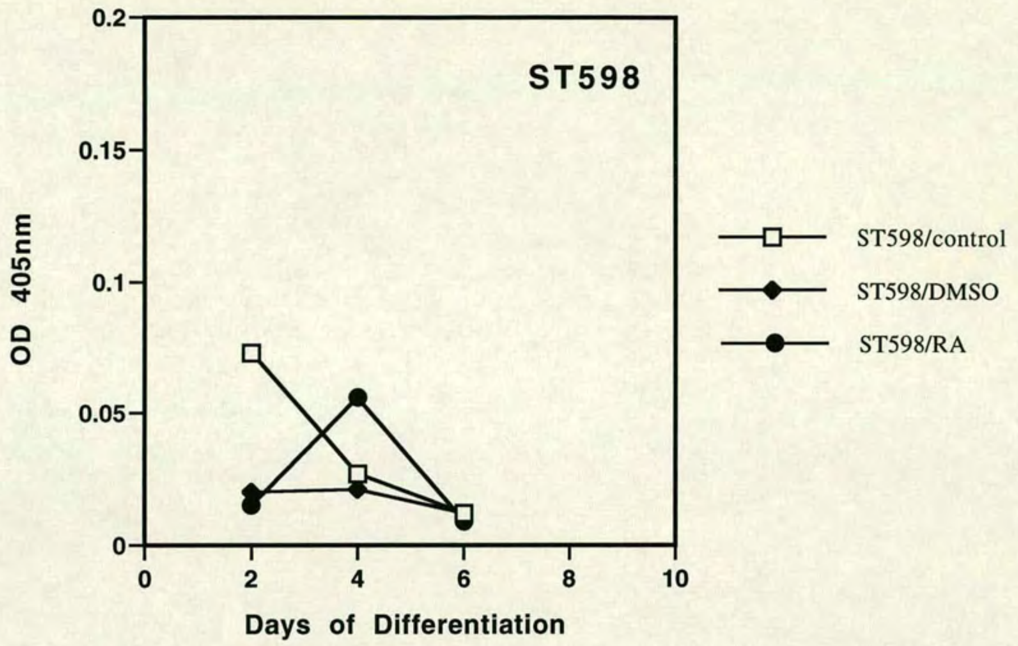
Figure 4.3: beta-gal activity assay of the gene trap lines ST598, Zin40, I114, R68 over a time course of differentiation. The control ES cell line EFC-1 and the parental ES cell lines CGR8 and R1 were included to assess background levels.

4.1.4 Effects of morphogenic factors on ß-galactosidase activity in gene trap cell lines during *in vitro* differentiation

Influences of morphogenic factors DMSO or RA on ß-gal activity in gene trap cell lines are shown in Figures 4.4.1 and 4.4.2 and summarized in Table 4.1. ES cell aggregates of each gene trap cell line were exposed to DMSO or RA for the first 48 hours during *in vitro* differentiation and allowed to differentiate for a further time in suspension culture before used for the analysis of ß-gal activity. ß-gal activity observed in gene trap line ST598 derived EBs was suppressed after exposure to DMSO and a changed pattern in temporal ß-gal activity was observed after exposure to RA. In gene trap line Zin40, the high ß-gal activity observed in control EBs (note extended scale) was increased after exposure to DMSO and RA. A change in the temporal pattern of ß-gal activity was detected after exposure to DMSO, with a decline to levels from untreated EBs at day 4 of differentiation followed by a repeated increase of ß-gal activity at day 6 (Figure 4.4.1).
The gene trap cell line I114 showed no responsiveness in ß-gal activity to exposure of DMSO or RA. Gene trap line I114 was originally identified in an inductive gene trap screen as being induced by RA, however, this screen assayed undifferentiated ES cells and did not monitor ß-gal activity during *in vitro* differentiation of ES cells into EBs (Forrester et al, 1996). Similarly, the gene trap line R68, was originally identified as a cell line whose ß-gal activity is reduced after exposure to RA (Forrester et al, 1996). ß-gal activity during *in vitro* differentiation of R68, after exposure of ES cell aggregates to RA, showed an initial 3-fold increase in ß-gal activity and maintained at an increased level during further differentiation, compared to the untreated counterpart EBs. A changed temporal pattern of ß-gal activity in R68 line derived EBs was also observed by DMSO, with initially high ß-gal activity declining to standard levels of untreated EBs after further differentiation (Figure 4.4.2).
If the gene trap lines used for these experiments had been included in a gene trap screen of 'anonymous' gene trap clones none of these gene trap lines would have

been chosen for further analysis after applying the preselection criteria. The expected characteristics of a gene trap clone for further analysis would have been temporal high ß-gal activity in a time window at around day 4 of *in vitro* differentiation, induced after exposure to DMSO and suppressed after exposure to RA. These ß-gal activity features in conjunction with the appearance of primitive haematopoietic progenitors, as defined by CFU-A analysis, were thought to be characteristics of a trapped gene potentially involved in early haematopoietic development.

The spatial *in vivo* reporter gene expression patterns of the gene trap lines I114 and ST598 indicated involvement of their trapped genes during early haematopoietic development. However, continuing investigations of *in vivo* reporter gene expression patterns of I114 and ST598 confirmed that both lines are not involved/restricted to haematopoietic development during embryogenesis, despite spatial expression in yolk sac and fetal liver (Forrester et al, 1996; Skarnes et al, 1995; Skarnes W, personal communication).



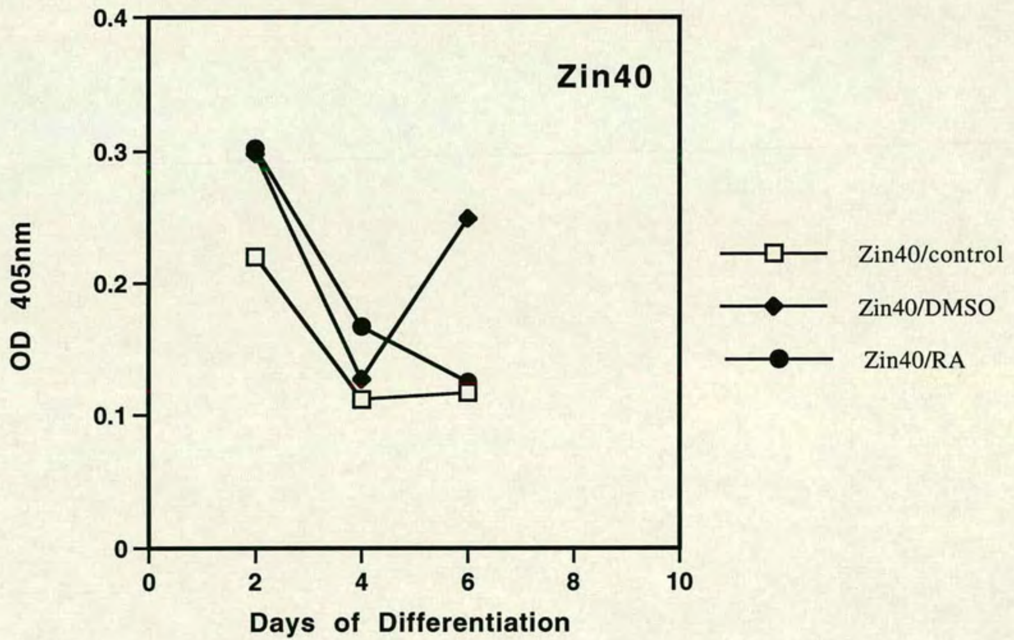
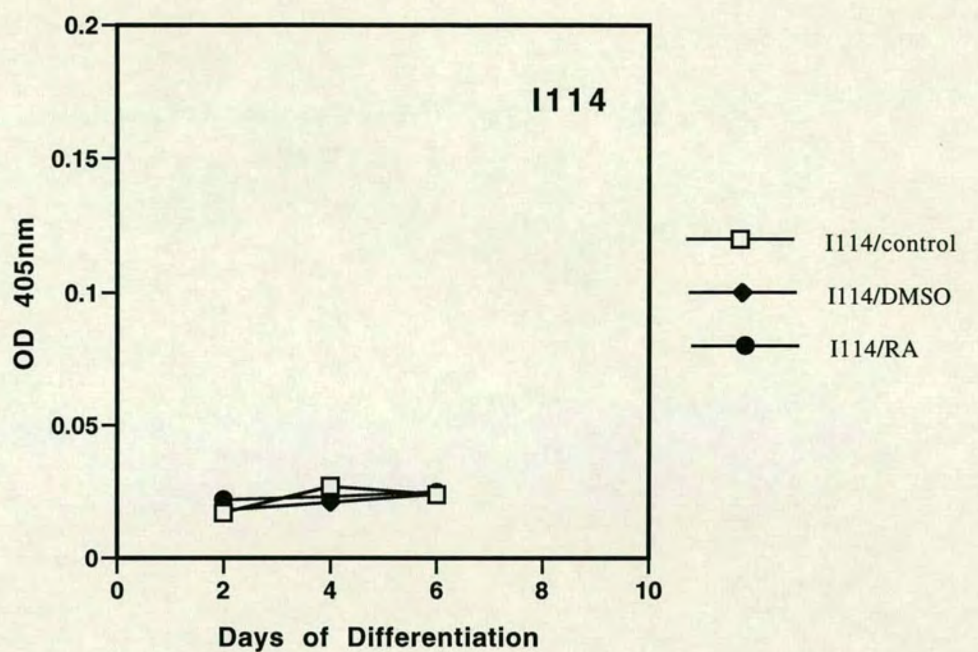


Figure 4.4.1: beta-gal activity assay of the 'gene trap' lines ST598 and Zin40 after induction with DMSO or RA over a time course of differentiation (parental ES cell line CGR8).



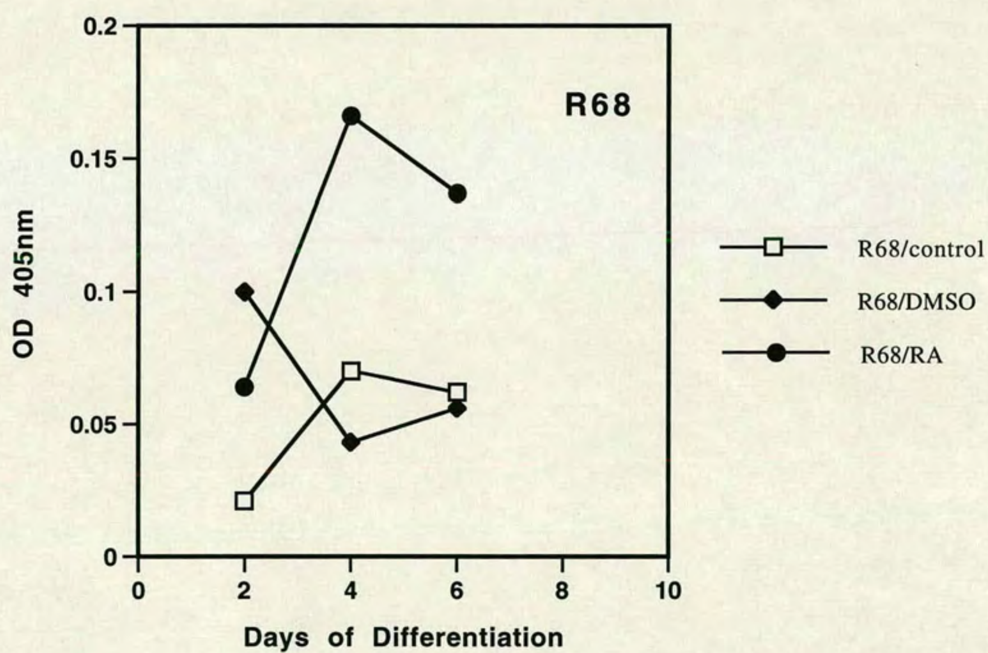


Figure 4.4.2: beta-gal activity assay of the 'gene trap' lines I114 and R68 after induction with DMSO or RA over a time course of differentiation (parental ES cell line CGR8).

Table 4.1: Summary of morphogen exposure on ES cell lines and gene trap cell lines.

Cell line	*in vivo* Expression	**Effect of morphogens on CFU-A profile**		**Effect of morphogens on ß-gal activity (OD 405nm)**	
		DMSO	RA	DMSO	RA
EFC-1 (1)		+++	---	nr	nr
CGR8 (2)		+++	---	nr	nr
R1 (3)		+++	---	nr	nr
I114	YS, FL	+++	---	=	=
R68	fetal heart	+	---	*	+
ST598	YS, FL	*	-	-	*
Zin40	ubiquitous	*	---	*	+

(1) Control ES cell line; (2) Parental ES cell line for ST598 and Zin40;

(3) Parental ES cell line for R68 and I114;

+++ Enhanced; + Enhanced; - Reduced; --- Inhibited; = No effect;

nr, Not relevant; * Changed, see text;

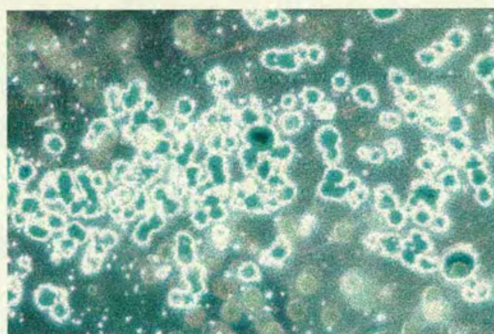
4.1.5 Spatial ß-galactosidase expression patterns during *in vitro* differentiation of gene trap cell lines

The spatial ß-gal expression in whole EBs derived from gene trap lines was assessed by qualitative X-Gal staining. Time course EBs of each gene trap line were stained using X-Gal as a colour substrate for active ß-gal fusion protein which results in a blue precipitate. The individual staining patterns between time points and gene trap lines were compared visually using an inverted microscope (Beddington et al, 1989). An example of a ß-gal expression pattern during *in vitro* differentiation is shown in Figure 4.5.

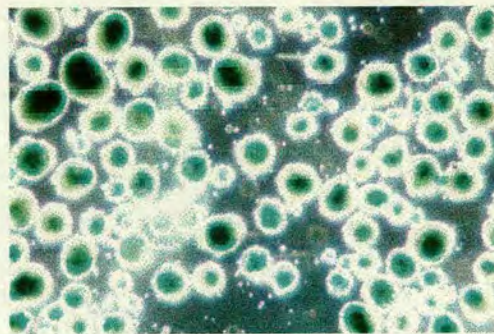
Each gene trap line displayed an individual staining pattern for X-gal (summarized in Table 4.2) and subpopulations of EBs with various ß-gal protein expression patterns could be identified within an EB population. This may be seen as evidence for the heterogeneous differentation of EBs into different lineages.

Analysis of ß-gal activity (see Sections 4.1.3-4.1.4) made it possible to detect temporal changes, however, information about the spatial expression patterns is required to obtain comprehensive information about the expression pattern of a trapped gene. With the exception of the gene trap line I114, all gene trap lines showed temporal and spatial changes in their staining patterns for ß-gal protein. Down regulation of staining for ß-gal may indicate that the trapped endogenous gene is of importance during early development but not at later stages. Morphological identification of cells expressing ß-gal fusion protein within an EB could indicate possible expression patterns of the trapped gene *in vivo*.

X-gal staining of gene trap line EBs, exposed to either DMSO or RA during *in vitro* differentiation was difficult to assess since quantitative analysis of the spatial staining pattern was not possible (data not shown).



day 0



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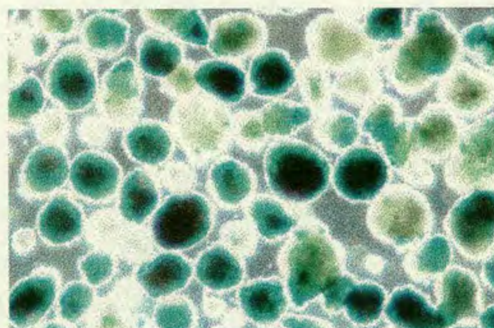

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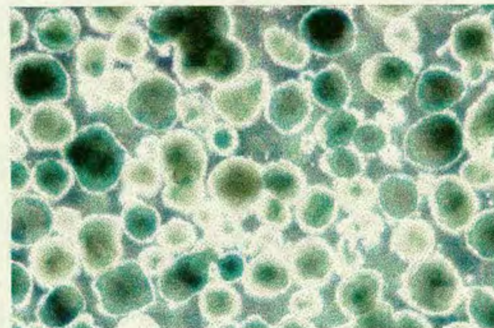

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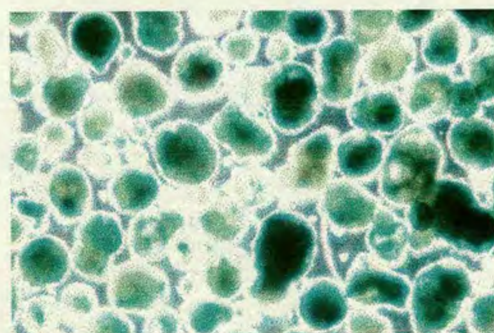


day 8

Figure 4.5: *LacZ* expression pattern in EBs derived from the gene trap cell line Zin40. A time course covering 8 days of *in vitro* differentiation was produced (Sections 3.1.6.4 and 3.1.7) and stained with X-Gal (Section 3.1.11). All undifferentiated ES cell aggregates (day 0) show ubiquitous staining for active ß-gal fusion protein. Upon differentiation (day 2-day 8) varying staining patterns for individual EBs within an EB population are detected, with some EBs staining ubiquitous, some EBs staining partially and some EBs showing no X-Gal staining (summarized in Table 4.2) (Magnification 100x).

Table 4.2: Summary of the spatial X-gal staining patterns in time course EBs from gene trap lines.

	Percentage of EBs showing X-gal staining for ß-gal fusion protein			
	Subpopulations of EBs defined by number of cells stained within an EB			
Gene trap line Age of EBs	All cells stained	Half of cells stained	Some cells stained	No visible staining
R68				
0 days	> 90	-	-	-
2 days	30 - 60	30 - 60	-	-
4 days	30 - 60	-	30 - 60	-
6 days	30 - 60	-	30 - 60	-
8 days	30 - 60	-	30 - 60	-
I114				
0 days	-	-	10 -30	60 - 90
2 days	-	-	10 -30	60 - 90
4 days	-	-	10 -30	60 - 90
6 days	-	-	10 -30	60 - 90
8 days	-	-	10 -30	60 - 90
ST598				
0 days	> 90	-	-	-
2 days	> 90	-	-	-
4 days	20 - 40	20 - 40	20 - 40	-
6 days	-	-	-	> 90
8 days	-	-	-	> 90
Zin 40				
0 days	> 90	-	-	-
2 days	20 - 40	20 - 40	20 - 40	-
4 days	20 - 40	20 - 40	20 - 40	-
6 days	20 - 40	20 - 40	20 - 40	-
8 days	20 - 40	20 - 40	20 - 40	-

4.2 Comparison of ES cell aggregate formation methods

Criteria for the practical aspects of a prescreen included the possible number of samples and the input of time and labour required for performing the screening procedures on a large scale. The routine culture techniques used for EFC-1 ES cells employed the hanging drop method for the formation of ES cell aggregates (Section 3.6.4; Figure 3.2). This allowed the production of an uniform population of aggregates and ensured a synchronized differentiation pattern during time course studies. However, the hanging drop method is a time consuming and labour intensive method which requires skillful cell culture procedures.

Simplification of the ES cell aggregate formation procedures for the initial screening of gene trap clones was examined by comparing various ES cell aggregate formation conditions to the hanging drop method, using the established ES cell line EFC-1. The practicability of simultaneous culture and production of ES cell aggregates from a large number of different gene trap clones (approximately 100 cell clones at the same time) was tested by using multi-well cell culture plates rather than tissue culture flasks.

4.2.1 The use of 'cellform'-coated plates for the formation of ES cell aggregates

When seeded at a high density in suspension culture, ES cells tend to aggregate rapidly by cell-cell collision and adhesion and form ES cell aggregates, rather than attaching to the plate substratum and spreading. However, the resulting ES cell aggregates tend to be heterogeneous in size and shape since the aggregation under these conditions takes places spontaneously, influenced only by the cell numbers seeded. To control and direct the spontaneous aggregation of undifferentiated ES cells towards the formation of an uniform ES cell aggregate population, plates coated with Cellform (poly (2-hydroxyethyl methacrylate)) were tested. Cellform had previously been shown to reduce adhesiveness of plastic plates when applied as an alcoholic solution (Folkman and Moscona, 1978). The preparation of Cellform-coated plates

was performed as described and used by others (B. Rosen, personal communication; Folkman and Moscona, 1978). The conditions for ES cell culturing, however, were adapted from cell culture procedures used routinely for the maintenance and haematopoietic differentiation of the ES cell line EFC-1 (see Sections 3.1.5 and 3.1.7). The maintenance of the haematopoietic differentiation potential of the ES cell line EFC-1, using the high density method in combination with Cellform (poly (2-hydroxyethyl methacrylate))-coated plates for the formation of ES cell aggregates, was assessed by CFU-A analysis. Comparison of size and shape of developing EBs and CFU-A colonies was performed visually using an inverted mircoscope.
The influence of cell density on the ability of ES cell aggregate formation was addressed by plating various concentrations of ES cells into plates. The suitability of plasticware for ES cell aggregate formation was tested by using tissue culture grade plastic plates or bacterial grade (non-tissue culture) plastic plates, coated with Cellform or uncoated. Single cell suspensions of undifferentiated ES cells were seeded at various cell densities, ranging from 0.3 x10^5 cells/ml to 1.0 x10^5 cells/ml, into tissue culture grade plastic dishes or bacterial grade plastic dishes, coated or not with Cellform. The ES cells were kept in FCS culture medium supplemented with LIF during this initial step of ES cell differentiation to provide conditions that support the formation of undifferentiated ES cell aggregates rather than culture conditions that induce ES cells to differentiate. Subsequent culturing of the ES cell aggregates was performed using procedures routinely used for hanging drop ES cell aggregates.

CFU-A analysis of EBs, developing from ES cell aggregates produced by either of the different methods and allowed to differentiate for 4 or 6 days in suspension culture, revealed that the ES cell line EFC-1 can maintain the ability to differentiate into haematopoietic lineages irrespective of the method that has been used to produce the ES cell aggregates (summarized in Table 4.3). However, comparison of EBs

and CFU-A colonies derived from ES cell aggregates formed by either of the different methods, showed distinct differences from EBs produced by the hanging drop method.

Whereas the hanging drop method provides well defined conditions for the production of ES cell aggregates (approximately 300 cells in an aliquot of 10 μl medium are allowed to settle in the hanging drop and form an aggregate) conditions for the formation of ES cell aggregates by either of the other methods is less well controlled. Thus, the hanging drop method allows the production of populations of ES cell aggregates with uniform size and shape and synchronized differentiation patterns. The high density method combined with or without Cellform-coated plates produced ES cell aggregates that varied in size and shape and showed less synchrony in differentiation depending mainly on the cell numbers plated. Apart from heterogeneity in size of ES cell aggregates it was noticeable that the suspension culture of high-density ES cell aggregates contained a large number of single cells and cell debris compared to the clear suspension culture medium from hanging drop ES cell aggregates.

Comparison of ES cell aggregates from Cellform coated and uncoated plates showed that the coating of plastic plates with Cellform supports the formation of ES cell aggregates that are similar in shape to ES cell aggregates produced by the hanging drop method. No significant differences between tissue culture grade and bacterial culture grade plastic plates were observed when plates were coated with Cellform, indicating that the Cellform coat effectively reduces the adhesiveness of plastic plates. Furthermore, no cytotoxic effects of the Cellform coat on ES cell viability was observed. Uncoated bacterial grade plastic plates appeared to have a lower adhesiveness than uncoated tissue culture grade plates and were therefore slightly more effective in preventing ES cells from attaching to the plate and spreading, compared to tissue culture grade plates.

The ES cell aggregates produced in either of these different conditions and allowed to differentiate for 4 to 6 days in standard differentiation conditions, were compared visually using a microscope, before their haematopoietic differentiation potential was assessed by CFU-A analysis. For the assessment of the ES cell aggregate formation conditions and the suitability of cell number density, characteristics such as size and shape, number and relative homogeneity of ES cell aggregates and EBs as well as the structure and size of positive and negative CFU-A colonies were taken into consideration and compared to 'hanging drop' ES cell aggregates, EBs and CFU-A colonies.
All sets of ES cell aggregates and EBs showed variations in size compared to those produced by the hanging drop method at the same time point during a time course of differentiation. Although differences in size were observed, the shape of ES cell aggregates formed on Cellform coated plates were round and compact, similar as observed from 'hanging drop' ES cell aggregates. However, many of the EBs formed on coated plates did not maintain their compact and dense structure. ES cell aggregates from uncoated plates showed a high tendency to attach to the plate substratum and developing EBs had uneven and rough shapes and surfaces. This was observed in all samples using uncoated plates and little influence of cell density was noticed.

High cell density (1.0×10^5 cells/ml) combined with coated plates resulted in a large number of very small ES cell aggregates that were able to develop into EBs, though size increase appeared to be slower than from ES cell aggregates that were formed from lower cell densities on coated plates. CFU-A analysis revealed the haematopoietic differentiation potential of an ES cell line can be maintained, independent of the method that was used for the formation of ES cell aggregation, although some conditions appear more suitable than others.
A general feature observed was the differences in size of positive CFU-A colonies from EBs, developing from 'high cell density' ES cell aggregates compared to

EBs from 'hanging drop' ES cell aggregates. Positive CFU-A colonies from 'hanging drop' derived EBs were on average ~2 mm larger and formed by whole EBs (data not shown) compared to positive CFU-A colonies of EBs developing from ES cell aggregates formed on plates. These appeared to be derived from small cell clusters or individual cells rather than from whole EBs, resembling the CFU-A profile obtained from disrupted EBs.

The production of ES cell aggregates using Cellform coated plates involved two simple steps; applying the alcoholic Cellform solution to plates and seeding of cells. This led to the formation of ES cell aggregates that were sufficient enough to be used for initial analysis of differentiation characteristics. Compared to this procedure, the hanging drop method is relatively time consuming and labour intensive, however, with the advantage that an uniform population of ES cell aggregates with high differentiation synchrony is obtained. The examination of different conditions for the formation of ES cell aggregates showed that ES cell aggregates can be produced by relatively simple culture procedures, which are suitable for the simultaneous production of ES cell aggregates from a large number of different cell clones. Though the ES cell aggregates and the developing EBs are heterogeneous and show less synchrony in differentiation than observed with the hanging drop method this method appears to be sufficient for monitoring ß-galactosidase expression of gene trap lines during a large screen. Nevertheless, the Cellform coated plate method was good enough to obtain preliminary information on the differentiation potential of respective cell clones. However, for studies that required homogeneous and synchronously developing EBs the hanging drop method should be the method of choice for the production of ES cell aggregates.

Table 4.3: Summary of CFU-A data from EBs derived from different conditions for ES cell aggregation. Data are from two independent experiments and are means from 3 plates at each timepoint. The data are expressed as the percentage of plated EBs forming positive CFU-A colonies.

Cell density and days of differentiation	Coated plates		Uncoated plates	
	Experiment I	Experiment II	Experiment I	Experiment II
Tissue culture grade plastics				
1.0×10^5 cell/ml				
4 days	22.0 ±6.9	28.1 ±4.6	10.9 ±8.5	4.9 ±2.2
6 days	10.1 ±7.6	nd	4.1 ±2.2	nd
0.5×10^5 cell/ml				
4 days	20.1 ±8.1	18.8 ±3.7	21.0 ±8.0	22.0 ±6.0
6 days	13.5 ±3.9	11.3 ±3.3	14.2 ±6.3	23.8 ±4.3
0.3×10^5 cell/ml				
4 days	27.9 ±9.4	nd	10.7 ±2.8	nd
6 days	10.7 ±3.9	nd	16.3 ±1.9	nd
Bacterial grade plastics				
1.0×10^5 cell/ml				
4 days	12.9 ±10.0	7.8 ±2.2	8.9 ±6.8	7.3 ±1.0
6 days	6.1 ±1.7	nd	1.6 ±1.6	10.7 ±3.1
0.5×10^5 cell/ml				
4 days	15.4 ±1.6	9.9 ±1.3	9.3 ±7.9	4.2 ±1.5
6 days	9.8 ±4.5	23.4 ±5.0	0 ±0	8.5 ±2.5
'Hanging drop' EBs				
4 days	nr	nr	26.9 ±0.8	13.6 ±4.0
6 days	nr	nr	15.6 ±8.2	9.1 ±3.1

± standard deviation; nd not done; nr not relevant;

4.3 Spatial analysis of gene expression in embryoid bodies by whole-mount *in situ* hybridization

Temporal and spatial gene expression analysis is an important approach for the study of developmentally regulated genes. Spatial information of gene transcripts within a cell can provide evidence for cellular heterogeneity within a tissue, or identify domains of expression that subdivide otherwise uniform regions of a developing embryo. For instance, localization of brachyury transcripts in mouse embryos gave critical clues as to how embryonic mesoderm develops (Herrman, 1991).

Until the establishment of whole-mount *in situ* hybridization techniques for widespread application, most information about embryonic gene expression has come from RT-PCR analyses or two-dimensional sections. However, embryos are complex three-dimensional entities and analysis of unsectioned embryos is clearly a more advantageous approach for the analysis of gene expression patterns. Non-isotopic *in situ* hybridization to whole embryos has become a useful and informative alternative for localizing transcripts.

The application of such a methodology to an *in vitro* model could form the basis for gene expression studies in particular differentiation systems. ES cell differentiation *in vitro* resembles *in vivo* development and has been shown to be a useful model for the study of haematopoietic development (Schmitt et al, 1991, Wiles et al, 1991; McClanahan et al, 1993; Keller et al, 1993; Hole et al, 1996).

The ES cell culture system used in this study has been shown to harbour cells with long-term reconstitution potential in lethally irradiated mice, indicating that primitive HSCs arise during *in vitro* differentiation (Hole et al, 1996). Analysis of the expression of genes known to be involved in haematopoiesis during a time course of *in vitro* differentiation revealed well defined expression patterns (Hole et al, 1996; Menzel U, Diplomarbeit, 1994). However, RT-PCR analysis uses pooled RNA from a population of cells from single EBs or a population of EBs which prevents the analysis of gene expression patterns in individual cells.

Analyses from a number of studies have also presented evidence of haematopoietic gene expression, both in undifferentiated ES cells and during the process of differentation (Schmitt et al, 1991, Wiles et al, 1991; McClanahan et al, 1993; Keller et al, 1993).

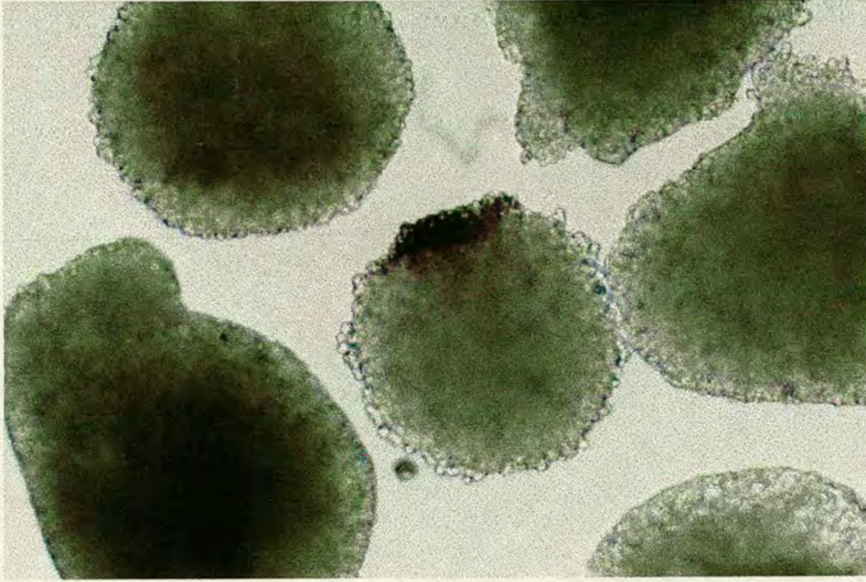
Spatial analysis of the expression of these haematopoietic genes during *in vitro* differentiation of ES cells has not been reported to date. Whole-mount *in situ* hybridization of EBs from various time points during the *in vitro* differentiation of ES cells was performed to obtain information of the spatial expression patterns and to complement RT-PCR analyses.

4.3.1 Spatial localization of globin transcripts in EBs analyzed by whole-mount *in situ* hybridization

Previously carried out RT-PCR analysis of the ES cell system has revealed that globin expression can be detected at high levels from day 4 of differentation which coincides with the presence of primitive cells with potential for long-term survival and multi-lineage reconstitution of lethally irradiated mice. Since it was not possible to conclude from RT-PCR analysis whether the high signal was the result from a large number of cells expressing low levels of globin transcripts, or from few cells expressing high levels of globin transcripts whole mount *in situ* hybridization for globin transcripts was performed.

Embryoid bodies, day 4 or day 6, were hybridized with fluorescein-labelled riboprobes and hybridized probes were detected by alkaline phosphatase conjugated anti-fluorescein antibodies. The colour substrate NBT/BCIP was utilized for the colorimetric visualization of target mRNA/riboprobe hybrids, which develops into dark blue/brown signal.

Figure 4.6.1-4.6.2 shows day 6 EBs hybridized for globin expression. Globin expression appears localized within a small domain of an EB and differences in colour intensity indicate differing levels of transcript levels within cells. However, it was very difficult to distinguish and identify EBs stained for globin expression since the relatively large size of EBs only allowed the use of low magnification (160x, 300x). Approximately 2% of day 6 sample EBs (2 EBs out of 115 EBs) were identified to be stained for globin expression. This is much less than was expected from CFU-A analysis and from the high level of globin expression from RT-PCR analysis. In day 4 sample EBs, no EB could be identified for globin expression.



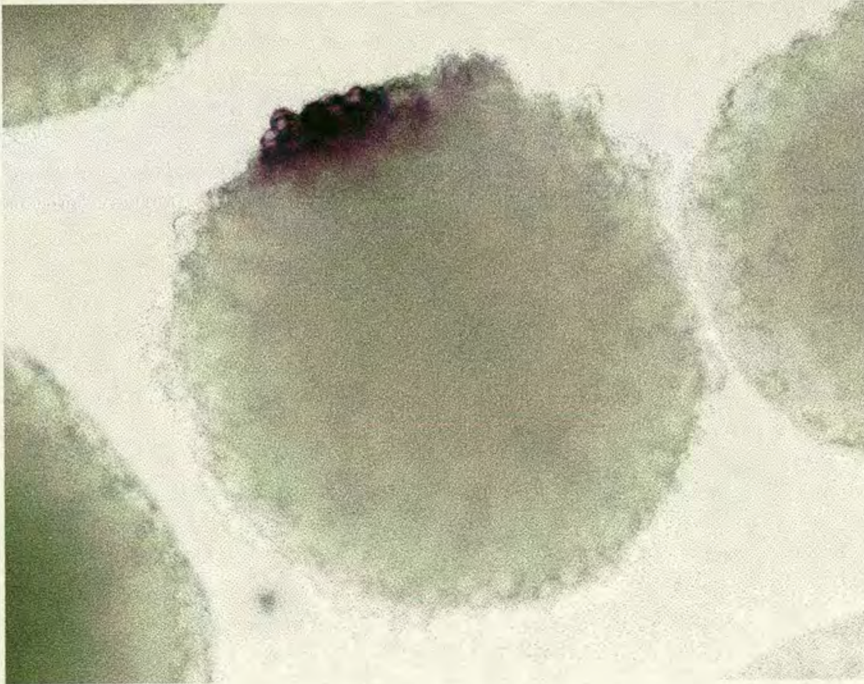


Figure 4.6.1: Whole-mount *in* situ hybridization analysis for globin expression in EBs. EBs allowed to differentiate for 6 days were fixed and hybridized with antisense riboprobes specific for globin transcripts. Signal was detected by incubation with alkaline-phosphatase conjugated antibodies against the probe label fluorescein and NBT/BCIP staining. Globin expression was detected in a discrete area within EBs. Images show the same EBs at different magnifications (top, 160x; bottom, 300x).

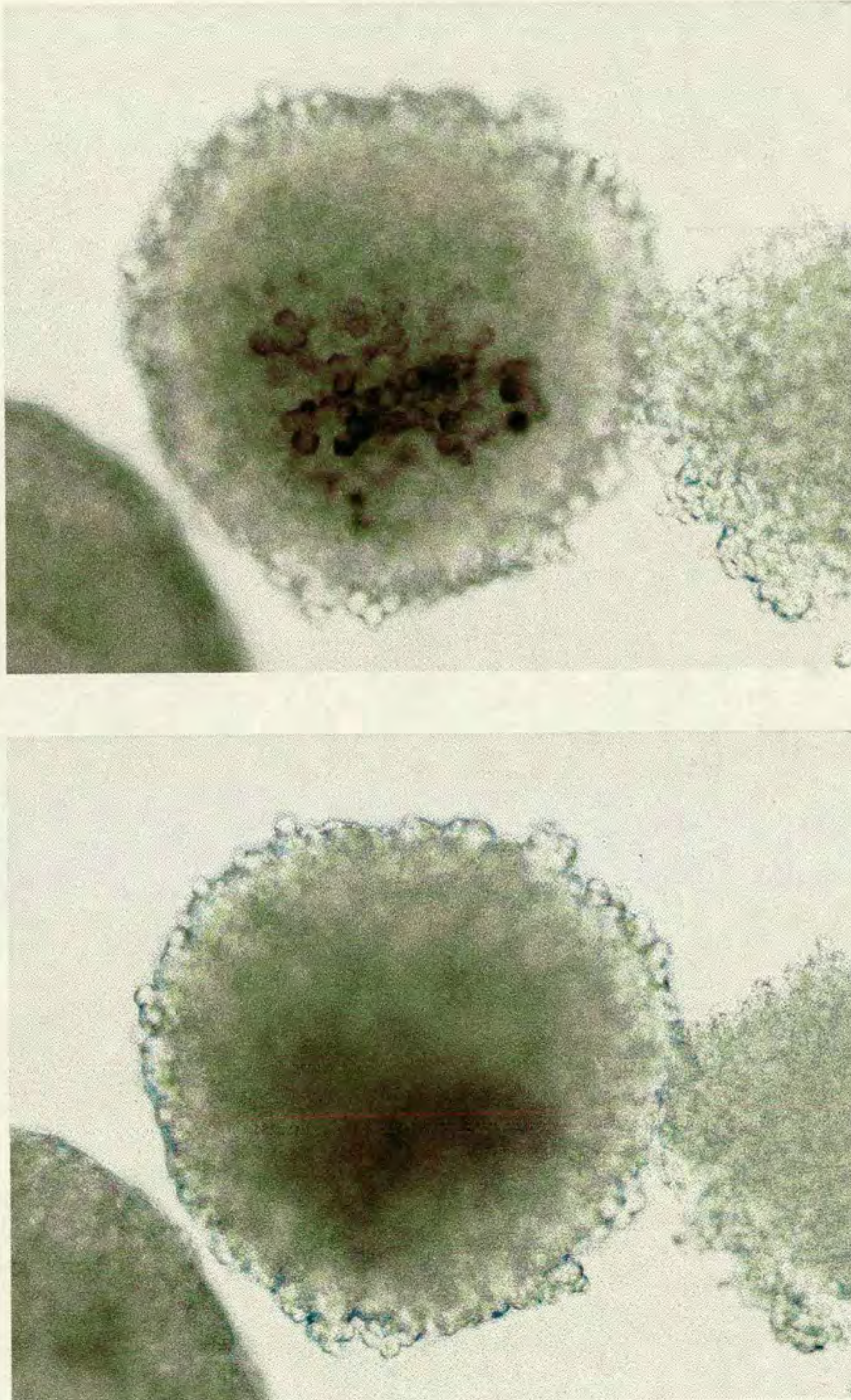


Figure 4.6.2: Whole-mount *in situ* hybridization analysis for globin expression in EBs. Day 6 EBs were processed as described in Figure 4.6.1. Two images of the same EBs are shown to illustrate the difficulties encounterd when EBs were assessed after colorimetric signal detection. The large size of the EBs made focus adjustment difficult and limited the assessment to the use of low magnification (Magnification 300x).

4.3.2 Two colour whole-mount *in situ* hybridization of EBs

Two colour whole-mount *in situ* hybridization was attempted for the analysis of co-expression patterns of haematopoietic genes and the intended use of whole-mount *in situ* hybridization methodology for the screening of ES cell lines containing gene trap constructs. Co-expression and co-localization in gene trap ES cell lines of the inserted reporter gene *lacZ* and a gene, known to be involved in haematopoiesis and expressed during ES cell differentiation, was thought to be suggestive for the potential involvement of the trapped endogneous genes in early haematopoietic development. Co-expression of known haematopoietic genes in EBs, was anticipated to reveal information about possible interrelationships during development, e.g. the receptor-ligand pair *c-kit/SCF*.

EBs, allowed to differentiate for 4 days or 6 days, were hybridized with DIG-labelled riboprobes specific for *SCF* transcripts and fluorescein-labelled riboprobes specific for *c-kit* transcripts. The hybridization solution used for each EBs sample contained both riboprobe types. Colorimetric signal detection utilized peroxidase-conjugated anti-DIG antibodies with the colour substrate DAB for *SCF* transcripts and alkaline phosphatase-conjugated anti-fluorescein antibodies with the colour substrate Fast Red for *c-kit* transcripts.

Figure 4.7.1 shows EBs hybridized with riboprobes specific for *c-kit* or *SCF* transcripts. Expression of *c-kit* and *SCF* is indicated by light redish colour for *c-kit* and brownish colour for *SCF*. Previously carried out time course studies of EBs using RT-PCR analysis detected transcripts for *SCF* in undifferentiated ES cells and throughout differentiation. Signal for *c-kit* expression by RT-PCR analysis was detected from day 5 of differentation onwards (Hole et al, 1996; Menzel U, Diplomarbeit 1994). However, it was not possible to obtain detailed information of the likely co-expression and possible co-localization of *c-kit* and *SCF* transcripts due to limitations of the microscopic equipment available for experimentation and the assessment of hybridized samples.

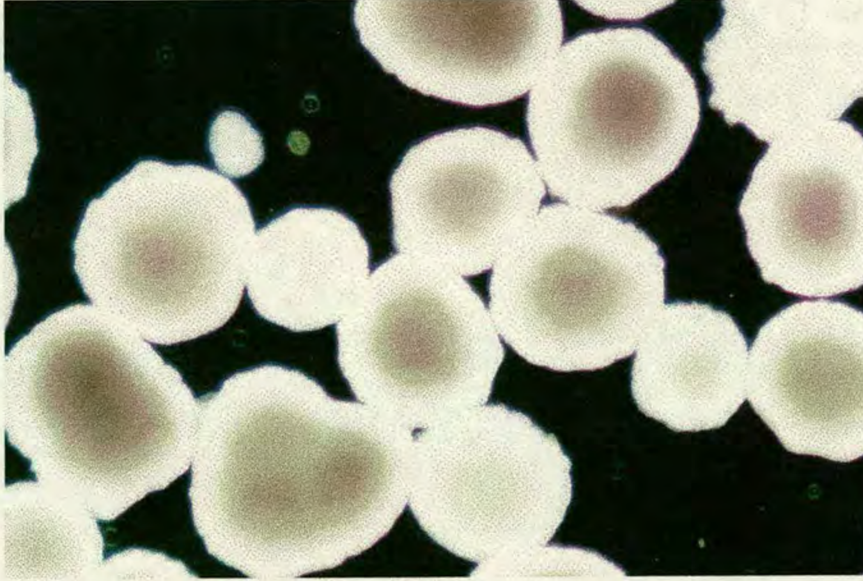
EBs hybridized with single riboprobe type specific for c-kit transcripts are shown in Figures 4.7.2. Some EBs hybridized with *c-kit* specific riboprobes showed staining indicating the presence of transcripts. Whole-mount *in situ* hybridization for *SCF* transcripts did not result in a staining pattern that indicated the presence of transcripts (data not shown). A possible interpretation for the lack of observable signal could be that cells expressing *c-kit* and/or *SCF* transcripts are located within EBs, thus not identifiable by normal microscopy. However, methodological shortcomings are also likely to account for the limited information possible to gain from whole-mount *in situ* hybridization experiments of EBs. Attempts to detect transcripts for the haematopoietic transcriptions factors *SCL* or *vav* by whole-mount *in situ* hybridization using appropriate specific DIG-labelled riboprobes did not result in identifiable signals (data not shown).

Non-invasive sectioning using confocal microscopy was thought to be a method for the three-dimensional analysis of gene expression patterns in EBs. Although comprehensive efforts and much time were expended to improve the whole-mount *in situ* hybridization conditions to be able to combine this technique with confocal microscopy, these attempts were not successful (see Material and Methods, Sections 3.2.5-3.2.5.4). Specimens used for *in situ* hybridization as a whole are often subsequently sectioned to obtain detailed information of the gene expression patterns. Thus, a possible alternative approach could be sectioning of specimens prior to *in situ* hybridization.

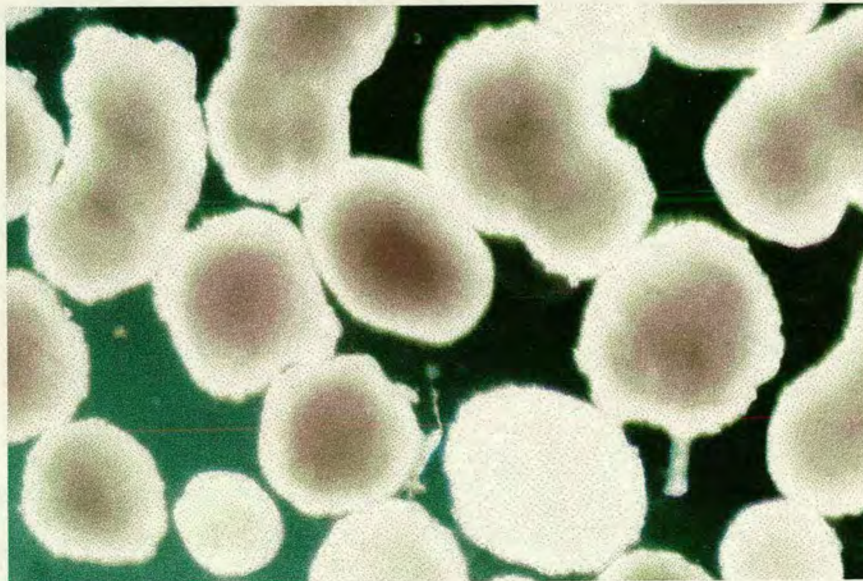
Whole-mount *in situ* hybridization of EBs using colorimetric signal detection made it possible to gain information for globin expression but was not so successful for the analysis of other haematopoietic genes, which were expected to be expressed in EBs. Since EBs do not display morphological orientation compared to mouse embryos, sectioning prior to hybridization was the strategy of choice to further analyze spatial gene expression in EBs. Successive sections were taken for the possibility to compare and overlap expression patterns and to obtain details of co-expression.

4.3.3 Whole-mount *in situ* hybridization of EBs derived from ES cell lines containing gene trap insertions

As mentioned above, two colour *in situ* hybridization of EBs derived from gene trap ES cell lines was considered as a methodology for the screening of a gene trap clone library. Studies addressing the correlation of ß-galactosidase expression and activity during *in vitro* differentiation and *in vivo* development of gene trap lines are described in Sections 4.1.3-4.1.5. To assess whether corresponding transcript levels to ß-galactosidase expression can be detected, whole mount *in situ* hybridization with EBs derived from the gene trap lines Zin40 and ST598 was attempted with riboprobes specific for *lacZ* transcripts. Day 4 or day 6 EBs of each gene trap line were hybridized with DIG-labelled antisense riboprobes for *lacZ* transcripts and alkaline phosphatase conjugated anti-DIG antibodies with NBT/BCIP as colour substrate were used for signal detection. Control samples for the hybridization conditions included EBs hybridized with labelled antisense riboprobes specific for actin transcripts (Figure 4.7.3). For both gene trap lines, it was expected to obtain corresponding staining patterns as observed with *in situ* ß-galactosidase staining (see Section 4.1.5). However, no obvious staining for *lacZ* transcripts was observed in both gene trap lines (Figure 4.7.4). This unexpected inability to detect transcripts for *lacZ* could arise from low numbers and/or low stability of fusion transcripts from the *lacZ* reporter gene and the trapped endogenous gene. However, methodological shortcomings can not be excluded.

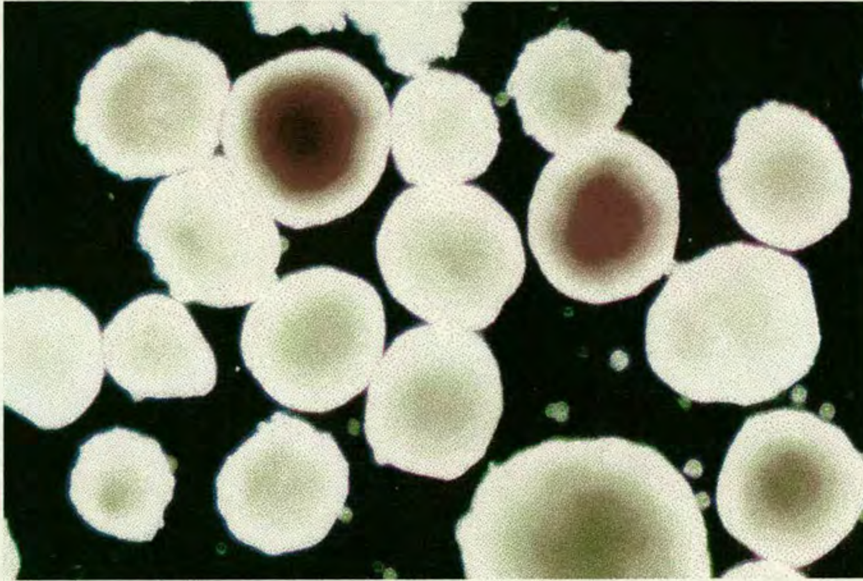


Day 4 EBs simultaneously hybridized with antisense riboprobes specific for c-kit and SCF.
c-kit expression is indicated by light red staining. SCF expression is indicated by brown staining

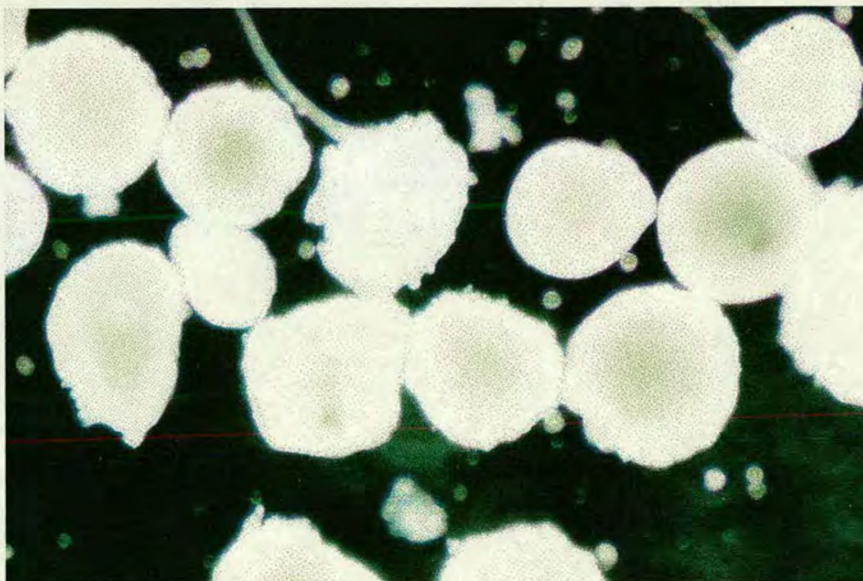


Day 6 EBs simultaneously hybridized with antisense riboprobes specific for c-kit and SCF.
c-kit expression is indicated by light red staining. SCF expression is indicated by brown staining

Figure 4.7.1: Whole-mount *in situ* hybridization analysis for c-kit and SCF expression in EBs. EBs were simultaneously hybridized with fluorescein-labelled riboprobes specific for c-kit expression and DIG-labelled riboprobes specific for SCF expression. DAB colour substrate was utilized for the detection of SCF expression and FastRed for c-kit expression (Magnification 100x).

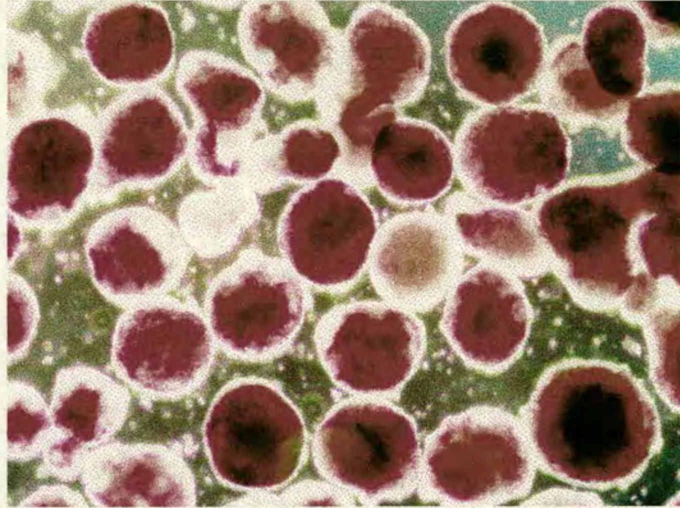


Day 6 EBs hybridized with antisense riboprobes specific for c-kit. c-kit expression is indicated by the red staining.

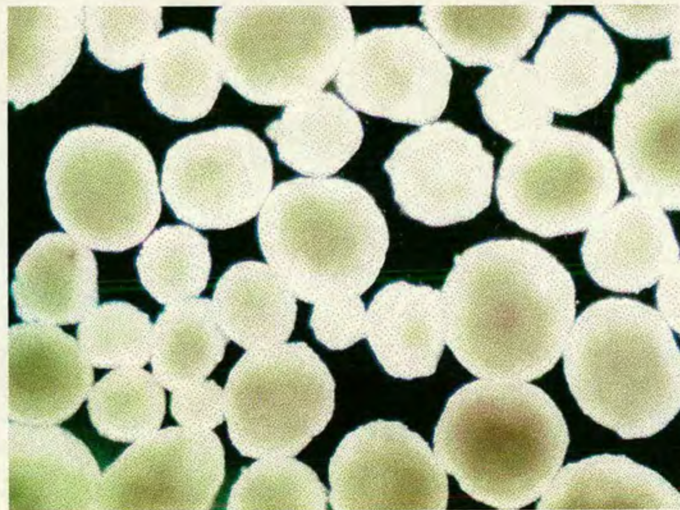


Day 6 EBs exposed to labelled sense riboprobes corresponding to sense sequence of c-kit to address background caused by non-specific binding by probes.

Figure 4.7.2: Whole-mount *in situ* hybridization analysis for c-kit expression. EBs were hybridized with fluorescein-labelled antisense riboprobes. FastRed colour substrate was utilized to detect signal for probe-target hybrids (top). A control reaction for unspecific probe binding was performed by incubating EBs with labelled sense riboprobes corresponding to c-kit sequence (bottom) (Magnification 100x).

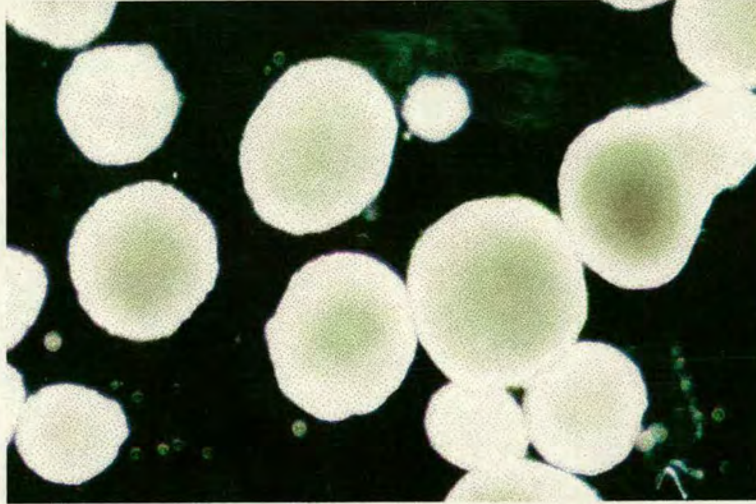


Day 6 EBs hybridized with antisense riboprobes specific for actin. Actin expression is indicated by the red/purple staining.

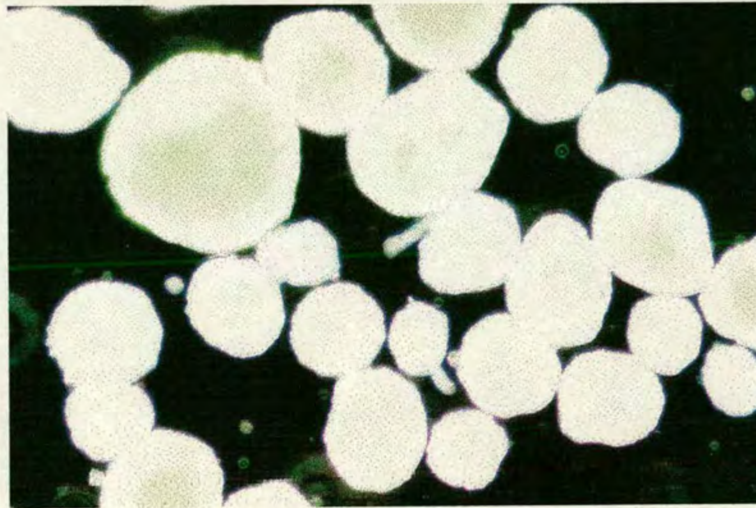


Day 6 EBs exposed to labelled sense riboprobes corresponding to sense sequence of actin to address background caused by non-specific binding of probes.

Figure 4.7.3: Whole-mount *in situ* hybridization analysis of Actin expression in EBs derived from the gene trap cell line ST598. EBs were hybridized with DIG-labelled antisense riboprobes. NBT/BCIP colour substrate was utilized to detect signal for pore-target hybrids (top). A control reaction for non-specific probe binding was performed by incubating EBs with labelled sense riboprobes corresponding to actin sequence (bottom) (Magnification 100x).



Day 4 EBs hybridized with antisense riboprobes specific for ß-gal. Staining patterns of EBs were expected to be similar to X-Gal staining for ß-gal fusion protein (Table 4.2).



Day 4 EBs exposed to labelled sense riboprobes corresponding to sense sequence of ß-gal to address background caused by non-specific binding by probes.

Figure 4.7.4: Whole-mount *in situ* hybridization analysis of ß-gal expression in EBs derived from the gene trap cell line ST598. EBs were hybridized with DIG-labelled antisense riboprobes. NBT/BCIP colour substrate was utilized to detect signal for probe-target hybrids (top). A control reaction for unspecific probe binding was performed by incubating EBs with labelled sense riboprobes corresponding to ß-gal sequence (bottom) (Magnification 100x).

4.4 Spatial analysis of gene expression in EBs by *in situ* hybridization of sectioned EBs

To make a fuller assessment of the spatial expression of haematopoietic genes during commitment to haematopoietic lineages *in vitro*, *in situ* hybridization was performed on sectioned EBs. Whole mount *in situ* hybridization of EBs (described in Section 4.3.1) demonstrated spatial restricted expression for globin. Although the whole mount *in situ* hybridization methodology was not as informative as anticipated when combined with confocal microscopy, colorimetric signal detection illustrated that in order to permit a more precise analysis of expression, sectioning of the EBs would be required. Whole mount *in situ* hybridization with subsequent sectioning for more precise analysis has been reported to work best for strong signals, however, a higher overall sensitivity is provided by *in situ* hybridization on sectioned specimens (Decimo et al, 1995).

Three time courses of EBs, each covering 8 days of differentiation, were used for gene expression analysis. One time course consisted of EBs allowed to differentiate under standard conditions, the other two time courses consisted of EBs exposed to morphogenic factors. In one time course, the EBs were induced with DMSO, in the other with RA. The EBs were embedded in wax and 7 μm sections were cut using a standard rotary microtome. Cryostat sectioning, attempted as an alternative approach to obtain sections of EBs without invasive pretreatment, was found not to be suitable for the experimental analysis intended. The expression of haematopoietic genes was studied by *in situ* hybridization with specific DIG-labelled antisense riboprobes and subsequent colorimetric signal detection. This was carried out by utilizing alkaline phosphatase conjugated anti-DIG antibodies with NBT/BCIP as colour substrate. In addition to routine time points, covering two day periods, EBs differentiated for 3 days or 5 days were included in these studies. The transient presence of primitive multilineage haematopoietic precursors at day 4 of *in vitro* differentiation in this

ES cell system was previously detected by long term reconstitution of lethally irradiated recipients (Hole et al, 1996). It was therefore of special interest to analyze the gene expression during this temporal window.
Furthermore, a subtractive cDNA library produced from day 5 and day 3 EBs identified novel genes with developmentally regulated expression patterns. The use of these genes as riboprobes for *in situ* hybridization could allow precise analysis of their spatial expression patterns and comparison to expression patterns of known haematopoietic gene (Graham G, Hole N, and Ansell J; personal communication).

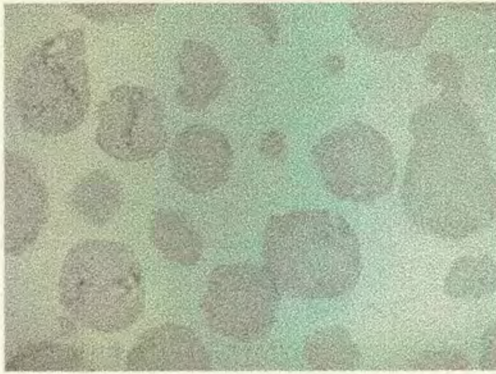
4.4.1 Effects of DMSO or RA on the spatial expression of globin during *in vitro* differentiation

The temporal expression pattern of globin in this ES cell system had previously been analyzed by qualitative RT-PCR analysis. Fetal and adult type ß-globin were detectable in EBs with a sharp onset of fetal ß-globin expression at day 4. Adult type ß-globin transcripts were detected at very low level at day 3 but from day 4 onwards high levels were detected (Hole et al, 1996; Menzel U, Diplomarbeit 1994).
In a semi-quantitative RT-PCR analysis ß-globin transcript levels of untreated EBs were compared to transcript levels in DMSO or RA induced EBs. A marked increase in ß-globin transcript levels was observed in DMSO treated EBs (Doostdar L, 1997).
Since these changes described above were observed during or around day 4 of *in vitro* differentiation it was of particular interest to investigate how the signal for globin expression is composed on a cellular level.
Time course EBs of untreated or induced EBs were sectioned, hybridized with antisense riboprobes specific for alpha-globin expression, and riboprobe-globin mRNA hybrids were detected by a colorimetric signal detection assay. Adult alpha-globin transcripts have been observed to be co-expressed with embryonic zeta-globin transcripts within the same cells from the onset of erythropoiesis

in vivo (Leder et al, 1992). The use of riboprobes specific for alpha-globin transcripts therefore made it possible to follow globin expression during *in vitro* differentiation using one type of probe. However, to distinguish between embryonic and adult type globin expression within EBs further analysis using embryonic and adult types of globin probes are required.

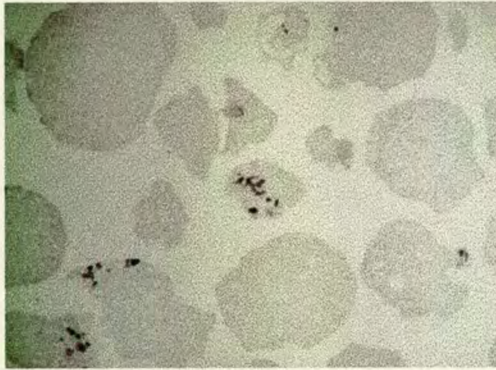
The results from these globin expression studies are illustrated in Figures 4.8.1-4.8.4 and 4.9, and summarized in Table 4.4. The temporal signal pattern for globin expression by *in situ* hybridization was in accordance to globin expression patterns found in this ES cell system using other experimental means. At day 2 of differentation no globin expression was detected in untreated or induced EBs. However, by day 3 signal for globin was detectable in EBs from standard or induction culture conditions. About 20% of untreated or DMSO induced EBs showed staining for globin expression and ~9% of EBs exposed to RA were found to express globin. In most of these positive EBs only few cells (<10 cells) were found to express globin. After 4 days of differentiation the effects of DMSO on CFU-A formation in EBs, routinely observed as an increase in CFU-A colony numbers, appeared to be mirrored in a corresponding increase in EBs expressing globin. The number of EBs expressing globin was two-fold increased in EBs induced by DMSO compared to untreated EBs. The effects of RA during *in vitro* differentiation of EBs when analyzed by the CFU-A assay result in a reduced number of CFU-A colonies. The percentage of day 4 EBs staining positive for globin expression after induction with RA was at a similar level as observed in untreated EBs (~30%). However, it must be noted, EBs exposed to RA during *in vitro* differentiation contained very few globin expressing cells per EB compared to untreated EBs (Table 4.4; Figure 4.9).
A constant increase in the number of EBs with globin expressing cells was observed in untreated EBs over the time course of *in vitro* differentiation analyzed. In DMSO induced EBs, a peak in numbers of globin expressing EBs was observed at day 5,

with ~80% of EBs staining positive for globin expression, which was reduced to a level (~60%) below that of untreated EBs at day 8 of differentiation. The number of globin expressing EBs in RA induced EBs remained at a similar level (~30%) over the period of 4-6 days of differentation and showed an increase at day 8 (~50%).
The opposing effects of the morphogenic factors DMSO and RA on haematopoietic *in vitro* differentation of EBs, as observed by CFU-A analysis are reflected in numbers of globin expressing EBs. This is well illustrated in Figure 4.9 where data obtained from these time course studies display the opposing effects of DMSO and RA on the percentage of EBs that contain globin expressing cells.
Especially the differences between these time courses over the time period of day 3 to day 6 are very illustrative. In untreated EBs a constant increase in numbers of EBs with globin expressing cells was observed with ~20% positive EBs at day 3 and ~70% positive EBs at day 8 of differentiation. In DMSO induced EBs a peak more than two-fold increased to untreated EBs was observed at day 5 with 80% positive EBs. In RA induced EBs a stagnation in numbers at ~30% was observed, during the time period when significant changes in globin expression were observed in untreated and DMSO induced EBs. The effects of DMSO on globin expression in EBs were not obvious until day 4, whereas EBs exposed to RA showed a reduction in globin expressing EBs from the onset of detectable globin expression. These observations are suggestive that DMSO does not alter the temporal pattern of haematopoietic differentiation of ES cells *in vitro* but influence the number of cells that commit to the haematopoietic lineage. Effects of RA exposure appear to be exerted earlier during *in vitro* differentiation.
The effects of DMSO and RA on globin expression during haematopoietic differentiation *in vitro* suggest that changes in globin expression may correspond to observed changes in CFU-A numbers.

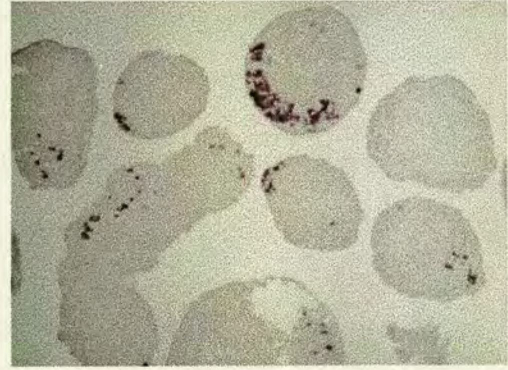


day 2

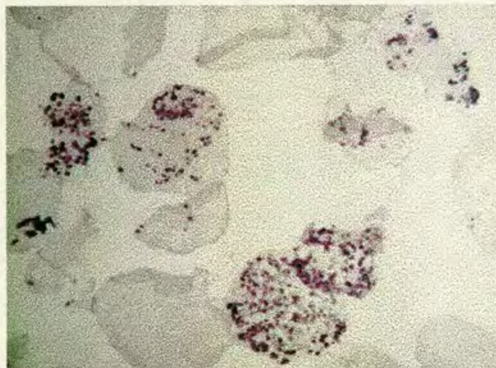
day 3



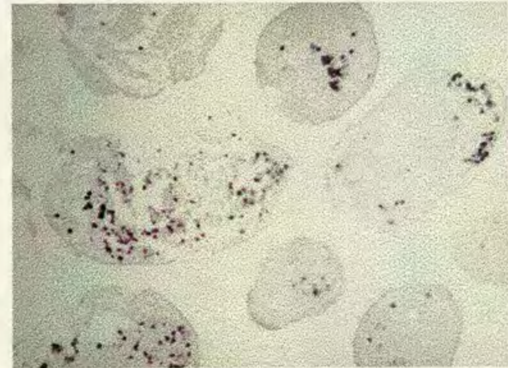
day 4



day 5

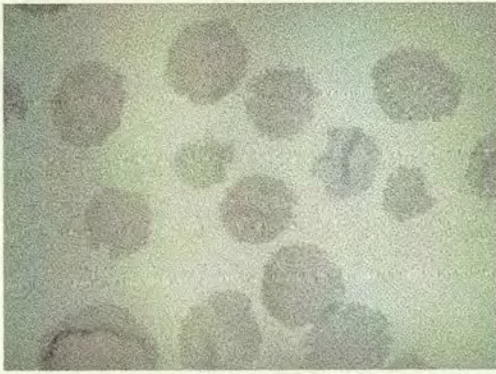


day 6



day 8

4.8.1: *In situ* hybridization analysis of globin expression in EBs during *in vitro* differentiation.
A time course of EBs (Section 3.1.6.4) was wax-sectioned and hybridized with DIG-labelled antisense riboprobes specific for alpha-globin. Signal was visualized with alkaline-phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate. Globin positive EBs are identified by blue/purple colour change. Globin transcripts were detectable from day 3 of differentiation onwards with the number of globin expressing EBs increasing over time (Magnification 160x).



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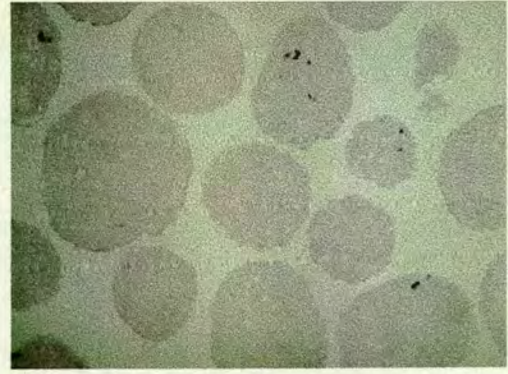
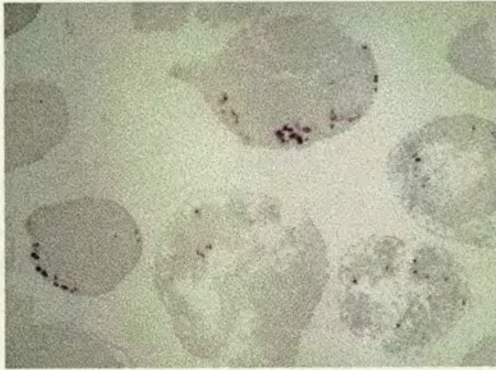


day 3



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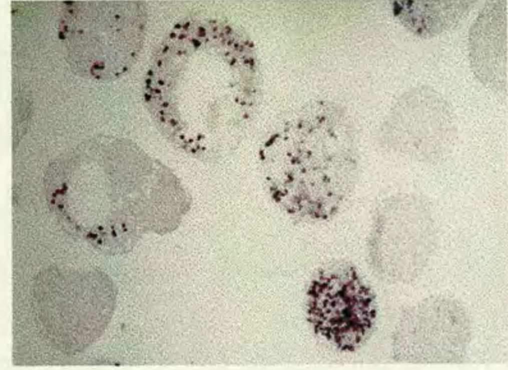

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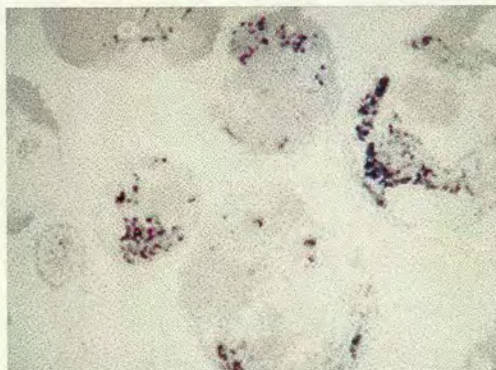
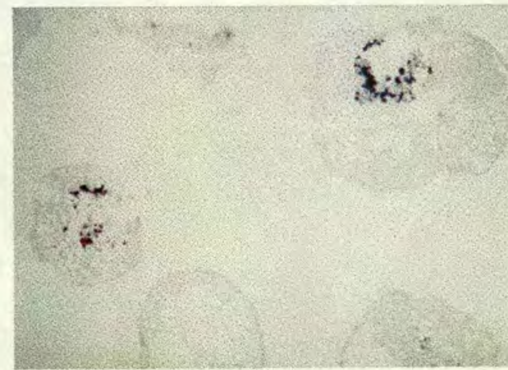


day 6

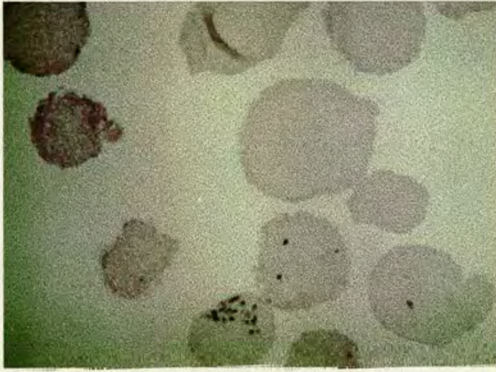


day 8

4.8.2: *In situ* hybridization analysis of globin expression in EBs induced by DMSO during *in vitro* differentiation. A time course of DMSO induced EBs (Section 3.1.6.4) was wax-sectioned and hybridized with DIG-labelled antisense riboprobes specific for alpha-globin. Signal was visualized with alkaline-phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate. Globin positive EBs are identified by blue/purple colour change. Globin transcripts were detectable from day 3 of differentiation onwards with the number of globin expressing EBs increasing over time (Magnification 160x).

day 2

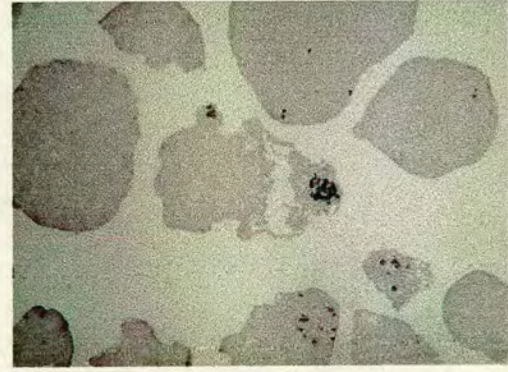
day 3



day 4

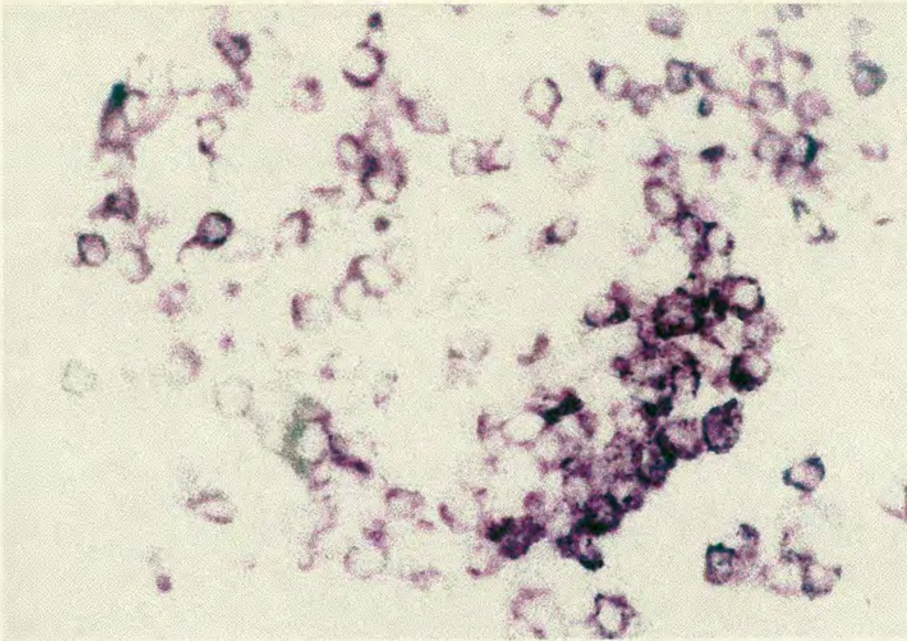
day 5

day 6

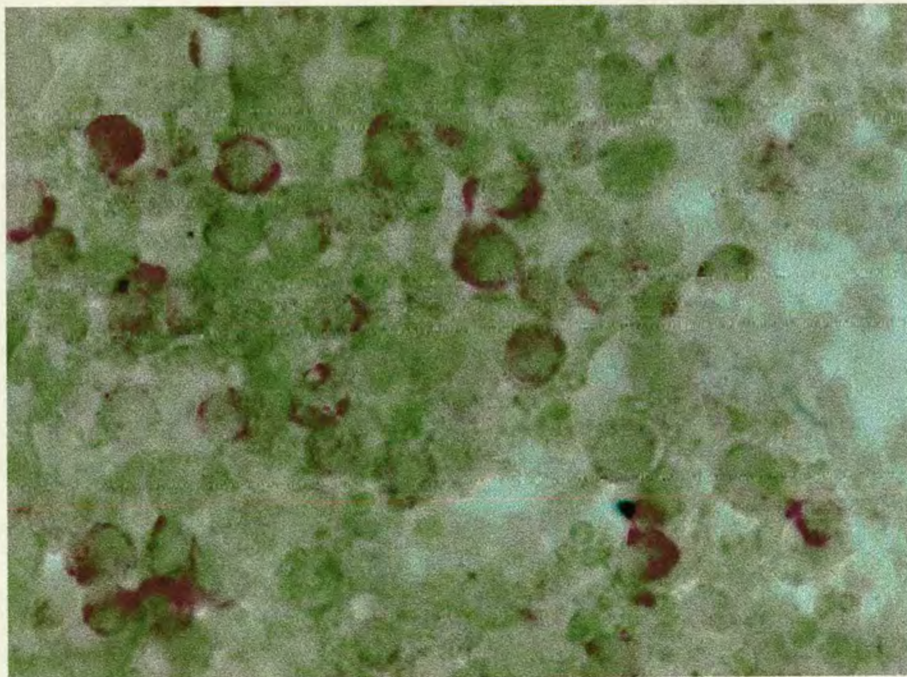


day 8

4.8.3: *In situ* hybridization analysis of globin expression in EBs induced by RA during *in vitro* differentiation. A time course of RA induced EBs (Section 3.1.6.4) was wax-sectioned and hybridized with DIG-labelled antisense riboprobes specific for alpha-globin. Signal was visualized with alkaline-phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate. Globin positive EBs are identified by blue/purple colour change. Globin transcripts were detectable from day 3 of differentiation onwards (not obvious in day 3 image presented) with the number of globin expressing EBs increasing over time (Magnification 160x).



Signal for globin expression stained with NBT/BCIP colour substrate.



Signal for globin expression stained with FastRed colour substrate.

Figure 4.8.4: *In situ* hybridization of sectioned day 6 EBs with antisense riboprobes specific for alpha-globin transcripts demonstrating the possible use of different colour subtrates. Wax-sectioned EBs were hybridized with DIG-labelled globin riboprobes and probe-target hybrids were detected by incubation with alkaline-phosphatase conjugated anti-DIG antibodies. Signal was developed by utilizing NBT/BCIP colour substrate (top) or FastRed colour substrate (bottom) (Magnification 1000x). Corresponding hybridizations using Fluorescein labelled riboprobes showed similar staining (data not shown).

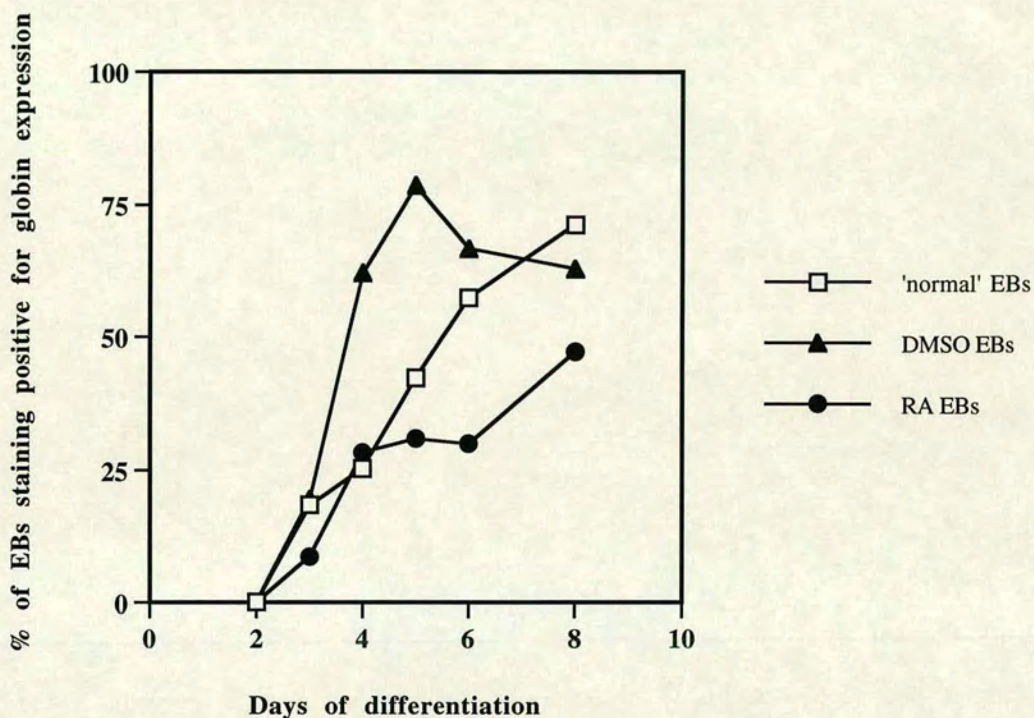


Figure 4.9: Time course analysis of globin expression in sectioned EBs from untreated EBs and induced EBs. The number of EBs staining positive for globin expression is shown as percentage of screened EBs (>100 EBs were screened for globin expression for each time point). Time is expressed as number of days EBs were allowed to differentiate in suspension culture. The graph shows a comparison of untreated EBs, DMSO induced EBs and RA induced EBs.

Table 4.4: Summary of EBs staining positive for globin expression in untreated and induced time course EBs of sectioned EBs hybridized for globin expression

Time course type / Age of EBs	% of EBs staining positive for globin expressing cells	% of positive EBs having >10 cells stained for globin	% of EBs with >10 cells stained for globin having cells located in a restricted area	CFU-A data
'normal' EBs				
2 days	0	-	-	9.79±4.3 #
3 days	18.36	6.38	100	20.0±1.8
4 days	25.30	15.48	100	13.55±4.0
5 days	42.26	36.60	75.59	6.0±4.8
6 days	57.46	44.48	86.13	9.09±2.8
8 days	71.23	59.62	100	25.12±8.9
DMSO induced EBs				
2 days	0	-	-	17.53±4.3
3 days	19.51	6.94	100	17.21±6.0
4 days	62.04	37.31	98	28.76±17.7 #
5 days	78.60	68.3	94.54	29.34±7.7
6 days	66.67	78.9	96.67	33.13±3.6 #
8 days	62.81	68.0	100	36.54±6.9 #
RA induced EBs				
2 days	0	-	-	0
3 days	8.6	-	-	4.43±1.4
4 days	28.26	17.95 *	100	7.76±1.8
5 days	30.88	11.11	100	1.55±2.2
6 days	29.89	12.07	100	1.26±1.8
8 days	47.19	20.23	100	0

Numbers were obtained from sectioned EBs with a minimum sample size of 100 EB sections from each time point.
* non of these positive EBs had > 35 cells stained for globin expression
CFU-A data: The number of EBs forming CFU-A colonies is expressed as a percentage of plated EBs (clonal efficiency)
total number of EBs < 50

4.4.2 Spatial expression of haematopoietic genes during *in vitro* differentiation

A number of haematopoietic genes were used for *in situ* hybridization of sectioned time course EBs covering 8 days of *in vitro* differentiation to study the spatial expression pattern of these genes. The temporal expression of most genes analyzed in this study has been studied by RT-PCR analysis or northern blot analysis, however, studies regarding the spatial expression have not been reported to date (Schmitt et al, 1991; Wiles et al, 1991; McClanahan et al, 1993; Keller et al, 1993; Hole et al, 1996). In order to obtain details of the genetic events that may control mesoderm and haematopoietic commitment during early *in vitro* differentiation, genes known to be involved at various stages during haematopoietic development and/or haematopoiesis were chosen for analysis. Genes used for time course studies by *in situ* hybridization included the mesoderm marker gene brachyury, the receptor tyrosine kinase *Flk-1* as developmental marker for endothelial and haematopoietic development, the haematopoietic transcription factor *SCL* and the haematopoietic surface antigen *CD34* as early haematopoietic markers, and the haematopoietic receptor/ligand pair *c-kit/SCF*. Genes used for *in situ* hybridization on sections of day 6 and/or day 8 EBs were the haematopoietic transcription factor *vav,* the mesoderm marker*goosecoid,* and the growth factors *BMP-4* and *TGF-ß1*. A novel zinc finger protein (gfi-1b), identified by subtractive cDNA analysis of day 3 and day 5 RNA from EBs of this ES cell system, was used for *in situ* hybridization on sections of day 5 and day 6 EBs as preliminary analysis (Graham G, Hole N and Ansell J; personal communication). *In situ* hybridizations with actin riboprobes were included as positive control for experimental procedures. The results of these *in situ* hybridization experiments are summarized in Table 4.5. Images of spatial expression during *in vitro* differentation of some genes are shown in Figures 4.10.1-4.10.8.

Signals for gene transcripts of the mesoderm marker *brachyury*, a putative transcription factor, were observed throughout the time course analyzed. The

signal intensity differed between time points and appeared heterogeneous within EB populations from day 4 of differentiation onwards (see Figure 4.10.1 and Table 4.5). These observations are consistent with RT-PCR analysis carried out with this ES cell system (Doostdar, 1997) and a study using whole-mount *in situ* hybridization to detect brachyury expression in EBs (Johansson and Wiles, 1995). Varying levels for brachyury transcripts have also been observed in a study in which single EBs were analyzed by RT-PCR (Kennedy et al, 1997).

Hybridization with antisense riboprobes specific for the receptor tyrosine kinase *Flk-1*, a kinase expressed during early haematopoietic development *in vivo*, resulted in stained EBs at each time point analyzed, however, at various intensities. A low signal intensity was observed in day 2 EBs and increased intensity was found in day 3 EBs. A slightly weaker signal was observed in day 4, day 5 and day 6 EBs. The signal for *Flk-1* expression appeared down regulated in day 8 EBs (Figure 4.10.2 and Table 4.5). The temporal expression levels detected for *Flk-1* during *in vitro* differentation of ES cells is consistent with findings in previous studies (Kabrun et al, 1997; Vittet et al, 1996). Using RT-PCR analysis, *Flk-1* was not expressed in undifferentiated ES cells but expressed after 3 days of ES cell differentiation. RT-PCR analysis of single EBs identified variations in *FLK-1* expression within an EB population (Kennedy et al, 1997).

The helix-loop-helix transcription factor *SCL* has been reported to be essential for haematopoietic development (Kallianpur et al, 1994, Shivdasani et al, 1995; Robb et al, 1995). Signals for *SCL* expression in EBs were observed throughout the time course analyzed (Figure 4.10.3 and Table 4.5). The staining intensity for *SCL* transcripts was consistently high in all time points with a transient lower signal in day 3 EBs. The signal intensity appeared heterogeneous within the EB populations. In a previous study, analysis of *SCL* expression during *in vitro* differentiation of ES cells by RT-PCR was detected from day 4 of differentiation onwards (Keller et al, 1993). Differences in culture systems and/or sensitivity of the experimental approach

adopted for analysis may explain the differing observations for the onset of *SCL* expression during ES cell differentiation *in vitro*. RT-PCR analysis of single EBs found variations in SCL transcript levels and EBs were identified which did not express *SCL* (Kennedy et al, 1997).

The *CD34* cell surface antigen, is a stem cell-associated glycoprotein and has been used for the enrichment of HSCs in humans and mice (Krause et al, 1994). *CD34* expression has been recognized to diminish as haematopoietic cells mature and is thought to have an important role in early haematopoietic development (Young et al, 1995; Wood et al, 1997). Signals for *CD34* expression during ES cell differentiation were found at high intensity in day 2 EBs and with diminished intensity in day 5 and day 8 EBs. Transcripts for *CD34* could not be detected in day 3 and day 4 (Figure 4.10.4, Table 4.5). This is contrary to a previous study were *CD34* expression was analyzed by RT-PCR and found to be consistently expressed in undifferentiated ES cells and during a time course of EB development (McClanahan et al, 1993).

The expression of the haematopoietic receptor/ligand pair *c-kit/SCF* has been associated with HSCs and/or early progenitors but both genes are also widely expressed in non-haematopoietic cells (Ikuta and Weissman, 1992, Fleischman, 1993). During ES cell differentiation, signals for the expression of the receptor tyrosine kinase c-kit were detected throughout the time course analyzed, however, at low levels from day 4 of differentiation onwards (Figure 4.10.5, Table 4.5). The signal intensity found for the expression of the c-kit ligand *SCF* was low in day 2 EBs and was slightly increased in day 3 EBs. Signals for *SCF* transcripts were undetectable in day 4 EBs but were present again in day 5 EBs and then throughout the time course analyzed (Figure 4.10.6, Table 4.5). The expression patterns for *c-kit* and *SCF* have previously been analyzed in this ES cell system by RT-PCR. No transcripts were detectable for *c-kit* before day 8 of differentiation by RT-PCR analysis and *SCF* transcripts were detected throughout ES cell differentation (Hole et al, 1996; Menzel U, Diplomarbeit 1994).

The differing findings for *c-kit* and *SCF* expression patterns may be due to differences in methodological sensitivity. In other ES cell systems, *c-kit* was found to be expressed in undifferentiated ES cells and consistently during *in vitro* differentiation (McClanahan et al, 1993; Keller et al, 1993; Schmitt et al, 1991). Corresponding expression patterns for *SCF* were found in these studies at later stages of *in vitro* differentiation (> day 8) but observations during early events of differentiation varied between ES cell systems (McClanahan et al, 1993; Schmitt et al, 1991; Keller et al, 1993).

Labelled riboprobes specific for the haematopoietic transcription factor *vav* or the mesoderm marker gene *goosecoid*, were used for *in situ* hybridization on sections of day 6 and day 8 EBs. Evidence for the expression of *vav* transcripts was indicated in both time points analyzed. Signal for *goosecoid* expression was not detectable in day 6 EBs but indicated in sections of day 8 EBs (Table 4.5). Expression of the growth factor *BMP-4* was investigated in day 6 and day 8 EBs and expression of *TGF-ß1* in day 6 EBs. Expression of both growth factors were indicated in the time points analyzed (Table 4.5). The indicative signals for *vav*, *goosecoid*, *BMP-4*, and *TGF-ß1* expression in day 6 and day 8 EBs require further analysis for confirmation.

Preliminary *in situ* hybridization analysis for the spatial expression pattern of a novel zinc-finger protein encoding gene (gfi-1b), which was identified by the use of subtractive cDNA methodology, was performed on sections of day 5 and day 6 EBs. The gfi-1b gene had been found to be strongly expressed in day 5 EBs but not in day 3 EBs (Graham G, Hole N, and Ansell J; personal communication).

Signals for the presence of gfi-1b transcripts at very high levels, similar to the signal intensity for the mesoderm marker *brachyury*, were detectable in both time points analyzed. Similar to *brachyury* expression, signal for gfi-1b was found at a high intensity in almost all cells within an EB section. However, control hybridizations of EBs sections with labelled ribonucleotides corresponding to the sense sequence of the gfi-1b gene produced a high background signal (Figure 4.10.7).

No problems, caused by background staining in control hybridizations, were encountered for any of the other riboprobes used in this *in situ* hybridizations analysis. An example for a control hybridization with sense riboprobes is shown in Figure 4.10.8. Sections of day 6 EBs were hybridized with antisense riboprobes for actin or incubated with sense riboprobes corresponding to the actin sense sequence. A possible cause of the unspecific hybridization events in control experiments for gfi-1b expression may derive from the specific sequence structure of the gfi-1b gene. Further analysis of the expression pattern of the gfi-1b gene over a time course of *in vitro* differentiation is required to confirm observations from preliminary northern blot analysis.

4.4.3 Two colour *in situ* hybridization of sectioned EBs

The spatial expression of the haematopoietic gene globin displayed a distinctive pattern during *in vitro* differentiation (see Section 4.4.1). The well-defined onset of globin expression, which coincides with other haematopoietic characteristics in this ES cell system, may have the potential to be explored for further examination of the genetic control mechanisms of haematopoietic development during *in vitro* differentiation. To assess whether co-expression and co-localization patterns of novel and/or known genes with globin can be utilized to obtain information about haematopoietic differentation *in vitro*, two colour *in situ* hybridization experiments on sectioned EBs were performed.

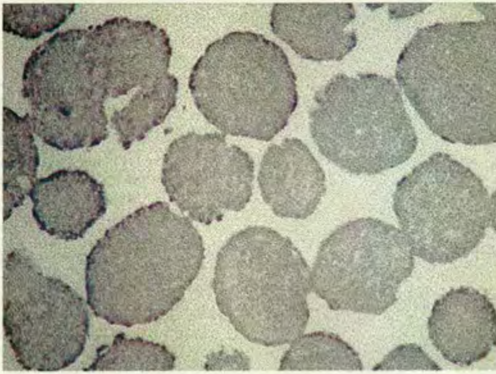
For initial two colour hybridization experiments sections of day 6 EBs were used for the simultaneous hybridization of DIG-labelled riboprobes specific for globin and fluorescein-labelled riboprobes specific for actin. Hybridization events for globin transcripts were detected by alkaline phosphatase conjugated anti-DIG antibodies and signal developed by the NBT/BCIP colour reaction. Signal for actin expression was subsequently detected by using alkaline phosphatase conjugated anti-fluorescein antibodies with Fast Red as colour substrate. The staining pattern obtained for globin and actin co-expression is shown in Figure 4.11. Whereas signals for globin expression were corresponding to previous expression patterns obtained from hybridization experiments with one riboprobe type, the signal intensity for actin expression was reduced. Signal intensity for actin expression obtained with DIG-labelled riboprobes with NBT/BCIP as colour substrate is shown in Figure 4.10.8. However, the two-colour staining pattern obtained indicates that two-colour *in situ* hybridization may be a feasible approach for co-expression analysis. Alternatively *in situ* RT-PCR may provide a means to analyze co-expression.

Table 4.5: Summary of signal intensity for haematopoietic gene expression in EBs.

	Intensity of colorimetric signal after in situ hybridization						
	Days of differentiation						
	2 days	3 days	4 days	5 days	6 days	8 days	Controls
actin (1)	++++	++++	++++	++++	++++	++++	-
globin *(2)	-	+	++	+++	+++	+++	-
brachyury (3)	++	+++	++++	++++	++++	+++++	-
Flk-1 (4)	++	++++	+++	+++	+++	++	-
SCL (5)	+++	++	+++	+++	+++	++++	-
CD34 (6)	++++	-	-	++	nd	+	-
c-kit (7)	++	+	+	+	+	+	-
SCF (8)	+	++	-	+	+	++	-
vav (9)	nd	nd	nd	nd	±	±	-
gsc (10)	nd	nd	nd	nd	-	±	-
BMP-4 (11)	nd	nd	nd	nd	±	±	-
TGF-ß1 (12)	nd	nd	nd	nd	±	nd	-
gfi-1b (13)	nd	nd	nd	++++	++++	nd	+++

globin* see Section 4.4.1 for data of induced time course studies
nd not determined
Individual controls for each riboprobe consisted of a labelled ribonucleotide sequence corresponding to the sense sequence of the particular gene. This labelled sequence was used for in situ hybridization on sections of day 6 or day 8 EBs.
Results are expressed as a qualitative measure of riboprobe/target mRNA hybrids as measured by colour intensity after colorimetric signal detection

(1) Biben et al, 1996; (2) Leder et al, 1992; (3) Wilson et al, 1995; (4) Yamaguchi et al, 1993; (5) Kallianpur et al, 1994; (6) Wood et al, 1997; (7) Keshet et al, 1991; (8) Keshet et al, 1991; (9) Ogilvy et al, 1998; (10) Blum et al, 1992; (11) Jones et al, 1991; (12) Dickson et al, 1995; (13) Graham, G., Hole, N. and Ansell, J., personal communication.



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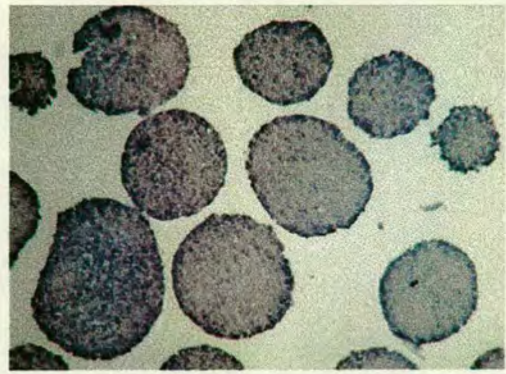

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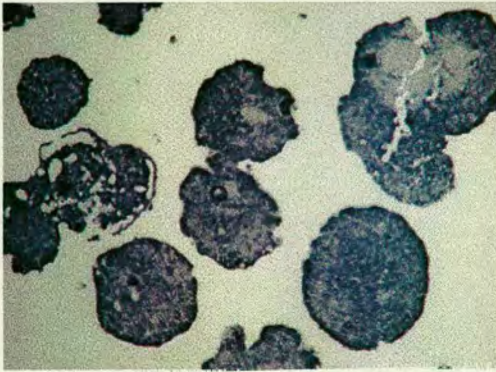

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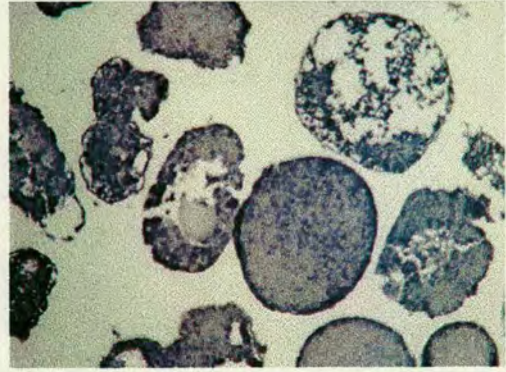

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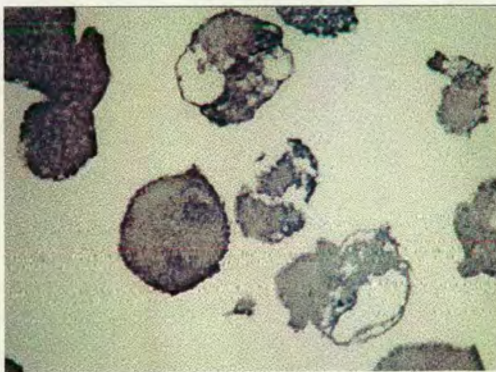

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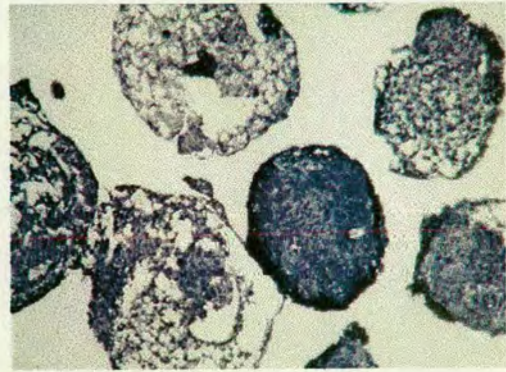
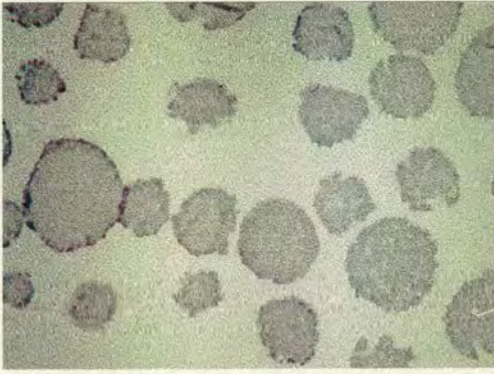


day 8

4.10.1: *In situ* hybridization analysis of *brachyury* expression in EBs during *in vitro* differentiation. A time course of EBs was wax-sectioned and hybridized with DIG-labelled antisense riboprobes specific for *brachyury*. Signal was visualized with alkaline-phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate. Signal for *brachyury* transcripts in EBs is visible by blue/purple colour change (Magnification 160x).



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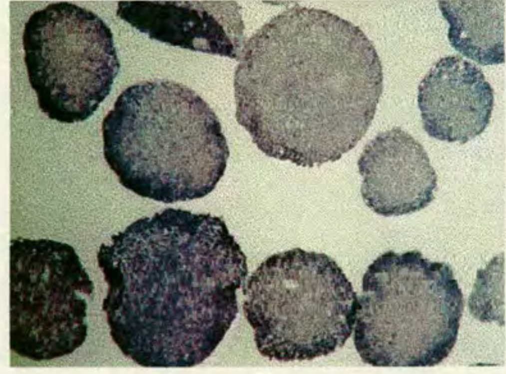

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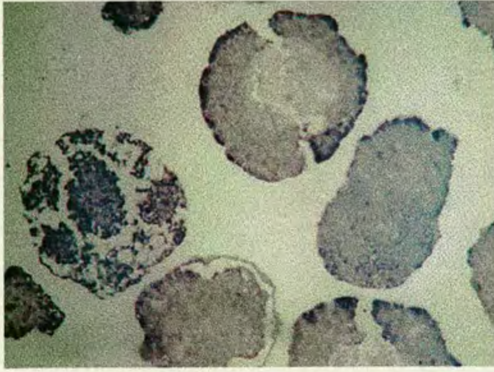

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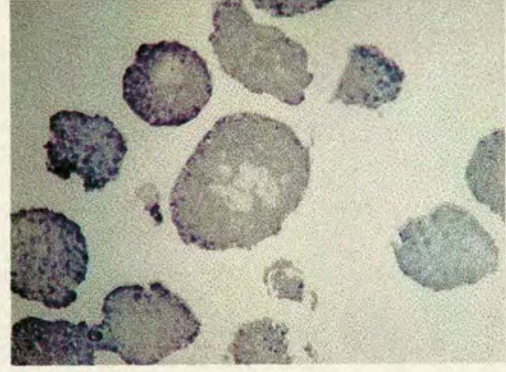

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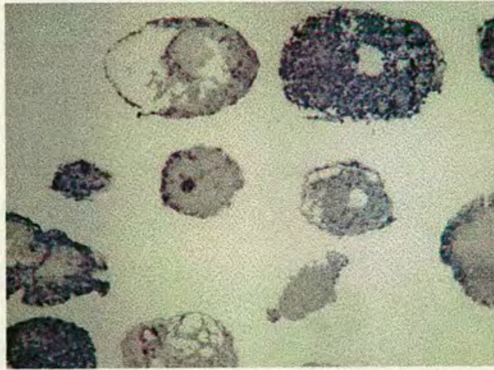

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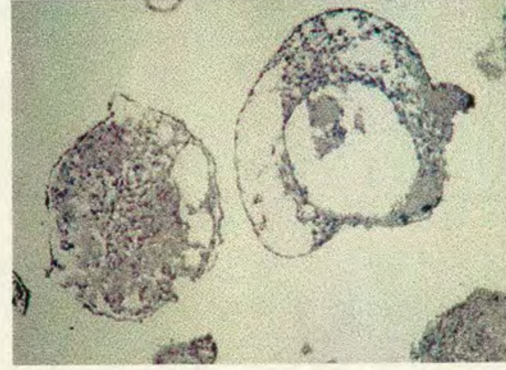
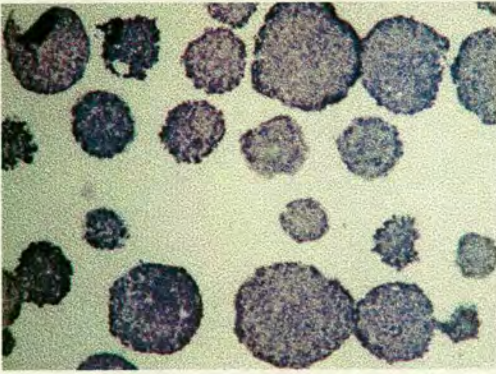


day 8

4.10.2: *In situ* hybridization analysis of *FLK-1* expression in EBs during *in vitro* differentiation. A time course of EBs was wax-sectioned and hybridized with DIG-labelled antisense riboprobes specific for *FLK-1*. Signal was visualized with alkaline-phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate. Signal for *FLK-1* transcripts in EBs is visible by blue/purple colour change (Magnification 160x).



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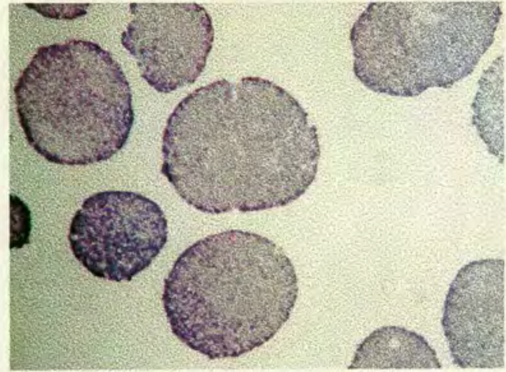

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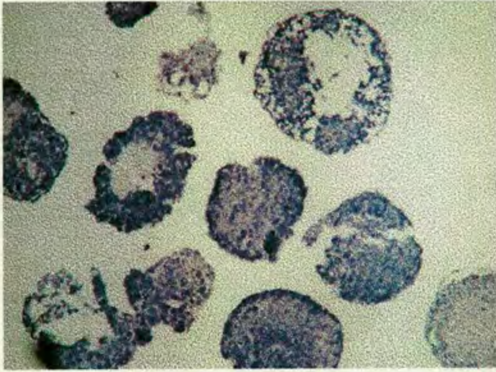

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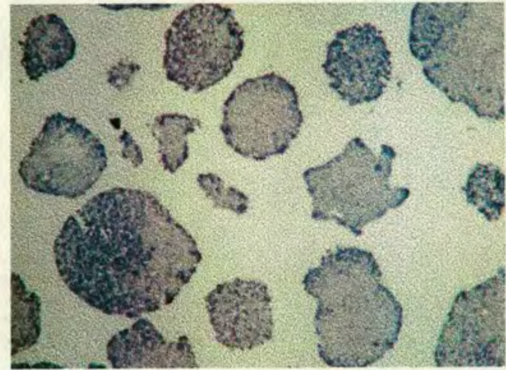

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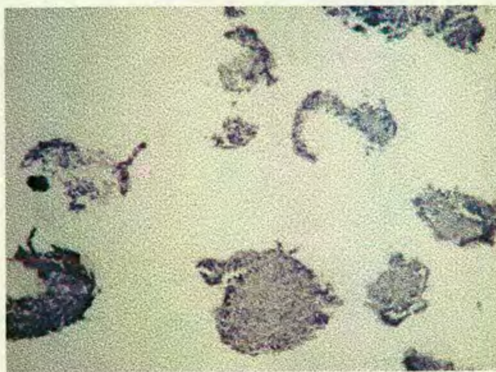

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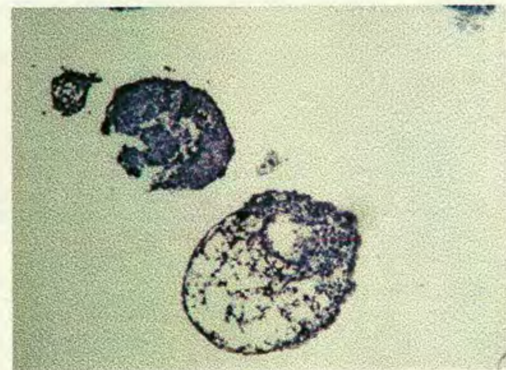
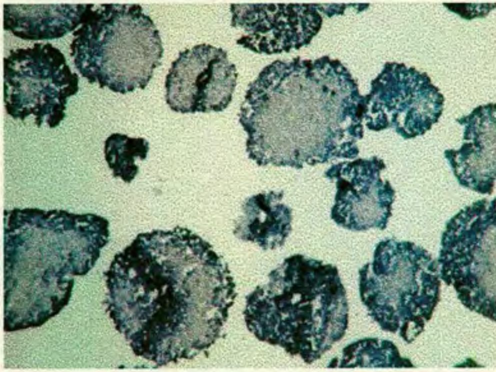


day 8

4.10.3: *In situ* hybridization analysis of *SCL* expression in EBs during *in vitro* differentiation. A time course of EBs was wax-sectioned and hybridized with DIG-labelled antisense riboprobes specific for *SCL*. Signal was visualized with alkaline-phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate. Signal for *SCL* transcripts in EBs is visible by blue/purple colour change (Magnification 160x).



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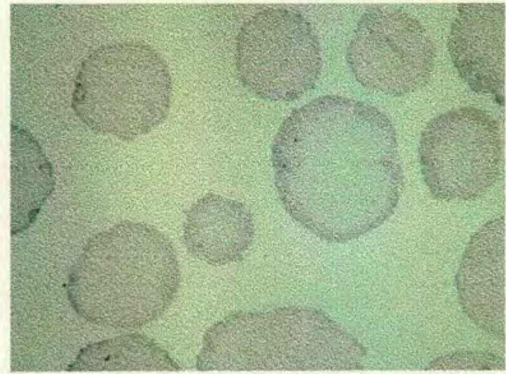

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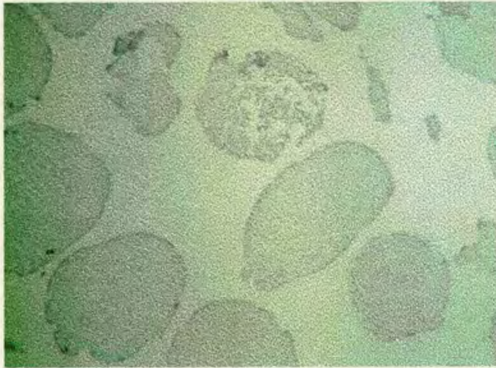

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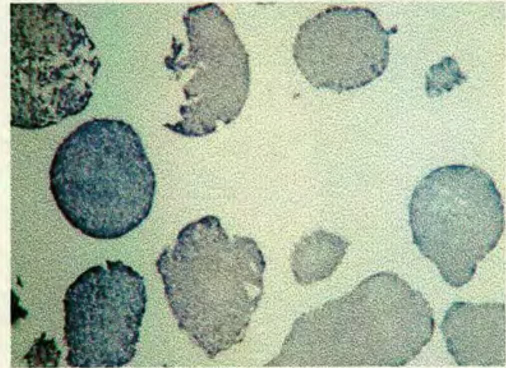

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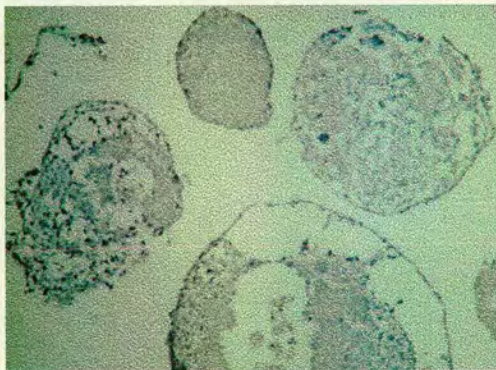
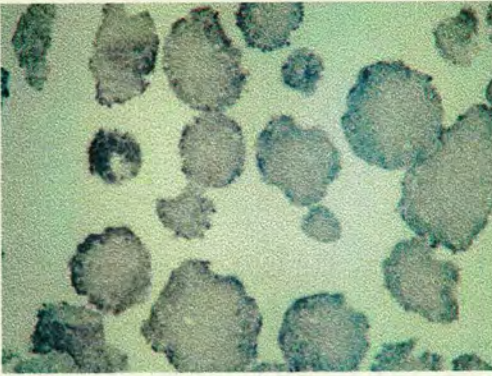


day 8

4.10.4: *In situ* hybridization analysis of *CD34* expression in EBs during *in vitro* differentiation. A time course of EBs was wax-sectioned and hybridized with DIG-labelled antisense riboprobes specific for *CD34*. Signal was visualized with alkaline-phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate. Signal for *CD34* transcripts in EBs is visible by blue/purple colour change (Magnification 160x).



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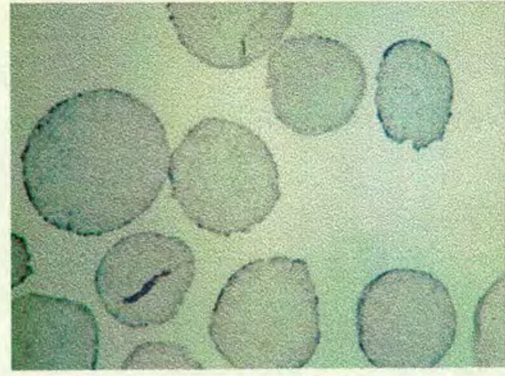

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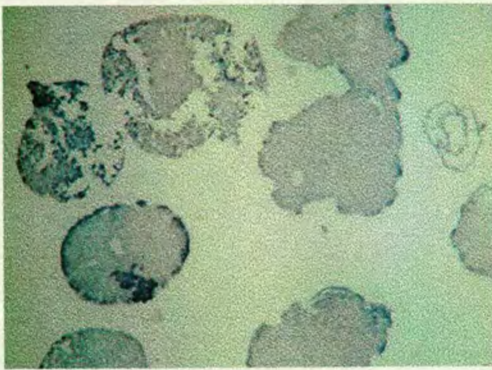

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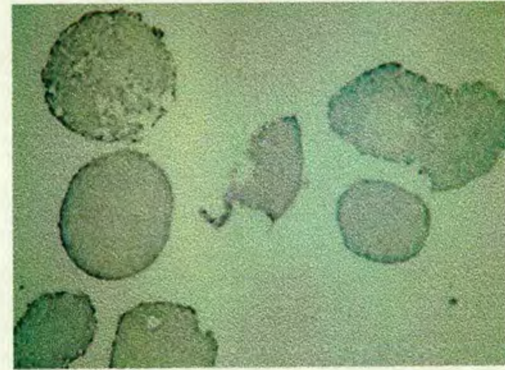

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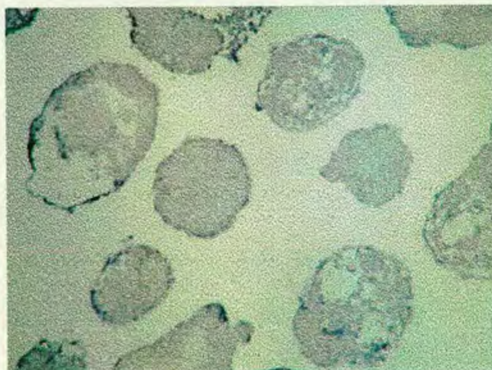

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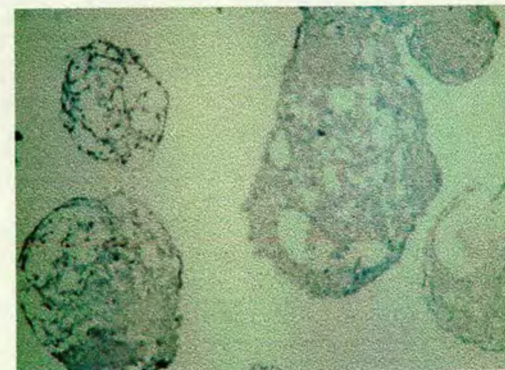
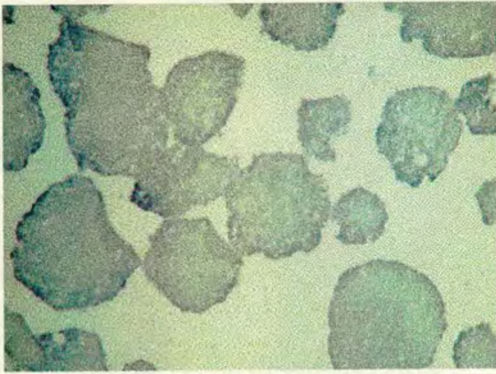


day 8

4.10.5: *In situ* hybridization analysis of *c-kit* expression in EBs during *in vitro* differentiation. A time course of EBs was wax-sectioned and hybridized with DIG-labelled antisense riboprobes specific for *c*--kit. Signal was visualized with alkaline-phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate. Signal for *c-kit* transcripts in EBs is visible by blue/purple colour change (Magnification 160x).



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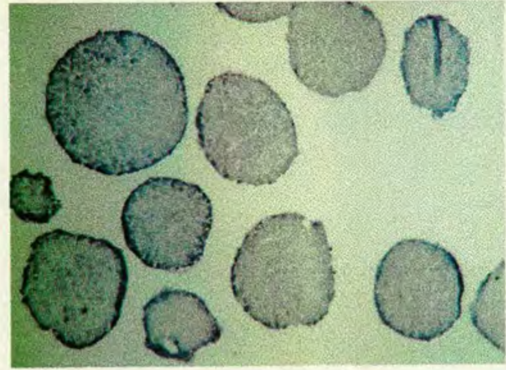

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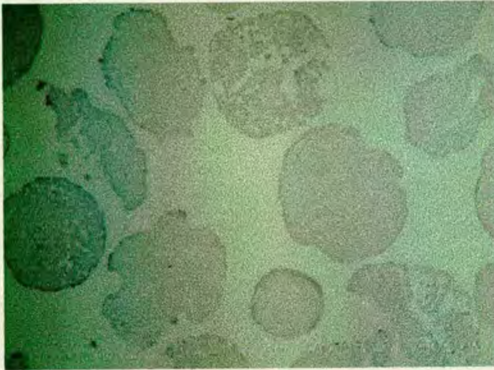

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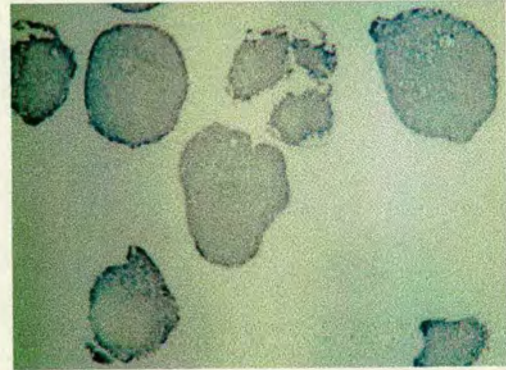

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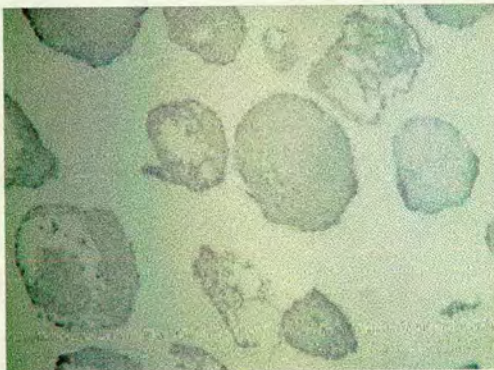

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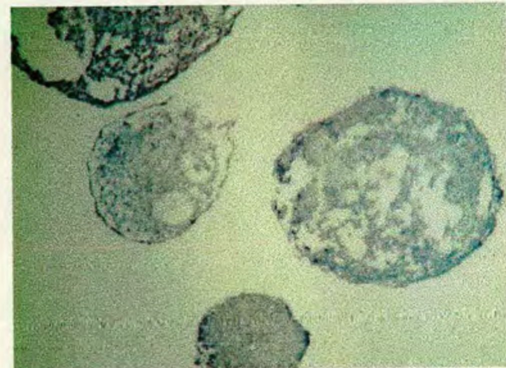
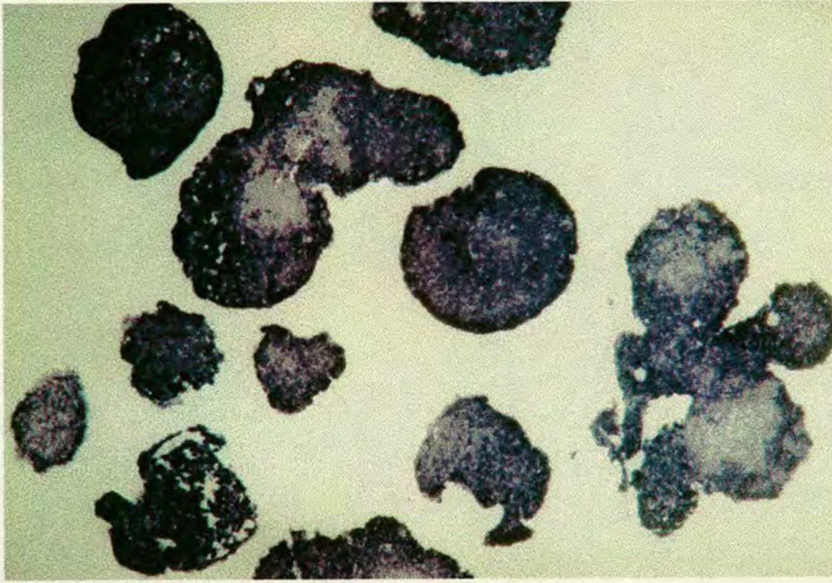
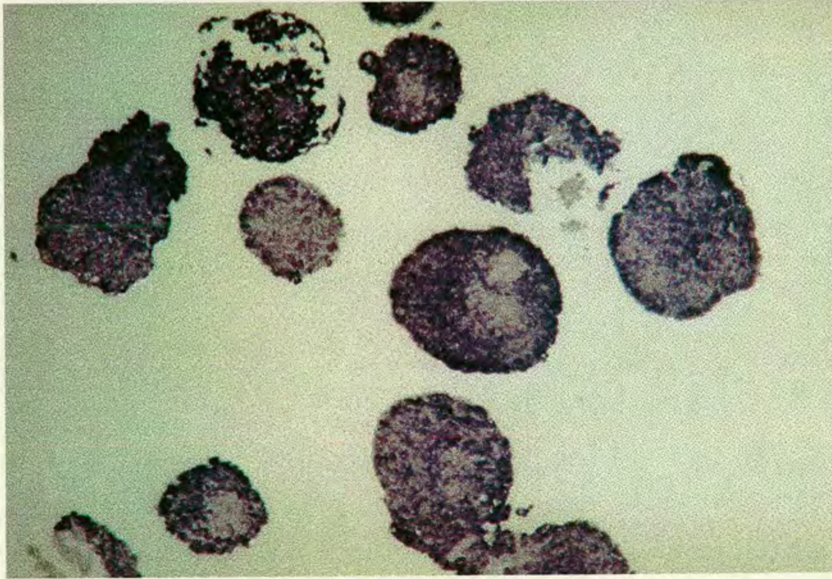


day 8

4.10.6: *In situ* hybridization analysis of *SCF* expression in EBs during *in vitro* differentiation. A time course of EBs was wax-sectioned and hybridized with DIG-labelled antisense riboprobes specific for *SCF*. Signal was visualized with alkaline-phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate. Signal for *SCF* transcripts in EBs is visible by blue/purple colour change (Magnification 160x).

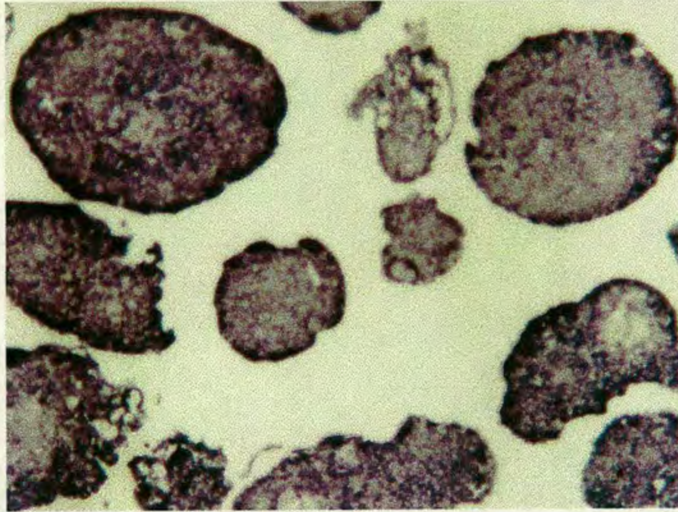


Singal obtained for *gfi-1b* expression after hybridization with antisense riboprobes for *gfi-1b.*



Background signal caused by unspecific binding of sense riboprobes corresponding to sense sequence of *gfi-1b.*

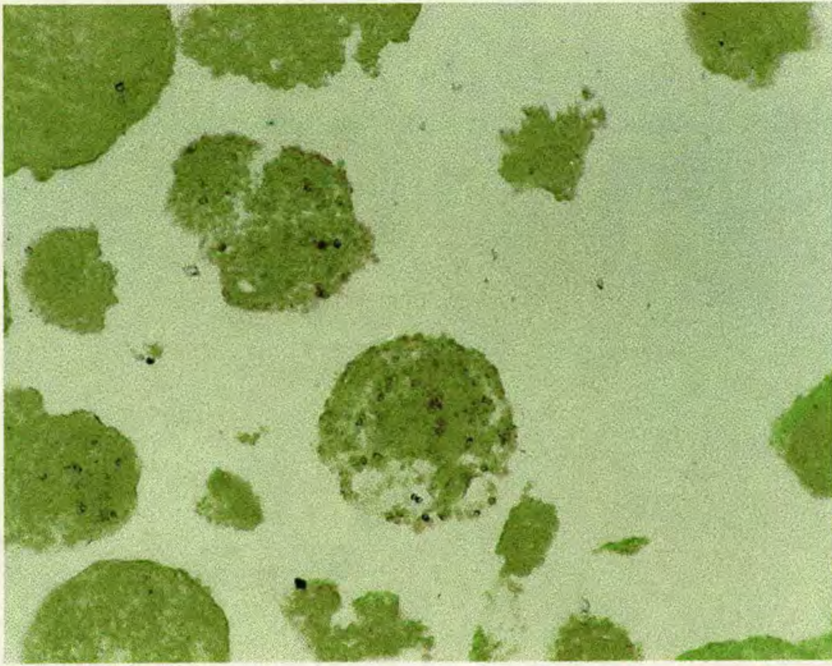
Figure 4.10.7: In situ hybridization analysis for the expression of zinc finger protein gfi-1b. Sectioned day 5 EBs were hybridized with DIG-labelled antisense riboprobes specific for gfi-1b (top) or with sense ribopbrobes corresponding to sense sequence of gfi-1b (bottom). Signal was visualized with alkaline-phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate (Magnification 160x).



Singal obtained for *actin* expression after hybridization with antisense riboprobes for *actin*.

Background signal caused by unspecific binding of sense riboprobes corresponding to sense sequence of *actin*.

Figure 4.10.8: *In situ* hybridization analysis for *actin* expression. Sectioned day 6 EBs were hybridized with DIG-labelled antisense riboprobes specific for *actin* (top) or with sense ribopbrobes corresponding to sense sequence of *actin* (bottom). Signal was visualized with alkaline-phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate. *In situ* hybridization with *actin* antisense probes and sense probes corresponding to actin sense sequence served as positive controls for *in situ* hybridization experiments (Magnification 160x).



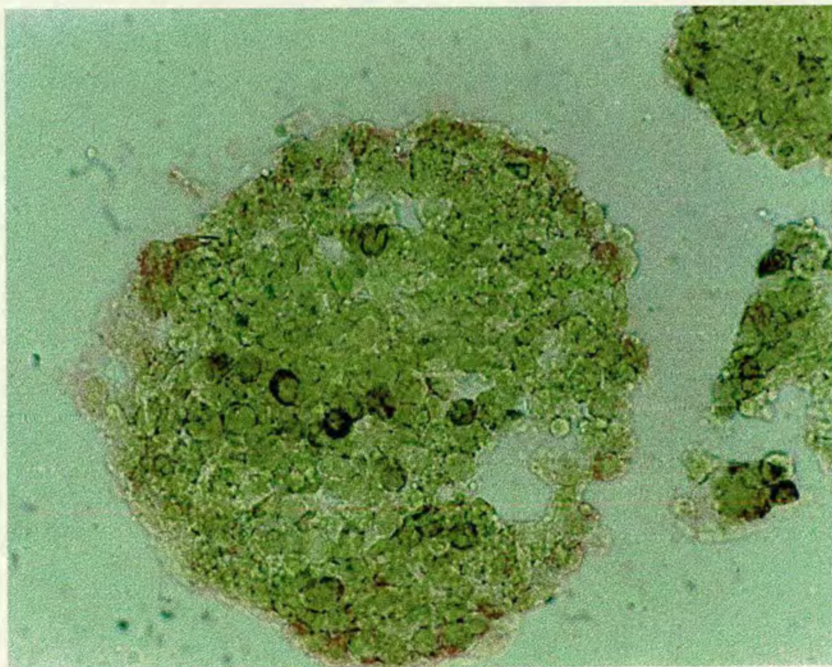


Figure 4.11: Two-colour *in situ* hybridization for *globin* and *actin* expression. Sectioned day 6 EBs were simultaneously hybridized with DIG-labelled antisense riboprobes specific for globin transcripts and with Fluorescein-labelled antisense riboprobes specific for actin transcripts. Signal for globin was detected with alkaline phosphatase conjugated anti-DIG antibodies and NBT/BCIP colour substrate and actin signal was subsequently detected with alkaline phosphatase conjugated anti-Fluorescein antibodies and FastRed colour substrate. Signal for globin transcripts is indicated by brown colour and for actin transcripts by red colour. (Magnification: top 160x; bottom 300x)

5. DISCUSSION

5.1 *In vitro* differentiation, an alternative approach for the preselection of developmentally regulated genes in gene trap clones

In the present study an *in vitro* gene trap prescreening approach for the identification of genes that may be involved in mesodermal and early haematopoietic commitment was assessed. The prescreening strategy involved *in vitro* differentiation of ES cell lines containing gene trap insertions and their exposure to morphogenic factors during differentiation. This approach was based on observations of the properties of an established ES cell system for *in vitro* haematopoiesis which has been reproducibly shown to produce transplantable HSCs (Hole et al, 1996). Although spatial identification and isolation of these HSCs within the EBs has not been achieved to date, they can be 'separated' on a temporal basis since the developmental pattern of haematopoietic commitment in this ES cell system is well defined. Characterization of the haematopoietic differentiation potential in this system has also demonstrated that the morphogenic factors DMSO and RA have an opposing influence on haematopoietic commitment (Doostdar, 1997). These effects were investigated on ES cell lines containing gene trap integrations to assess whether the exposure to morphogenic factors may be of use *in vitro* as a directed screen for a gene trap library.

Efforts to establish alternative screening strategies prior to *in vivo* analysis have increased with advances in gene trap vector design and the possibility of using a gene trapping approach as a routine methodology (Evans et al, 1997). Various *in vitro* strategies have been devised to improve the efficiency of the identification of gene trap clones of interest. Some are based on refined gene trap vector design to direct the vector insertion into particular classes of genes (Skarnes et al, 1995), some on *in vitro* induction assays (Forrester et al, 1996) and some on *in vitro* differentiation

(Baker et al, 1997). Furthermore, *in vitro* screening of gene trap clones based on sequence information has been reported (Zambrowicz et al, 1998; Holzschu et al, 1997). By using culture conditions optimized for *in vitro* haematopoiesis it was expected to determine whether these conditions are widely applicable on various ES cell lines or restricted to the ES cell line for which they were optimized. To address the correlation of *in vitro* and *in vivo* reporter gene expression and the reliability of this screening strategy, gene trap cell lines previously characterized for their tissue-specific *in vivo* expression were used for this pilot study.

The general applicability of this prescreening strategy for haematopoietic genes was demonstrated by using the CFU-A assay to assess haematopoietic commitment combined with an assay to monitor ß-gal activity during *in vitro* differentiation. Time course studies with various ES cell lines and gene trap cell lines revealed that all cell lines contained haematopoietic differentiation potential as defined by the CFU-A analysis (Figure 4.1). The first appearance of primitive haematopoietic progenitors was consistently at around day 4-6 of *in vitro* differentiation. This suggested a common pattern for haematopoietic commitment in ES cell lines and demonstrated that the culture conditions used to support *in vitro* haematopoiesis were not restricted to the ES cell line EFC-1. It also indicated that monitoring ß-gal activity in a time window between day 3 and day 6 may potentially allow genes involved in early haematopoietic commitment to be identified. The induction studies with DMSO and RA indicated that these morphogenic factors exert their opposing effects on haematopoietic commitment in a relative common fashion under the culture conditions used (Figures 4.2.1-4.2.4). The selection of DMSO and RA for the use in a prescreening strategy for haematopoietic genes was entirely based on induction studies previously carried out on the ES cell system EFC-1 (Doostdar, 1997). Therefore, the analysis of the effects of DMSO and RA on ES cell lines and gene trap cell lines was limited in the present study to the influences on CFU-A numbers and ß-gal activity. The analysis of haematopoietic commitment by CFU-A analyses during *in vitro*

differentiation and after exposure to DMSO and RA was completed by monitoring temporal ß-gal activity in the gene trap cell lines to examine how the reporter gene expression patterns corresponded to CFU-A data (Figures 4.3, 4.4.1 and 4.4.2; Table 4.1). Potentially interesting gene trap integrations for haematopoietic development were expected to show onset of ß-gal activity or increase from low level ß-gal activity at around day 4 of differentiation. This would then coincide with the appearance of primitive haematopoietic progenitors as defined by CFU-A analysis. Further indication of their involvement in haematopoietic development would be a significant increase in ß-gal activity after exposure to DMSO and suppression after exposure to RA during this time period.

Although the gene trap cell lines used in this pilot study were selected on the basis of their spatial *in vivo* reporter gene expression pattern, and two lines were thought to be potential positive controls for involvement in haematopoietic development, none of the gene trap cell lines would have been chosen for further analysis if included in a pre-screen of 'anonymous' gene trap clones. However, continuing investigations that analyzed the gene trap cell lines which displayed *in vivo* reporter gene expression patterns restricted to yolk sac and fetal liver identified the trapped genes as not being involved in haematopoiesis (Forrester et al, 1996; Skarnes et al, 1995).

Thus, the indications in this *in vitro* analysis that the gene trap cell lines I114 and ST598 are not involved in haematopoietic development are consistent with results from *in vivo* studies.

The spatial ß-gal expression patterns during *in vitro* differentiation were examined by an *in situ* X-gal staining assay (Figure 4.5; Table 4.2). Although changes in ß-gal expression over time and/or between gene trap lines could be observed, the evaluation of these expression patterns was limited. *In situ* staining for ß-gal protein allows the location of functional ß-gal fusion protein expressing cells within EBs to

be identified but does not provide the means to determine the cell type(s) of these cells. Therefore, only limited information was obtained for the spatial *in vitro* reporter gene expression patterns from individual gene trap cell lines.

Although *lacZ* is the most commonly used reporter gene, with the temporal and spatial expression patterns in cells being easily monitored by the *ß-gal* activity and the X-gal *in situ* staining assays, limitations occur (Figures 4.3, 4.4.1, 4.4.2 and 4.3; Tables 4.1 and 4.2). For instance, enzyme activity cannot be used as a direct measure of transcript levels. This restricts quantitative analysis of reporter gene expression levels to relative comparisons within groups of samples assayed in sets. Furthermore, possible differences in the stability of *lacZ* fusion transcripts and proteins make temporal and quantitative analysis complicated (Forrester et al, 1996). Translational repression of fusion transcripts may further obscure the analysis of transcriptional activity of trapped endogenous genes (Baker et al, 1997). An early widespread ß-gal expression pattern that becomes restricted at later stages during differentiation has been observed as a common pattern in gene trap screens (Friedrich and Soriano, 1991; Skarnes et al, 1992; Wurst et al, 1995; Baker et al, 1997). Thus, in a screen for genes involved in early haematopoietic development a large 'background' of non-haematopoietic gene traps may reduce the efficiency of such a screen.

In addition to assay related limitations, the heterogeneity of an EB population further complicates the evaluation of results from ß-gal expression assays. Because ß-gal activity is assayed from a pooled population of EBs, it is not possible to distinguish between for example, many cells expressing low levels of ß-gal or few cells expressing high levels of ß-gal. Although these short-comings of the ß-gal activity assay can be partially overcome by the *in situ* X-gal staining assay, some limitations remain since cell type distinction on the basis of morphological characteristics is not possible during early *in vitro* differentiation of ES cells.

In vitro differentiation of ES cells has been exploited for the *in vitro* preselection of gene trap clones with novel genes expressed in neurons, glia cells, myocytes, and chondrocytes (Baker et al, 1997). Selection criteria for further analysis were non-constitutive reporter gene expression during differentiation but expression in selected cell types after a certain period of differentiation. In these experiments gene trap cell lines were allowed to differentiate and then assayed for co-expression of ß-gal and antigens specific for neurons, glia cells, myocytes, and chondrocytes. Three novel genes, potentially specific for the cell lineages of interest, were identified by this screen, clearly demonstrating the success of the applied screening strategy. However, other methods of isolating differentially-expressed genes, e.g. cDNA subtraction (Lee et al, 1992) and differential display (Liang and Pardee, 1992) have been reported to be more efficient. For instance, a subtractive cDNA library derived from EB RNA at different time points of differentiation has isolated >100 novel clones related to haematopoietic differentiation (Graham G, Hole N, and Ansell J; personal communication).

There are obvious similarities between the *in vitro* pre-screen for neurogenic and myogenic genes (Baker et al, 1997) and the screening strategy employed in the present study. However, a crucial difference, apart from screening for different cell lineages, are the developmental stages analyzed. Baker et al. concentrated on the identification of genes expressed in differentiated neuronal cell lineages with overt morphological phenotypes, at a relatively late stage in development. The screening of differentiated cells allowed the use of qualitative ß-gal analysis which make co-expression studies feasible. In the present study the focus was on the identification and characterization
of genes involved in the very early haematopoietic commitment of differentiating ES cells. Whilst terminally differentiated cells are relatively easy to identify by morphological and/or antigenic characteristics, primitive progenitor cells are more difficult to identify. For instance, to date there is no specific phenotypic marker

known to be unique for HSCs. In addition to the lack of an overt phenotype, primitive progenitors are rare in numbers compared to differentiating and mature cells. Since the aim in this study was to design an *in vitro* pre-screen to identify gene trap cell lines potentially involved in early haematopoietic development, screening of co-expression patterns utilizing X-gal staining and labelled antibodies was not feasible. However, for the identification of genes expressed in terminally differentiated haematopoietic cells, co-expression of ß-gal and haematopoietic antigens could have been assessed by the analysis of cells composing haematopoietic CFU-A colonies.

Two aspects that have to be considered when deciding on a pre-screening strategy are specificity and practicability. Often a workable compromise between specificity and practicability has to be found. Specificity for haematopoietic differentiation was addressed in the present study by using culture conditions that have been established for *in vitro* haematopoiesis of ES cells and by using the CFU-A assay. Practicability for the simultaneous screening of a large number of gene trap cell lines was assessed by testing different methods of forming ES cell aggregates.

Culture conditions can have a great influence on the differentiation potential of ES cells which is also dependent on the number of cells differentiating within an EB (Maltsev et al, 1993). Therefore, the simplification of cell culture procedures was tested using the well characterized ES cell line EFC-1 and compared to standard culture procedures established for *in vitro* haematopoiesis.

The significance of the three-dimensional structure of EBs in providing an appropriate microenvironment for haematopoietic progenitors is implied by the importance of the haematopoietic microenvironment during haematopoietic development *in vivo*. Recently, a dominance of the developmental stage of the haematopoietic microenvironment over the developmental stage of injected HSCs has been demonstrated in transplantation studies in mice (Geiger et al, 1998). Experimental evidence for the importance of the microenvironment *in vitro* has

been obtained by examining the haematopoietic differentiation potential of disrupted EBs using the CFU-A assay. Disrupted EBs appear to lose the ability to form CFU-A colonies, indicating the dependency of haematopoietic progenitors on an appropriate stromal microenvironment during *in vitro* differentiation (Hole et al, 1996). The ES cell aggregates formed by culturing on Cellform-coated plates were critically assessed for their morphological structure and their haematopoietic potential after *in vitro* differentiation. Although the developing EB population was not as uniform in size and differentiation synchrony than 'hanging drop' EBs, the ES cell aggregates produced on Cellform-coated plates were able to develop into EBs that could form CFU-A colonies (Table 4.3) and were sufficient for initial characterization of gene trap cell lines. The use of Cellform-coated plates for the formation of ES cell aggregates would therefore allow the simultaneous analysis of a large number of gene trap cell lines compared to *in vivo* screening in chimeric embryos.

As mentioned before, the feasibility of using a large number of gene trap clones for analysis is one of the most important requirements for a successful screening strategy. This has been achieved for gene trap screens assaying undifferentiated ES cells for ß-gal activity, for which the analysis of several thousand gene trap cell lines has been reported (Wurst et al, 1995; Forrester et al, 1996). However, screens that utilize *in vitro* differentiation are at a comparably small scale with a few hundred gene trap cell lines used for screening (Baker et al, 1997; Scherer et al, 1996). Using the culture procedures described in this study it would have been feasible to screen a few hundred gene trap cell lines by *in vitro* differentiation.

The insertion of a gene trap construct mutates and tags the genes for subsequent cloning, making identification of the endogenous gene possible by rapid amplification of cDNA ends (RACE) (Townley et al, 1997). The relative ease identifying the endogenous sequence in gene trap cell lines has been exploited in a gene trapping approach for the automated identification of sequence tags from the mutated genes (Zambrowicz et al, 1998). In an attempt to have all genes in ES cells as potential

targets a vector construct that traps and mutates genes regardless of their expression status was used. This expression-independent method for sequence identification has been used to produce a library of ES cell gene trap clones in which sequence-tagged mutations are described. In another gene trap study a library was generated for sequence information in the form of expressed sequence tags (ESTs) and cDNA sequences (Evans et al, 1997). Gene trap clone libraries represent a functional genomic resource, as they contain sequence information of already mutated genes and thus provide opportunity to rapidly produce mouse mutants for gene function analysis (Evans et al, 1997; Zambrowicz et al, 1998).

Expression patterns of novel sequences can be examined by molecular analysis, for instance, northern blots or *in situ* hybridization to wild type embryos. The successful use of sequence-based gene trap screens has recently been reported (Chowdhury et al, 1997; Townley et al, 1997; Holzschu et al, 1997).

Despite the application of the gene-trapping approach by a number of groups, mentioned above, some reservations about its overall advantages still apply. For instance, selective pressures generated by vector design, vector delivery, target size and gene accessibility are likely to produce some level of bias in the relative representation of trapped genes (Skarnes, 1993). Furthermore, limitations associated with reporter gene expression assays also complicate the analysis of gene trap clones. Several reports have been published that assess the potential of *in vitro* differentiation for pre-screening of gene trap libraries, however, the successful application of such screens on a large scale (>1000 gene trap clones) has to be awaited (Baker et al, 1997; Gajovic et al, 1998; Voss et al, 1998). To date it may appear that the most successful application of gene trapping is the production of ES cell gene trap libraries as a data bank for sequence information and potential source of ES cell clones for functional studies.

Although the effects of DMSO and RA had shown opposing effects on haematopoietic differentiation of ES cells (Doostdar, 1997) it is likely that these factors may also

exert other effects on ES cell differentiation, which could confuse the 'readout' of this screen. Furthermore, the generalized rationale behind the prescreening strategy that developmentally regulated gene expression may code for developmentally important molecules may be too simplified. Widespread and/or constitutive expression of a gene does not preclude tissue-specific functions. For instance, the transcription factor *SCL* has been identified as essential for haematopoiesis, however, expression was observed in the developing nervous and skeletal systems and in the vascular endothelium (Robb et al, 1996; Kallianpur et al, 1994; Green et al, 1992).

In summary, the experiments performed in the present pilot study to assess the feasibility of an *in vitro* pre-screening strategy of gene trap cell lines for the identification of genes important during haematopoietic development has the potential for a large scale screen. Clearly an identified haematopoietic gene trap integration would be the most convincing proof for the applicability of this *in vitro* screening strategy. The evidence presented in this study indicates that this screening strategy may be used to enrich for gene trap integrations involved in early haematopoietic commitment. However, given the amount of work it would take to screen even 1000 gene trap clones by this method, it is doubtful whether enough data could be generated to make meaningful conclusions about the haematopoietic involvement of gene trap clones.
Previous studies on the ES cell line used in the present study demonstrated that globin expression may serve as a marker for haematopoietic activity during ES cell differentiation *in vitro* (Hole et al, 1996; Menzel U, Diplomarbeit, 1994) and furthermore, it was found that the effects of DMSO and RA were reflected in globin expression levels (Doostdar, 1997). Thus, co-expression analysis of reporter gene and globin may provide a more sophisticated and efficient strategy to screen a gene trap library for haematopoietic genes. A screen specific for haematopoietic gene

trap integrations would thus involve analysis of co-ordinate temporal and spatial expression of globin and the reporter gene by two-colour *in situ* hybridization. This *in situ* hybridization approach could also be effectively used to screen for candidate genes involved in haematopoiesis generated by cDNA subtraction. Using the precise time window of *in vitro* differentiation of the ES cell system used, a subtractive cDNA library was produced from EBs at day 3 and day 5 of differentiation. This approach has produced >500 differentially expressed clones over the 3 to 5 day differentiation period which coincides with the onset of haematopoietic differentiation and has been found to be a rich source of known and novel haematopoietic genes (Graham G, Hole N, and Ansell J; personal communication).

5.2 Analysis of the spatial expression of haematopoietic genes in EBs

The temporal expression pattern of haematopoietic genes during *in vitro* differentiation of ES cells has been extensively studied by a number of groups (Schmitt et al, 1991; Wiles et al, 1991; Keller et al, 1993; McClanahan et al, 1993; Hole et al, 1996).
In these studies methods such as RT-PCR and northern analysis were used to detect transcripts specific for haematopoietic genes. Although the application of these techniques allows one to follow temporal gene expression patterns during *in vitro* differentiation the cells expressing the genes and their spatial relationships cannot be identified by methods that analyse RNA from total cell populations. Such studies have not been reported to date.
In order to identify areas of haematopoiesis within EBs and to assess the possible feasibility of cellular co-expression analysis in EBs, whole-mount *in situ* hybridization experiments were performed utilizing riboprobes. As described in Section 4.3.1, whole-mount *in situ* hybridization of EBs using riboprobes specific for the detection of globin transcripts combined with colorimetric signal detection demonstrated that globin expressing cells are localized in small discrete areas within an EB (Figures 4.6.1-4.6.2). Differences in transcript levels within individual cells was identified (Figure 4.6.2), which may indicate differences in developmental stages of the erythroid lineage. However, preliminary investigations were unable to detect globin protein at this stage of *in vitro* differentiation (Hole N and Ansell J, personal communication). Although globin transcripts were readily detected, the number of EBs staining positive for globin expression was unexpectedly low (2%).
Previous observations in this ES cell system suggested an association between globin expression and the formation of CFU-A colonies (Hole et al, 1996). It was therefore expected that globin expression would be observed in 40-60% of EBs.

EBs developing in suspension culture have been found to have an outer rind of endodermal cells and an inner core of ectodermal cells that develop into mesoderm

(Bradley, 1990). Thus, the localization of haematopoietic cells should be expected within an EB rather than in the outer cell layer. This localization of mesodermal cells within an EB (Bradley, 1990) may explain the low proportion of EBs with globin transcripts when using whole mount *in situ* hybridization as a detection method. The three dimensional complex structure and the relative large size of EBs hampered the spatial analysis of gene expression in whole EBs. For instance, day 6 EBs are ~80 μm in size which limited the examination of hybridized EBs to the use of low magnification (300x) (Figure 4.6.1-4.6.2).

Whole-mount *in situ* hybridization of EBs without subsequent sectioning was performed in a study to monitor mesoderm commitment during differentiation of ES cells cultured in chemical defined medium (Johansson and Wiles, 1995). These investigators analyzed *brachyury* expression by whole-mount *in situ* hybridization to confirm RT-PCR studies and to determine the number of EBs differentiating into mesoderm lineages. Transcripts specific for *brachyury* were reported to be localized in clusters of cells within the EBs and signal intensity was described as heterogeneous. Depending on culture conditions, the number of EBs staining positive for brachyury expression varied. Approximately 90% of all EBs grown in medium containing serum showed signal for brachyury expression, compared to 5-10% of all EBs grown in chemical defined medium. No detailed analysis of the *brachyury* expressing cells within EBs was reported (Johansson and Wiles, 1995).

The *in situ* hybridization experiments on whole EBs emphasized the need to section the EBs to obtain more detailed information about the spatial gene expression patterns. In a study analyzing gene expression during endoderm differentiation, embryoid bodies derived from an embryonic teratocarcinoma cell line were used as an *in vitro* model (Becker et al, 1992). In order to establish whether a position-dependent differentiation pattern existed within embryoid bodies, whole-mount *in situ* hybridization was performed. Evidence in support for the assumed position-dependent

endoderm differentiation during early lineage commitment in differentiating embryonic teratocarcinoma cells was obtained only after the hybridized embryoid bodies had been sectioned (Becker et al, 1992).

In situ hybridization can be combined with colorimetric or fluorescent signal detection for labelled probe-target RNA hybrids. The use of fluorescence molecules as probe labels provides the means for the potential use of confocal microscopy for signal expression analysis. In order to take advantage of non-invasive optical sectioning by confocal laser scanning microscopy in the present study, attempts were made to combine whole-mount *in situ* hybridization of EBs with fluorescent signal detection. As described in Section 3.2.5, the use of confocal microscopy for the analysis of spatial gene expression in EBs was hampered by the high autofluorescent background. Confocal laser scanning microscopy has been reported to have become a practical research tool with increasing applications for the studies of tissue and cellular organization (Bacon et al, 1991). However, the use of confocal laser scanning microscopy requires complex adjustments for each experimental system and may be more suitable for some systems than for others. To the author's knowledge there has been no report to date in which whole-mount *in situ* hybridization and confocal laser scanning microscopy has been combined for spatial gene expression analysis during *in vitro* differentiation of ES cells.

In a study using *in vitro* differentiation of ES cells as an *in vitro* model for early erythropoiesis, confocal laser scanning microscopy was used to analyze the viability of cells within an EB (Gassmann et al, 1995). Lethal cell staining of EBs was performed using a fluorescent dye which stains only dying or dead cells and a series of consecutive optical sections were analyzed for fluorescence. Some weak staining was observed, but it was reported that almost all cells within the EB were viable. In contrast to gene expression analysis by whole-mount *in situ* hybridization as described in the present study, lethal cell staining does not require fixation and prolonged incubation in inorganic solutions of EBs.

These analytical and methodological differences may explain for the different experiences in applying confocal laser scanning microscopy to EBs.
Analysis of gene expression patterns by whole-mount *in situ* hybridization with radio-isotopic probes was reported for EBs differentiated under culture conditions optimized for ES cell differentiation into neuronal and skeletal muscle cell lineages (Rohwedel et al, 1994). ES cell aggregates allowed to differentiate under such specific culture conditions grow in a relative two-dimensional fashion by spreading on the plate substratum. Thus, cell samples for the analysis by whole-mount *in situ* hybridization were comparable to monolayers of cells or sectioned samples without the complication of complex three-dimensional structures. *In situ* hybridization of EB outgrowths was reported to detect corresponding expression of myogenic factor genes as observed *in vivo* (Rohwedel et al, 1994).
Culture conditions optimized for ES cell differentiation into haematopoietic cell lineages as used in the present study (described in Sections 3.1.5-3.1.9) support the growth of individually intact EBs in suspension culture. EBs grown under these conditions form three-dimensional structures which makes whole-mount *in situ* hybridization with radio-isotopic probes unsuitable as a method for gene expression analysis. Radio-isotopic hybridization has been described as only appropriate for monolayers of cells or sectioned material (Rosen and Beddington, 1993).

Information about the precise localization of gene transcripts in cells and tissues can give crucial clues for the identification of molecular mechanisms during differentiation. Since the spatial gene expression analysis of whole EBs was limited, efforts were concentrated on using the alternative approach of *in situ* hybridization on sectioned EBs over a time course of differentiation. The results obtained from *in situ* hybridization experiments of sectioned EBs revealed unique details of the spatial expression pattern for globin transcripts and identified an earlier onset of globin expression than detected by RT-PCR analysis (Figures 4.8.1-4.8.4, 4.9; Table 4.4).

In situ hybridization experiments with riboprobes for other haematopoietic genes provided information of their expression patterns during *in vitro* differentiation of ES cells (Figures 4.10.1-4.10.7; Table 4.5).
The distinctive signals for the localization of globin transcripts within EBs clearly illustrates the discrepancy between the number of EBs expected to express globin and the number of EBs expressing globin by whole-mount *in situ* hybridization as a methodological shortcoming (Figure 4.6.1-4.6.2) and confirms the mesoderm localization within EBs. In contrast to globin expression detected by whole-mount *in situ* hybridization, *in situ* hybridization on sections detected the expected proportion of globin expressing EBs. Most globin expressing cells were localized within an EB (Figures 4.8.1-4.8.3) which is in accordance to the observed organization of EBs with an outer rind of endodermal cells and an inner core of ectodermal cells (Bradley, 1990).

The analysis of globin expression over a time course of 8 days of EB differentiation demonstrated the localization of globin transcripts within EBs and provided evidence for the presence of globin transcripts at day 3 of differentiation (Figures 4.8.1-4.8.3; Table 4.4). The ability to detect globin transcripts in day 3 EBs illustrated the relatively higher sensitivity of the *in situ* hybridization approach compared to RT-PCR analysis for the detection of rare transcripts. Previous RT-PCR analyses for globin expression in this ES cell system in association with the temporal characterization of haematopoietic gene expression, the effects of exposure to DMSO and RA on haematopoietic commitment and the transplantation of EBs into lethally irradiated recipients, detected globin transcripts at day 4 of ES cell differentiation but not day 3 (Hole et al, 1996; Menzel U, Diplomarbeit, 1994; Doostdar, 1997).
This lack of sensitivity is most likely related to the 'dilution effect' of transcripts in total RNA samples and the heterogeneity of the EB cell population used for RNA extraction.

In situ hybridization for globin expression in sectioned EBs from either DMSO or RA induced EBs produced for the first time evidence of the effects of these morphogenic factors at a cellular level. The opposing effects of DMSO and RA, respectively, on haematopoietic commitment, have been studied by semi-quantitative RT-PCR and differential protein expression analysis (Doostdar, 1997). Although some changes in gene and protein expression patterns, which could be attributed to the exposure to morphogenic factor were detected, these studies used pooled total RNA or protein extract samples from a heterogeneous EB population. This experiment therefore did not allow identification of the effects of DMSO or RA at a cellular level. The detection of globin expressing cells in untreated and induced day 3 EBs is remarkable since previous studies suggested day 4 of differentiation as the concurrent onset of globin expression, CFU-A appearance and transient presence of transplantable HSCs. Transplantation studies identified day 4 of differentiation as the critical time point in which multilineage long-term repopulating HSCs are present in EBs. Transplantation of cells from day 3 EBs resulted in death of recipients. Cells from EBs at day 5 of differentiation were able to support long-term survival of irradiated animals but did not result in detectable reconstitution with ES cell derived progeny (Hole et al, 1996). The detection of globin transcripts in day 3 EBs, however, indicates the presence of primitive progenitors committed to the erythroid lineage (Figure 4.8.1; Table 4.4). Although the detection of transcripts does not necessarily indicate the presence of functional protein, it is however likely.

The appearance of primitive progenitors prior to transplantable HSCs closely resembles the temporal order of appearance of haematopoietic cells within the embryo *in vivo*. A reverse hierarchical order for haematopoietic cells during embryogenesis has been proposed which was based on the observations that differentiated haematopoietic cells and progenitors can be detected between day 7 pc and day 8.5 pc, whereas fully potent adult repopulating HSCs are not found until

day 10 pc (Dzierzak et al, 1998). The first detectable haematopoietic cells *in vivo* are primitive erythrocytes at day 7.5 pc. The presence of globin expressing cells within EBs at day 3 of differentiation *in vitro* suggests these EBs may correspond to developmental stage day 7.5 pc *in vivo*. The absence of adult repopulating HSCs in day 3 EBs further supports the correspondence between the sequence of developmental events *in vitro* and *in vivo*. The appearance of transplantable HSCs in day 4 EBs would therefore correspond to the presence of HSCs in the AGM region *in vivo* at day 9 pc. The indication from the temporal order of appearance that ES cell derived HSCs may be similar to AGM-HSCs is supported by observations from transplantation studies. The earliest HSCs in the AGM region are inefficient at long-term multilineage haematopoietic reconstitution (Medvinsky et al, 1996).
ES cell-derived HSCs are able to provide long-term reconstitution albeit at low level (Hole et al, 1996; Ansell J; personal communication). The haematopoietic activities observed during day 3 and day 4 of *in vitro* differentiation may therefore resemble the temporal development of primitive and definitive haematopoiesis *in vivo*.
The absence of adult repopulating HSCs but presence of survival supporting progenitor cells in day 5 EBs may indicate that EBs are not able to provide a fully competent microenvironment for the expansion of HSCs as observed in the AGM region. The number and efficiency of repopulating cells in the AGM region increases significantly by day 11 pc (Medvinsky and Dzierzak, 1996). However, although a deficiency in expansion of ES cell-derived HSCs appears to occur in day 5 EBs, an expansion of haematopoietic activity at the level of committed progenitors can be detected in globin expression and in CFU-A numbers (Table 4.4, Figure 4.8.1). This lack of potential in EBs to expand the number of ES-cell derived HSCs may possibly be overcome by co-culture of EBs and AGM regions or the use of conditioned medium from AGM cultures.
Globin gene expression has been widely used in ES cell systems as a molecular marker to monitor developmental stages and cell-lineages during haematopoietic

differentiation *in vitro* (Nakano et al, 1996; Hole et al, 1996, Keller et al, 1993; Schmitt et al, 1991; Wiles et al, 1991; Doetschman et al, 1985). Members of the ß-globin gene family are often used to distinguish between fetal and adult stages, since these globin types are regulated in a tissue-specific and developmentally specific manner. The alpha globin cluster consists of the embryonic zeta-gene and two adult alpha-genes. In the present study, specific alpha-globin riboprobes were used for the analysis of globin expression. Adult alpha-globin gene transcripts have been found to be co-expressed with embryonic zeta-globin gene transcripts. The co-expression of embryonic zeta-globin and adult alpha-globin occurs in the same cells and from the onset of erythropoiesis (Leder et al, 1992). Therefore the use of alpha-globin specific probes in this study allowed the use of one type of probe to follow the globin expression pattern during haematopoietic differentiation of EBs. The relevance of globin as a differentially expressed gene during *in vitro* differentiation is emphazised by the isolation of the zeta-globin gene in the cDNA subtraction library mentioned above.

The CFU-A data that correspond to these globin expression analyses (Table 4.4) somewhat contradict the suggestion that globin expression marks the onset of haematopoietic activity during ES cell differentiation. Although CFU-A colonies were detected at day 2 of differentiation, it must be noted these CFU-A data do not give any qualitative information about the type of colony. The CFU-A assay was used in the present study as a rapid screen for haematopoietic differentiation and colonies were scored by eye on size and structure. In a qualitative analysis of colonies formed in the EB-derived CFU-A assays three colony types have been observed (Hole et al, 1996). It may be possible the early colonies detected were not necessarily derived from primitive haematopoietic progenitors. CFU-A progenitors have been found to share many properties with CFU-S progenitors. A definitive description of CFU-A progenitors would require more detailed morphological

analysis, replating assays and an assessment of responsiveness to MIP-1alpha (Lorimore et al, 1990).

Comparison of globin expression patterns from untreated and DMSO or RA induced EBs demonstrated differences in the numbers of globin expressing EBs. However, no obvious changes in the arrangement of the globin expressing cells within EBs was observed (Figures 4.8.1-4.8.3, 4.9; Table 4.4). This observation is consistent with previously carried out CFU-A analyses on untreated and induced EBs, which suggested that DMSO and RA do not alter the temporal pattern of haematopoietic differentiation but influence the number of EBs that commit to haematopoiesis (Doostdar, 1997). Although no common pattern for globin expression could be deduced from these sectioned EBs it was interesting to observe that globin expressing cells were located within EBs either as clusters of cells or isolated, scattered cells. The arrangement of globin expressing cells in clusters may resemble blood islands in yolk sac during embryogenesis *in vivo*. The scattered globin expressing cells may indicate the location of primitive progenitors or may be an *in vitro* artefact. The understanding of these globin expression patterns is complicated because EBs do not display 'morphological landmarks' as do developing embryos. Due to the lack of morphological clues other means need to be used to gain information from these spatial globin expression patterns about the molecular events during *in vitro* haematopoiesis. One approach by which this could be achieved is the analysis of
co-expression of globin with other haematopoietic genes and/or markers associated with stromal cells of the haematopoietic microenvironment. The first haematopoietic cells to appear during embryogenesis *in vivo* are erythrocytes which reflect the commitment of mesoderm to an haematopoietic fate. Thus these haematopoietic cells can provide an avenue by which to examine the development of the haematopoietic system. The resemblance of haematopoietic *in vitro* differentiation of ES cells to

in vivo development and haematopoiesis is well documented (Doetschman et al, 1985; see for review Keller, 1995). Therefore, the globin expression patterns in EBs may provide a promising route for the examination of haematopoietic differentiation and the identification of the HSCs *in vitro*.

The quantitative comparison of globin expressing EBs between untreated and induced EBs shows some prominent features (Table 4.4; Figure 4.9). As mentioned before, no globin transcripts were detectable in day 2 EBs but from day 3 onwards in each time course. It is noteworthy that in untreated EBs and DMSO induced EBs similar levels (~20%) of globin expressing EBs were detected at day 3, whereas in RA induced EBs the number is reduced to about half (~10%) that of untreated EBs. This observation suggests that RA acts at an earlier stage of ES cell differentiation than DMSO. The similarity in numbers of globin expressing EBs between untreated and DMSO induced EBs is also maintained when the positive EBs are subdivided into EBs with scattered globin expressing cells and EBs with clusters of globin expressing cells (>10 cells). As mentioned above, the most prominent feature that could be distinguished in the spatial expression patterns of globin in EBs was the arrangement of globin expressing cells in clusters or scattered single cells. Since this difference may hold some significance, positive EBs were subdivided into EBs with >10 globin expressing cells and EBs with <10 positive cells (Table 4.4). The precise meaning of these observations remains to be determined. By day 4 of differentiation, the numbers of globin expressing cells in DMSO induced EBs is two-fold increased over untreated EBs, clearly illustrating that DMSO exerts its effect between day 3 and day 4 of differentiation (Table 4.4). Changes in numbers of globin expressing EBs between later time points are at similar ratios in untreated and DMSO induced EBs. The observed increase in haematopoietic activity of EBs after exposure to DMSO are consistent to observations made in other cell systems. DMSO has been shown to induce erythropoiesis in murine erythroleukaemia (MEL) cells, which

represent plastic erythroid progenitors (Elefanty et al, 1996). On the basis of these observations it can be concluded that DMSO enhances globin expression within an EB population by the recruitment of progenitors to the erythropoietic lineage. Although the numbers of globin expressing EBs do increase in RA induced EBs over time, most positive EBs (>80%) have less than 10 cells expressing globin (Table 4.4). The reduction in haematopoietic activity in RA induced EBs in comparison to untreated EBs may result from inductive effects of RA for differentiation into other cell lineages. For instance, RA has been reported to induce the differentiation of neural lineages from ES cells (Wobus et al, 1994; Bain et al, 1995). Alternatively, RA may act on certain primitive progenitors and reduce the number of cells that commit to the haematopoietic lineage.

In order to confirm the haematopoietic identity of the globin expressing cells and to investigate whether a certain order in the location of globin expressing cells exists, co-localization studies of haematopoietic genes and globin need to be performed. For instance, at day 4 of differentiation, the time point when transplantable HSCs are present, only ~15% of EBs staining positive for globin expression have more than 10 cells expressing globin (Table 4.4). It will be of interest to investigate whether position-dependent differentiation for haematopoiesis in EB exists. To be able to reconstruct the molecular sequence of expression during haematopoietic differentiation temporal and spatial co-expression analyses will have to cover a wide range of haematopoietic genes including transcription factors, receptor kinases, cytokines, covering early and/or late developmental stages and early and/or late involvement in haematopoiesis. Preliminary experiments using globin and actin probes (Section 4.4.3) to explore the feasibility of two-colour *in situ* hybridization experiments suggest co-expression analysis as a promising approach to further unravel the haematopoietic organization within EBs.

Expression patterns of some genes known to be involved in mesoderm or haematopoietic development were analyzed by *in situ* hybridization with specific riboprobes (summarized in Table 4.5; Figures 4.10.1-4.10.7). Although all genes used for these *in situ* hybridizations on EBs are known to be involved in haematopoiesis *in vivo*, most have also been found to be expressed in non-haematopoietic cells *in vivo*, e.g. FLK-1, SCL, CD34, c-kit, SCF. This relatively wide spread expression in various cell lineages *in vivo* appears to be reflected in the expression patterns in sectioned EBs after *in vitro* differentiation. None of the genes analyzed produced a distinguishable staining pattern as detected for globin expression. Furthermore, the relative disorganized structure of EBs without obvious morphology makes interpretation of these staining patterns very difficult. Although broadly consistent with gene expression studies in EBs using RT-PCR analysis (Keller et al, 1993; Schmitt et al, 1991; McClanahan et al, 1993, Kennedy et al, 1997; Hole et al, 1996), whole mount *in situ* hybridization (Johansson and Wiles, 1995) and flow cytometric analysis for immunophenotypic characterization (Ling and Neben, 1997; Kabrun et al, 1997) direct comparison of the spatial expression pattern with these studies is difficult not only because of the different methodologies used but also because of variations in culture systems.

In order to be able to integrate the data for spatial expression of haematopoietic genes in EBs into the presently known molecular events occurring during haematopoietic differentiation further analysis will be required. Co-expression analysis of haematopoietic and microenvironmental genes by *in situ* hybridization or *in situ* RT-PCR techniques combined with analysis of their corresponding proteins is likely to provide more conclusive information for the haematopoietic structure within EBs.

6. Summary and Perspectives

In summary, the studies outlined for the analysis of molecular events during haematopoietic differentiation demonstrate the potential of the *in vitro* prescreening strategy of gene trap clones assessed and illustrate spatial gene expression analysis as a promising approach for the reconstruction of the molecular events.

The assessment of the *in vitro* prescreening strategy for the identification of genes potentially involved in haematopoiesis proved the applicability of the tested approach (Chapter 4.1). Although monitoring ß-gal activity during *in vitro* differentiation after exposure to DMSO or RA indicated its usefulness as a measure of whether a gene trap clone may be of interest and should be included for further studies, some objections remained. The strategy was based on observations with the ES cell line EFC-1 in which the temporal pattern of the appearance of haematopoietic progenitors has been well defined by CFU-A analysis and transplantation studies (Hole et al, 1996). Haematopoietic differentiation in this ES cell system has been found to be induced by exposure to DMSO and reduced by exposure to RA (Doostdar, 1997). Although the generality of these observations were shown on other ES cell lines (CGR8 and R1) in the present study (Figures 4.2.1-4.2.3; Table 4.1), given the considerable amount of time and work it would need to perform a large-scale screen (>1000 gene trap clones) some doubts about the value of this screen remain. Specificity for haematopoiesis was introduced in this prescreening strategy by the exposure to DMSO and RA. An increase of ß-gal activity after DMSO exposure and a reduction after RA exposure during the time period of 3 days and 6 days of differentiation would have resulted in the selection of the gene trap clone for further characterization by CFU-A analysis. However, the question that remained is how much information about the involvement in haematopoiesis can be gained from

ß-gal reporter gene expression patterns and whether alternative approaches for the prescreening of gene trap clones in a more direct manner are possible. The use of gene trap cell lines, selected on the basis of their spatial expression patterns in chimeric embryos confirmed the correctness of the rationale of the tested *in vitro* prescreening strategy. However, the use of a quantitative assay for reporter gene expression of a heterogeneous EB population caused limitations. As mentioned in Section 5.1, most *in vitro* prescreening strategies, employed and assessed on gene trap clone libraries, are based on qualitative reporter gene assays or on sequence information based techniques (Baker et al, 1997; Chowdhury et al, 1997; Townley et al, 1997; Holzschu et al, 1997; Gajovic et al, 1998; Voss et al, 1998).

RT-PCR analysis of gene expression during *in vitro* differentiation of the ES cell system EFC-1 identified globin as a potential marker for the onset of haematopoietic differentiation (Hole et al, 1996; Menzel U, Diplomarbeit, 1994). Therefore an alternative approach for the screening of gene trap clones, which is based on a qualitative measure, can be co-expression analysis of gene trap transcripts with globin. This was attempted by whole-mount *in situ* hybridization experiments.

Based on the observations in the present study assessing a *in vitro* prescreening strategy for gene trap clones having a 'standard' gene trap construct inserted, a splice acceptor site and a *lacZ* reporter gene (Section 4.1), an alternative gene trap strategy has been devised using a refined gene trap construct. In order to be able to identify trapped endogenous genes co-expressed with globin, the gene trap construct contains the globin locus control region (LCR) unit driving a more accessible reporter gene, the green fluorescence protein (GFP). This enables identification of differentiating cells that express GFP in a viable state and does not rely on enzymatic colour substrate assays. Preliminary studies have identified ES cell clones that show temporal GFP expression corresponding to the globin expression identified in the ES cell system EFC-1 (Cunningham A, and Ansell J; personal communication).

Parallel to the use of gene trap technology for the identification of genes involved during haematopoietic differentiation a cDNA subtraction approach has been used to identify genes that are differentially expressed during the onset of haematopoietic differentiation in EBs. cDNA of day 3 and day 5 were subtracted from each other and a cDNA library produced which has been shown to be a rich source of known and novel genes involved in haematopoietic differentiation (Graham G, Hole N, and Ansell, J; personal communication). This cDNA subtraction approach proved to be a more efficient and superior methodology for the identification of novel haematopoietic genes than gene trapping.

Therefore further investigations in the present study were concentrated on the analysis of spatial gene expression in EBs during haematopoietic differentiation (Section 4.4). Analysis of the globin expression pattern on sectioned EBs by *in situ* hybridization revealed unique details at the cellular level. Furthermore evidence for the effects of DMSO and RA at the cellular level is presented (Section 4.4.2). However, in order to fully understand the identified expression pattern for globin further analysis will be required. Although DMSO has effectively been shown to increase haematopoietic differentiation *in vitro*, parallel to molecular analysis further efforts should be made to optimize culture conditions to achieve 100% haematopoietic differentiation within an EB population.

The interpretation of the spatial expression pattern of other known haematopoietic genes was difficult, since most genes are also expressed in non-haematopoietic cells and due to the lack of 'morphological landmarks' within EBs. However, these analyses provide information to explore various possibilities to analyze the interrelation between haematopoietic and microenvironmental cells. For instance, co-expression analysis by *in situ* hybridization or *in situ* RT-PCR technology or immunophenotypic methodology.

Based on results from the present project it is proposed that a promising avenue to identify and characterize novel genes during haematopoietic development will

be by combining the cDNA subtraction approach for the isolation of genes differentially expressed during haematopoietic differentiation *in vitro* with the analysis of spatial expression in 'wild-type' EBs. This molecular *in vitro* characterization will provide information by which identified clones can be chosen for functional analysis *in vitro* and *in vivo* by a gene targeting approach. The use of gene targeting techniques will allow the production of a range of transgenic models by the use of varying vector constructs, e.g. knock out mutations, conditional gene mutations.
The results presented and the techniques explored in this project will be advantageous in devising further strategies for the identification and characterization of HSCs and the definition of molecular events involved in their establishment.

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PUBLICATIONS AND PRESENTATIONS

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Haematopoietic Differentiation from Embryonic Stem (ES) Cells. **U Menzel**, L Doostdar, H Taylor, N Hole , J Ansell; British Society for Haematology 37th Annual Scientific Meeting; Harrogate, UK, April 1997 (Poster Presentation)

Embryonic stem (ES) cells: *In vitro* Model for Haematopoietic Development. **U Menzel**, L Doostdar, H Taylor, G Graham, N Hole, J Ansell; Genetical Society/British Society for Developmental Biology Spring Meeting; Warwick UK, March 1997 (Poster Presentation)

Strategies for the Identification of Genes in Early Haematopoietic Development. **U Menzel**, L Doostdar, L McGarry, J Murray, H Taylor, G Graham, N Hole, J Ansell; Keystone Symposia: 'The Haematopoietic Microenvironment'; Taos USA, February 1996 (Poster Presentation)

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Molecular Control of Haematopoiesis Using an *In Vitro* Embryonic Stem Cell Differentiation Model. **U Menzel**, Scottish Developmental Biology Group, Autumn SDBG meeting in St. Andrews, UK, September 1995, (Oral Presentation)

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Gene trapping using embryonic stem cells: A strategy for the identification of genes involved in haematopoietic development. ¶

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ABSTRACT

Most screening strategies for integrations of gene trap constructs in embryonic stem (ES) cells employ *in vivo* techniques such as the production of chimaeric embryos and transgenic mice. Using an established ES cell system for *in vitro* haematopoiesis the design of an *in vitro* prescreen for 'gene trapping' events that may identify genes involved in mesodermal and early haematopoietic commitment has been investigated. Exposure of 'gene trap' lines to morphogenic factors such as dimethyl sulphoxide (DMSO) and retinoic acid (RA) which induce and suppress haematopoietic differentiation of ES cells respectively has shown that haematopoietic commitment in ES cell lines with gene trap integrations can be reproducible influenced. Changes in haematopoietic commitment can be detected using a colony forming assay for primitive progenitors *in vitro* (the CFU-A assay) and changes in *lacZ* expression can be detected with a simple ß-galactosidase activity assay. Coincidence in temporal patterns from these assays can be used to enrich for potential haematopoietic gene trap clones. These studies demonstrate the potential of this *in vitro* screening strategy for a large-scale prescreening assay that should allow us to enrich for the number of 'haematopoietic' genes to be studied in vivo.

INTRODUCTION

The Haematopoietic System

The mammalian haematopoietic system is a complex developmental system which can be viewed as a hierarchy of pluripotent, committed and maturing cell populations differentiating into lymphoid, myeloid and erythroid cell types. Terminally differentiated cells within this system are short-lived and must be continually replaced from pluripotent haematopoietic stem cells (HSCs) throughout the lifespan of the animal. The definition of HSCs is based on characteristics such as self-renewal capability, multilineage differentiation and the competence to rescue recipient animals from lethal irradiation and repopulate the entire host haematopoietic compartment. Both, differentiative and proliferative processes of HSCs are regulated and influenced by interactions with non-haematopoietic stromal cells providing a haematopoietic inducing microenvironment, also described as a stem cell 'niche' (1). Cells of the haematopoietic microenvironment are a major source of haematopoietic cytokines supporting growth and differentiation of haematopoietic cells. The importance of interactions between haematopoietic cells and their respective microenvironment is widely accepted. However, little is known of the precise nature of the inter- and intra-cellular signals involved in control of haematopoietic differentiation of HSCs.

Haematopoietic development remains difficult to analyse for a number of specific reasons: (i) HSCs exist in a very low frequency in haematopoietic tissues (2) and lack the expression of specific markers (3). (ii) HSCs display transient phenotypes during their various developmental stages and occur as resting and activated subsets in the haematopoietic system (3). (iii) HSCs migrate through diverse sites of haematopoiesis during their development before haematopoiesis is established in the bone marrow (BM) of the adult mouse. The mouse haematopoietic system derives from ventral mesoderm of the developing embryo, which is formed at day 6.5 post coitum (pc). During mouse embryogenesis diverse anatomical regions of the developing embryo show successive haematopoietic activity beginning at approximately day 7 of gestation in the yolk sac (YS) with the first visible differentiated haematopoietic cells of the erythroid lineage. Haematopoiesis shifts at midgestation to the fetal liver (FL) and later to the spleen and BM which becomes the major site of haematopoiesis in the adult. While the bone marrow of adult mice is clearly a major site for HSCs it is still undetermined where the stem cells arise during embryogenesis. Until recently it had been generally accepted that all HSCs in mammals originate from the YS and consecutively migrate to the sites of definitive haematopoiesis (4). This view was based on *in vitro* studies showing that YS cells can differentiate into various haematopoietic lineages and *in vivo* studies showing that YS contains spleen colony-forming units (CFU-S) from day 8 of gestation. However, although primitive haematopoiesis and committed haematopoietic progenitors can be detected in the YS as early at days 7 - 8.5 pc, the lack of definitive CFU-S progenitors and long term repopulating (LTR) HSCs in the YS until late day 9 pc and day 11 pc respectively has brought this widely held dogma into controversy.

In non-mammalian vertebrates the ontogenic source of the definitive adult haematopoietic system has been determined by orthotopic embryo grafting experiments. Two sites of haematopoiesis have been found to exist early in the embryonic development of birds and amphibians. In the avian and amphibian systems, the YS gives rise only to early, transitory haematopoiesis whereas the definitive adult HSCs in these vertebrates are exclusively of intra-embryonic origin derived from the mesodermal region containing the dorsal aorta (5). Experiments with the analogous area of the mouse embryo have been performed by two groups (6, 7). Godin et al. (6) investigated for a possible non-yolk-sac source of stem cells by grafting intra-embryonic paraaortic splanchnopleura from 10- to 18-somite (day 8.5 - 9.5 pc) mouse embryos under the kidney capsule of adult immunodeficient SCID mice. Lymphoid cell clones, which could differentiate into mature B cells, were obtained from YS and paraaortic splanchnopleura cell preparations but not from other tissues of the embryonic body. Their initial minute numbers increased in parallel in the YS and the paraaortic splanchnopleura, suggesting that their emergence in the two sites was simultaneous. The detection of multipotent HSCs in the body of the mouse embryo prior to liver colonization (between the stages of 8.5 - 9.5 days of gestation) by this *in vitro* approach led to the suggestion that HSCs appear in parallel in the paraaortic splanchnopleura and in the YS, where they represent a new generation of primitive HSCs (8). Medvinsky et al. (6) directly compared the CFU-S activity of the aorta, gonad, mesonephros (AGM) region with the YS and FL during embryogenesis and found that this intra-embryonic region contains CFU-S activity at a higher frequency than that in embryonic YS and that such activity appears in the AGM region before the FL. Furthermore, the AGM region has been shown to harbour adult type multipotent haematopoietic progenitors (CFU-S) and pluripotential LTR-HSCs at late day 10 pc, a time slightly earlier than in the YS and FL. While the results presented by Medvinsky et al. (6) indicate that the intra-embryonic AGM region is the most potent pre-liver site of definitive haematopoietic activity, the direct measurement of CFU-S and LTR-HSCs within different parts of the embryo as a means of identifying the primary source of definitive haematopoietic activity cannot exclude the active interchange of cells via the circulation and possible interstitial migration of embryos. Therefore, to examine the source of the definitive HSC in the mouse embryo, Medvinsky et al. (10) have developed a novel *in vitro* organ culture approach. By using isolated organ cultures they demonstrated that CFU-S progenitor activity is not only maintained but autonomously generated and increased in AGM cultures at day 10 and 11 pc while YS supports only weak CFU-S activity. At day 10 pc the AGM region exclusively initiates abundant LTR-HSC activity and is totally independent of influences from the YS and the FL at this time. This evidence suggests that the AGM region is the pre-fetal liver source of the adult definitive haematopoiesis in the mouse and strongly supports the view that the FL is seeded by HSCs generated in the AGM region (10).

Although the identification of an intra-embryonic source of haematopoietic activity in mice in the paraaortic splanchnopleura or AGM region has greatly advanced our knowledge of

haematopoietic development in mammals their *in utero* development still prohibits orthotopic embryo grafting experiments as performed in non-mammalian vertebrates. Therefore alternative methods are required to investigate the origin of the most primitive HSC and the order of appearance of embryonic (primitive) HSCs and adult (definitive) HSCs in mammals. A variety of suitable *in vitro* systems have been established by a number of groups using haematopoietic precursors derived from various explanted adult or fetal tissues to study haematopoietic development (10, 11). Various cell types e.g. long-term bone marrow cells, fetal liver cells and cell lines possessing a stable pluripotential embryonic phenotype have been demonstrated to be useful in studying cellular and molecular events of haematopoiesis *in vitro*. Although most of these *in vitro* systems do not allow the study of the complete developmental process of haematopoiesis they are usually very specific for certain sections of the developmental pathway. Taken together it should be possible to obtain a comprehensive knowledge of the cellular and molecular processes occurring during haematopoietic development *in vivo*.

Embryonic Stem Cells and Gene Trapping

Cell lines of an early developmental stage that possess a stable pluripotential embryonic phenotype can be obtained from early mouse blastocysts, embryo-derived teratocarcinomas and primordial germ cells (12). Embryonic stem (ES) cells are totipotent cells derived directly from the inner cell mass of preimplantation 3.5 day mouse blastocysts (13, 14) and represent primary cultures with a high differentiation ability *in vitro* and *in vivo*. They closely resemble their normal counterparts and are able to form derivatives of all three germ layers when reintroduced into mouse blastocysts (13, 15). By co-culturing with feeder cells or in a feeder-free system in the presence of leukaemia inhibitory factor/differentiation inhibitory activity (LIF/DIA) (16) ES cells can be kept in their undifferentiated state. However, if LIF is withdrawn ES cells will differentiate spontaneously into many cell types including those of the haematopoietic system (13-15, 17). The *in vitro* differentiation of ES cells results in the formation of embryoid bodies (EBs) with endodermal, mesodermal, and ectodermal layers (17). Several laboratories have taken advantage of this *in vitro* model system to study the early development of haematopoietic cells (18-23).

The capacity of ES cells to differentiate *in vitro* and the appearance of terminally differentiated haematopoietic cells indicates the intermediate presence of HSCs during ES cell differentiation. Identification and amplification of these ES cell-derived HSCs may provide an abundant source of haematopoietic cells for analysis and modification. The appearance of haematopoietic cells is routinely observed as islands of haemoglobinised erythroid cells or blood islands within the developing EBs (17). However, EBs also contain progenitor cells capable of giving rise to myeloid, erythroid and lymphoid cells (18-20, 22-25). The structure of EBs seems to provide an appropriate microenvironment for ES cell-derived haematopoietic differentiation and it can be assumed that interactions occurring in EBs resemble events between haematopoietic tissues and HSCs *in vivo*.

Reconstitution experiments using differentiated ES cells have been shown that long-term repopulation can be derived from ES cells (21, 25-28). However, results reported by these groups are not correspondent. Some workers reported lineage restricted ES cell-derived repopulation (21, 25) whereas others observed multi-lineage reconstitution (26-28). These contradictory reports demonstrate that the reconstitution of lethally irradiated mice by ES cell-derived haematopoietic cells is very much depending on the respective ES cell system used and long-term multi-lineage repopulation may be cell line and/or passage number dependent. Data presented by groups who report multi-lineage repopulation (26-28) indicate that one prerequisite for appropriate haematopoietic commitment by ES cells is the presence of exogenous stromal cells or intact EB formation providing an appropriate microenvironment for the emerging HSCs within EBs. Two groups (26, 27) using an ES cell stromal cell co-culture system and conditioned medium argue that the isolation of HSCs from differentiated ES cells appears to be dependent on these stromal cell co-culture conditions. However, Hole et al. (28) present evidence that ES cell differentiation *in vitro* in the absence of stromal cell culture or conditioned medium can give rise to multi-lineage haematopoietic progenitors capable of long-term reconstitution. Using an *in vitro* assay for the detection of primitive haematopoietic progenitors (the CFU-A assay) they have characterised the earliest time points at which haematopoietic commitment can be detected in their system and concentrated reconstitution studies around this critical time point. These authors present data suggesting the transient presence of multipotent, long-term repopulating haematopoietic progenitors within their ES cell system and argue that the transient and early presence of the primitive progenitors during ES cell differentiation may be a reason for the limited lineage reconstitution seen in other studies. By relying on the internal differentiation programme provided by the EBs themselves they may have identified more clearly the time point at which the earliest haematopoietic progenitors can be detected. The identification and amplification of this assumingly transient HSC phenotype derived during *in vitro* differentiation of ES cells may provide an alternative strategy for genetic analysis and modification of the early haematopoietic development and provide a potential source of HSCs.

One of the most powerful tools to study developmental processes is the application of genetics. Although genetic manipulation techniques are commonly used in model invertebrates it has been very difficult and demanding to apply similar techniques in mammals. Thus, most mutations in mice have arisen either spontaneously or were induced by radiation or chemical mutagenesis. The ability to mutate the mouse genome in a more directed and subtle manner has been revolutionised by the use of ES cells. ES cells have proven useful for the successful application of a number of mutagenic strategies, including analysis of spontaneous mutations, chemical mutagenesis, and insertional mutagenesis (reviewed in 29). It has been demonstrated that ES cells are amenable to genetic manipulation *in vitro* and that these genetically altered cells retain their differentiation potential and normality *in vivo* when transferred into the germ line of chimaeric mice (30). Genetic manipulation of ES cells *in vitro* (reviewed in 31,32) provides the

means to mutagenize populations of ES cells, which can be screened for specific genetic alterations and characterized *in vitro* prior to transfer into the mouse germline. Furthermore, *in vitro* differentiation enables examination of the phenotype of for example targeted haematopoietic genes without the need to go through the germline and to identify novel genes involved in haematopoietic development.

Gene trapping provides a method to identify and functionally characterize novel genes and involves the transcriptional activation of promoterless reporter gene constructs after appropriate integration into the genome. It does not depend on the availability of sequence information. Gene trap approaches have been successfully used to trap genes expressed in undifferentiated ES cells (33-35). The design and uses of the many different gene trap constructs has been reviewed by Skarnes (36) and Hill and Wurst (29). The two main types of gene trapping vectors differ in their requirements for reporter gene activation. Promoter trap vectors simply consist of a promoterless reporter gene the expression of which requires insertion of the vector into an exon of a gene. Gene trap vectors contain a splice acceptor sequence upstream of the reporter and integration of this type of vector within an intron of an endogenous gene is predicted to generate fusion transcripts through the use of the splice acceptor. Conventional gene trap vectors generally contain the *lacZ* gene which encodes for the bacterial ß-galactosidase (ß-gal) protein as a reporter and neomycin as a selectable marker, or the ß-geo gene which encodes for a directly selectable reporter protein, the lacZ-neomycin phosphotransferase fusion protein (33,34). The subsequent expression of the reporter gene in both cases is under the control of the transcriptional regulatory elements of the endogenous trapped gene and the active form of a functional ß-gal fusion protein in *lacZ* expressing cells can be easily identified employing various standard assays for ß-gal expression. By creating a fusion transcript with the endogenous gene, promoter trap and gene trap insertions have the potential to be mutagenic. As a portion of the interrupted endogenous gene is included in the fusion transcript, the mutated gene is accessible to molecular cloning using the 5' rapid amplification of cDNA ends (RACE) strategy (35). Gene trapping in ES cells offers a rapid method to identify and simultaneously mutate genes expressed during mouse development (33). In a random gene trapping approach the ES cell clones are selected on the basis of reporter gene expression in undifferentiated ES cells and then screened for expression in the developing embryos. Integrations that result in the expression of the reporter gene in the tissue or developmental pathway of interest can then be selected for further study. However, this is a particularly time consuming and expensive *in vivo* screening strategy (37) and clearly an *in vitro* prescreen that could select for integrations into genes expressed in cell lineages of interest would be very useful. Various approaches addressing *in vitro* prescreening of ES cell clones with gene trap insertions have been performed and two general strategies have been reported (38, 39). One strategy is a directed gene trap screen where gene trap integrations were selected *in vitro* on the basis of their response of the addition of the morphogen retinoic acid (RA) (39). As ES cells can differentiate into a large number of different lineages *in vitro* it should be possible to adapt this screening

strategy for any endogenous trapped gene that is expressed in a cell lineage of interest after they have been exposed to various experimental environments - growth factors, morphogens or specific feeder cells, for example. Generally, this screening strategy should allow the identification of genes that act downstream of any ligand-mediated pathway. Another strategy for *in vitro* prescreening of gene trap integrations in ES cells involved the design of a specific gene trap construct which will integrate into particular sequence sites to generate active ß-gal fusion proteins. The 'secretory trap' used by Skarnes et al. (38) relies on capturing the N-terminal signal sequence of an endogenous gene to generate an active ß-gal fusion protein.

In this manuscript we discuss a novel approach for the *in vitro* preselection of gene traps for integrations into genes important for the development of the haematopoietic system. The strategy being the induction of haematopoietic *in vitro* differentiation of ES cells by morphogens that have been shown to induce or suppress haematopoiesis respectively. Using established ES cell culture procedures which are suitable for haematopoietic differentiation *in vitro* and enable ES cells to maintain their potential of *in vivo* differentiation we have used ES cell lines containing gene trap integrations to design an *in vitro* prescreen specific for haematopoietic events. To address reproducibility and correlation of *in vitro* and *in vivo* reporter gene expression of this approach ES cell lines containing gene trap insertions selected on the basis of their spatial expression patterns in chimaeric embryos have been used for this study. Haematopoietic development *in vitro* was determined by CFU-A analysis and reporter gene activity was detected by a quantitative ß-gal assay. We have found that the ES cell lines containing gene trap insertions can maintain their potential of haematopoietic development *in vitro* and that responsiveness to haematopoiesis inducing morphogens can be monitored by a quantitative ß-gal assay.

MATERIAL AND METHODS

Embryonic Stem Cell Lines and 'Gene Trap' Lines

ES cell lines used were EFC-1 (40), R1 (41), and CGR8 (42). 'Gene trap' lines I114 and R68 contain conventional gene trap vectors with a splice acceptor sequence fused to the reporter gene *lacZ* and the bacterial neomycin-resistance gene driven by the phosphoglycerate-1 (*PGK1*) promoter. *In vivo* expression of I114 was found to be restricted to YS and FL, R68 was found to be expressed in fetal heart and various other areas (43). 'Gene trap' line ST598 contains a 'secretory trap' vector with the reporter gene ß-geo, which is a *lacZ*-neomycin phosphotransferase fusion gene linked to a splice acceptor sequence, a signal sequence and a transmembrane domain, which relies on capturing the N-terminal signal sequence of an endogenous gene to generate an active ß-gal fusion protein. *In vivo* expression of this 'gene trap' line has been found to be restricted to YS and FL (38, Skarnes, W., personal communication). 'Gene trap' line Zin40 contains a gene trap construct with a splice acceptor sequence linked to the ß-geo reporter gene and is ubiquitous expressed *in vivo* (Smith, A., personal communication).

Embryonic Stem Cell Culture and Embryoid Body Formation

ES cell lines and 'gene trap' lines were routinely passaged and maintained in an undifferentiated state under feeder-free culture conditions in the presence of leukaemia inhibitory factor (LIF), as described by Smith (44). All experiments described used cell lines of less than 30 passages. Embryoid bodies (EBs) were formed by using the hanging drop method as described (23, 28). In brief, ES cells were cultured as hanging drops (10µl) at a concentration of $3x10^4$ cells/ml in the presence of LIF for 48 hours in a humidified 5% CO_2 atmosphere. ES cell aggregates were harvested into petri dishes (10^3 aggregates/10 ml medium) containing ES cell culture medium lacking LIF and allowed to differentiate into EBs for varying periods of time (up to 8 days). Medium was replaced every two days.

Exposure of Embryoid Bodies to Morphogen

ES cell aggregates formed by the hanging drop method (see above) were exposed to 1.0% dimethyl sulphoxide (DMSO) or 10^{-8} M of all-*trans* retinoic acid (RA) for the first 48 hours of differentiation and allowed to differentiate for a further period (up to 8 days) before being assayed (Doostdar, L., Ansell, J.D. and Hole, N., personal communication).

The CFU-A Assay

The CFU-A assay is an *in vitro* assay that detects primitive haematopoietic progenitors (CFU-A) and was set up as described previously (45, 46). Briefly, a feeder layer consisting of 0.6% agar in alpha MEM with conditioned medium from two cell lines (AF1-19T, a source of GM-CSF, and L929, a source of CSF-1) was poured in 3 cm diameter tissue culture grade dishes (1 ml per layer). EBs (50 EBs/plate) were added to 0.3% agar in alpha MEM and added to the culture dish to form an upper layer. The dishes were incubated for 11 days at 37°C in a humidified atmosphere with $5\%O_2/10\%CO_2$.

Quantitative ß-Galactosidase Activity Assay

The detection of ß-gal activity in EBs from ES cell lines containing gene trap integrations was performed using a colorimetric assay as described by Eustice et al. (48). Briefly, EBs were lysed by three freeze/thaw cycles and the protein concentration of cell lysates was determined measuring the optical density at 280nm. ß-Gal activity assays were performed with equivalent amounts of protein (40 µg) using o-nitrophenyl-ß-D-galactopyranoside (ONPG) as substrate. Samples were incubated at 37°C over night and the absorbance at 405nm was determined using an Elisa reader.

ß-Galactosidase Expression Pattern in Embryoid Bodies

ß-Galactosidase activity can be assayed by *in situ* staining since the action of ß-gal on the exogenously added substrate X-gal produces an insoluble blue cleavage product which precipitates at the site of enzyme activity. The ß-gal staining assay was performed as described previously (47). In brief, EBs from ES cell lines containing gene trap integrations were fixed in 0.2% glutaraldehyde for 10 minutes and stained with X-gal (1mg/ml X-gal) for ß-gal expression.

RESULTS

Four ES cell lines with gene trap insertions selected on the basis of their spatial expression patterns in chimaeric embryos (I114; R68; ST598; Zin40), their respective parent ES cell lines (CGR8; R1) and a control ES cell line (EFC-1) were used to assess *in vitro* differentiation. Emphasis was laid on development into haematopoietic lineages and correlation of *in vitro* and *in vivo* reporter gene expression. All experiments were performed in sets, i.e. ES cells with gene trap insertions, their parental cell lines and the ES cell line EFC-1 as an internal control were assayed at the same time.

CFU-A analysis and responsiveness to morphogen factors.

The ability of EBs from 'gene trap' lines, their parent ES cell lines and a control ES cell line to form CFU-A colonies was followed over a time course of 8 days of *in vitro* differentiation (Fig. 1). The CFU-A analyses revealed that all cell lines used possess the ability to form CFU-A colonies indicating the presence of primitive haematopoietic progenitors within these developing EBs. Comparison of CFU-A profiles from these different cell lines showed that CFU-A colonies started to appear at around day 4-6 of *in vitro* differentiation which was consistent for all cell lines, suggesting a 'common developmental programme' for haematopoiesis induced by the removal of LIF. No substantial numbers of CFU-A colonies were detectable prior to day 4 of differentiation. Undifferentiated ES cell aggregates were unable to form haematopoietic colonies in the CFU-A assay, indicating the need for differentiation in an appropriate microenvironment within the EB prior to the appearance of primitive haematopoietic progenitors. The temporal pattern for the first appearance of primitive haematopoietic progenitors within the differentiating EBs from these different cell lines was precise and reproducible. Variation in onset of appearance and cloning efficiency of CFU-A colonies within cell lines between different experiments were within the consistency of this *in vitro* assay. Cloning efficiency was defined by the percentage of EBs plated that formed haematopoietic CFU-A colonies. Each cell line displayed a characteristic CFU-A pattern with all lines, except one (ST598), showing a continuous increase of CFU-A numbers over the 8 day time course followed.

Responsiveness to morphogenic factors dimethyl sulphoxide (DMSO) and retinoic acid (RA) respectively was assessed by exposure of ES cell aggregates to morphogenic factor for the first 48 hours of *in vitro* differentiation and further differentiation in suspension culture for up to 6 days before using these induced EBs for assaying. Previous studies have shown that haematopoietic commitment of the ES cell line EFC-1 can be influenced by the effects of morphogenic factors such as DMSO and RA which respectively increase or decrease the proportion of EBs which contain primitive haematopoietic progenitors forming CFU-A colonies (Doostdar, L., Ansell, J.D. and Hole, N.; personal communication). The opposing effects of these two morphogenic factors on different ES cell lines and cell lines with gene trap insertions were being exploited as a strategy for the design of a specific *in vitro* prescreen of gene trap insertions to identify genes which may be involved in controlling haematopoietic differentiation.

Results from CFU-A analyses assessing the inductive response of EBs to morphogenic factors on haematopoietic commitment during ES cell differentiation are shown in figures 2.1 and 2.2. These studies revealed differences between original ES cell lines and 'gene trap' lines to exposure to morphogenic factors. Whereas the three ES cell lines (CGR8, R1, EFC-1) displayed a 'common' response, the cell lines with gene trap insertions (I114, R68, ST598, Zin40) showed individual responses to morphogenic factors. Temporal and quantitative analysis of CFU-A formation from time course studies revealed that the opposing effects of DMSO and RA respectively are not ES cell line dependent (Fig. 2.1). All three original ES cell lines showed increased numbers of CFU-A colonies after exposure to DMSO (generally by 10-20%) with the increase starting at around day 4 of differentiation, 2 days after removal from exposure to morphogenic factor. However exposure of ES cell aggregates to RA suppressed haematopoietic commitment within differentiating EBs. EBs of ES cell lines exposed to DMSO not only showed an increase in haematopoietic commitment defined by the number of CFU-A colonies but also resembled the temporal CFU-A pattern of their untreated counterparts. No significant differences in CFU-A numbers between treated and untreated EBs were detected directly after exposure to morphogenic factor at 2 days of differentiation suggesting differentiation within the EBs is a prerequisite for the appearance of primitive haematopoietic progenitors. ES cell lines with gene trap insertions (I114, R68, ST598, Zin40) showed varying responses to exposure to morphogenic factors such as DMSO and RA respectively. This diversity is more likely to be caused by clonal variations in subclones of ES cells than by the insertions of gene trap constructs since integration of gene trap constructs in the genome results in heterozygousity at the site of insertion which can be compensated by the second allele. Although the 'gene trap' lines displayed no common response to exposure to DMSO or RA the temporal pattern of emergence of haematopoietic progenitors within EBs appeared unchanged suggesting 4 days as a crucial time point for haematopoietic commitment during *in vitro* differentiation. With the exception of DMSO treated Zin40 ES cell aggregates no significant numbers for CFU-A colony formation were detected in gene trap lines treated or untreated prior to day 4 of *in vitro* differentiation (Fig. 2.2). Time course studies of the temporal CFU-A formation pattern of EBs derived from Zin40 cells showed an earlier onset and increase in CFU-A colonies after DMSO treatment. However suppression of haematopoietic commitment in Zin40 EBs was detected after exposure to RA (Fig. 2.2 *D*). The 'gene trap' lines I114 and R68 followed their parental ES cell line R1 in their response to exposure to morphogenic factors with the numbers of CFU-A colonies increasing after exposure to DMSO and inhibition of haematopoietic commitment after exposure to RA (Fig. 2.2 *A,B*). Negative effects on CFU-A colony formation of both morphogenic factors DMSO and RA were observed when ES cell aggregates derived from ST598 cell were exposed to these morphogenic factors. Exposure to DMSO reduced the number of CFU-A colonies and exposure to RA inhibited the formation of CFU-A colonies during differentiation of EBs derived from ST598 cells (Fig. 2.2 *C*). The changes in haematopoietic commitment during ES cell differentiation after exposure to

morphogenic factors detected by CFU-A analyses can most likely be attributed solely to the morphogenic factors DMSO and RA respectively since the ES cell culture system used for these studies was a feeder-free system without supplementation of cytokines or conditioned medium.

Quantitation of the ß-Gal activity and responsiveness to morphogenic factors

Monitoring ß-gal activity of EBs derived from 'gene trap' lines allows the assessment of temporal reporter gene expression during *in vitro* differentiation. However, no information about spatial expression patterns, cell types and numbers of cells expressing active ß-gal fusion proteins can be obtained by this approach. Given the results from CFU-A analyses suggesting that day 4 is a crucial time point for haematopoietic commitment during ES cell differentiation *in vitro* and furthermore that haematopoietic commitment of ES cells can be influenced by exposure to morphogenic factors it was reasoned that ß-gal activity at around day 4 of differentiation after exposure to DMSO and RA respectively will most likely enable *in vitro* pre-screening of gene trap events potentially interesting for early haematopoietic development. Results shown from time course studies (Fig. 3) and from induction studies with DMSO and RA (Fig. 4 and Table 1) are representative data from an individual experiment. All experiments were repeated 3 times, however complete consistency between individual experiments was difficult to achieve since pooled EBs were used. Furthermore possible differences in stability of *lacZ* fusion transcripts and protein can complicate a temporal and quantitative analysis of ß-gal activity.

Temporal reporter gene expression over an 8 day time course in 'gene trap' lines, I114, R68, ST598, Zin40 was assessed using a quantitative ß-gal assay (Fig. 3). Parental ES cell lines (R1 and CGR8) and ES cell line EFC-1 were included as controls for definition of background levels in this ß-gal activity assay. Non significant background levels of ß-gal activity were seen in these controls. All four 'gene trap' lines showed individual patterns of ß-gal activity. Changes over time in ß-gal activity during differentiation were observed in 'gene trap' lines R68 and ST598 where ß-gal activity decreased after initial high activity indicating developmentally regulated expression of the trapped genes. Constitutive ß-gal activity was detected in 'gene trap' lines Zin40 and I114. However ß-gal activity in line Zin40 was found to be significant higher than in line I114, where detected ß-gal activity was only little above background.

Influences of morphogenic factors DMSO and RA respectively on ß-gal activity in 'gene trap' lines are shown in Figure 4 and summarized in Table 1. 'Gene trap' line I114 showed no responsiveness in ß-gal activity to exposure to DMSO or RA. Unresponsiveness to RA was surprising since line I114 was originally identified in an inductive gene trap screen as being induced by RA (43). However this gene trap screen used undifferentiated ES cells and did not allow differentiation of ES cells into EBs. Differences in assay system conditions may also be the reason for the unexpected positive response to exposure to RA of EBs derived from line R68. 'Gene trap' line R68 originally identified as a line whose ß-gal activity is reduced after exposure to RA (43) showed a 3-fold increase in ß-gal activity after exposure of ES cell

aggregates to RA. Temporal induction of ß-gal activity in R68 line derived EBs was observed by DMSO before reaching similar ß-gal activity levels as obtained from EBs not exposed to morphogenic factor. Activity of ß-gal observed in ST598 derived EBs showed complete repression of ß-gal activity after exposure to DMSO and a changed temporal ß-gal activity pattern after exposure to RA. In 'gene trap' line Zin40 high ß-gal activity was observed in control EBs (note extended scale) and was induced after exposure to DMSO and RA respectively however, there was also a change in temporal ß-gal activity after exposure to DMSO.

Temporal high ß-gal activity in a time window at around day 4 of *in vitro* differentiation, induction of ß-gal activity by exposure to DMSO and suppression after exposure to RA in conjunction with appearance of primitive haematopoietic progenitors as defined by CFU-A analysis were thought to be the characteristics of a potential candidate gene involved in early haematopoietic development trapped by a gene trap vector. Although the spatial *in vivo* reporter gene expression patterns of two 'gene trap' lines (I114 and ST598) indicated involvement of their trapped gene during early haematopoietic development non of the 'gene trap' lines showed the anticipated 'ideal' ß-gal activity pattern and responsiveness to DMSO and RA. However, continuing investigations of *in vivo* reporter gene expression pattern of I114 (43) and ST598 (38, Skarnes, W., personal communication) confirmed that both lines are not involved/restricted to haematopoietic development during embryogenesis despite spatial expression in YS and FL.

ß-Gal expression patterns in embryoid bodies derived from 'gene trap' lines.

Spatial *lacZ* expression in EBs derived from 'gene trap' lines was assessed by qualitative X-Gal staining (data not shown). Disperse *lacZ* expression was observed in EBs derived from lines ST598 and R68 respectively. Whereas ES cell aggregates of both 'gene trap' lines showed intense blue staining for active ß-gal 'spread' over the whole ES cell aggregates staining patterns for these lines changed after induction of differentiation by withdrawal of LIF. Almost all day 2 ST598 EBs showed 'global' reporter gene expression however at day 4 of differentiation spatially restricted reporter gene expression was observed with one third of EBs displaying 'global' reporter gene expression, one third showing small spots of restricted reporter gene expression and one third showing no staining for ß-gal. At day 6 and day 8 of differentiation none of the ST598 derived EBs showed obvious staining for ß-gal indicating that *lacZ* reporter gene expression was reduced. At day 2 of differentiation one half of EBs derived from line R68 displayed global reporter gene expression whereas the other half of EBs showed spots of restricted expression. From 4 days of differentiation onwards only EBs with 'global' reporter gene expression showing an increase in intensity on X-Gal staining and unstained EBs were present. In EBs derived from line I114 *lacZ* expression observed was restricted to very few EBs displaying small distinctive spots of blue stain for *lacZ* expression in defined regions of the EB. This expression pattern for *lacZ* was observed throughout the time course. All EBs

derived from Zin40 showed ubiquitous reporter gene expression resulting in intensely stained blue EBs throughout the time course consistent to *in vivo* expression patterns.

Although it was difficult to obtain specific details for spatial and temporal *lacZ* expression since this approach does not allow the identification of cell types and cell numbers expressing active ß-gal fusion protein such microscopical observation of ß-gal expression has confirmed temporal changes in ß-gal activity detected by quantitative ß-gal analysis.

DISCUSSION

In this study an established ES cell system for *in vitro* haematopoiesis was used for the design of an *in vitro* prescreen for gene trap events that may identify genes involved in mesodermal and early haematopoietic commitment. Morphogenic factors such as DMSO and RA respectively have been used as inducing agents to investigate if the opposing effects of these factors on haematopoietic commitment during *in vitro* differentiation of ES cells are effective on ES cell lines with gene trap integrations and therefore may be of use for a directed gene trap screen.

Using ES cells in gene trapping approaches has allowed the establishment of thousands of random reporter gene insertions. However, conventional screening strategies (reviewed in 37) which employ prescreening in chimaeric embryos prior to characterisation of gene trap insertions in transgenic animals are time consuming and technically demanding, restricting the number of clones potentially available for analysis. Thus, the rate limiting step for a gene trap approach is not the isolation and establishment of lines with gene trap insertions but subsequent screening and analysis. Although reporter gene expression in chimaeric embryos directly reflects expression patterns in transgenic animals (33) and is therefore considered to be the most convincing screening strategy to date an *in vitro* prescreening step prior to production of chimaeric embryos would be desirable. In a directed gene trapping screen conventional gene trap constructs are used for integration and established gene trap clones are then screened by induction to a specific factor for the identification of genes that lie downstream of ligand/receptor mediated signalling pathways. In one such directed gene trap screen (39) gene trap integrations were selected *in vitro* on the basis of the response of undifferentiated ES cells to the addition of the morphogen RA. Gene trap integrations responding *in vitro* to the inducer RA have been shown to be significantly enriched for integrations that displayed restricted reporter gene expression *in vivo* (43). Most *in vitro* screening approaches reported to date used undifferentiated ES cells containing gene trap integrations for the selection of clones to be used for further analysis *in vivo*, consequently not including a number of gene trap integrations which are not expressed in undifferentiated cells but are expressed during *in vitro* differentiation. Employing *in vitro* differentiation of ES cells for a screening approach enables gene trap integrations to be included that would not normally be identified in a screen using undifferentiated ES cells alone. Furthermore, such a screen would allow temporal changes of *lacZ* reporter gene expression during *in vitro* differentiation to be monitored and thus provide critical indications for specific developmental processes. The ES cell culture system used in this study has been shown to be an established system for *in vitro* haematopoiesis with a defined temporal pattern of haematopoietic activity within developing EBs (28).

Little is known about correlation of reporter gene expression during *in vitro* differentiation of ES cell lines with gene trap integrations into EBs and their *in vivo* expression patterns. Correlation of *in vitro* and *in vivo* reporter gene expression patterns were addressed in this study by using gene trap integrations previously characterised for their tissue-specific *in vivo*

expression. Interpretation of results was centred on the applicability of ES cell differentiation *in vitro* for large-scale selection of gene trap integrations expressed in haematopoietic lineages. By using the CFU-A assay for the detection of haematopoietic commitment combined with a ß-gal activity assay for monitoring reporter gene expression during *in vitro* differentiation the general applicability for screening of gene trap integrations in haematopoietic lineages was demonstrated. Time course studies investigating haematopoietic commitment of different ES cell lines and 'gene trap' lines during *in vitro* differentiation revealed that all cell lines contain the potential to form CFU-A colonies and that the time point of appearance of primitive haematopoietic progenitors is consistently at around day 4-6 after inducing differentiation by withdrawal of LIF (Fig. 1). The correspondence in the first appearance of CFU-A colonies suggests the presence of primitive haematopoietic progenitors within developing EBs for all cell lines tested. It is likely that these will be capable of long term rescue and multilineage reconstitution of lethally irradiated adult mice as it has been shown for the ES cell line EFC-1 (28). Furthermore, it demonstrates that this culture system enables a variety of ES cell lines to maintain their potential to differentiate into haematopoietic lineages. Induction studies with DMSO and RA showed a common response in the parental ES cell lines: increased *in vitro* haematopoiesis and similar temporal CFU-A patterns after exposure to DMSO and suppression after exposure to RA suggesting a general 'developmental programme' for haematopoiesis in ES cell lines (Fig. 2.1). In contrast, ES cell lines with gene trap integrations responded individually to DMSO and RA, however the general suppressive effect of RA on *in vitro* haematopoiesis was observed in all 'gene trap' lines used (Fig. 2.2). Since the gene trap lines are by definition highly selected sub-clones these differences are more likely caused by clonal variation of the original parents than by the integrations of gene trap constructs per se, although the positional effects of random integrants can not be ruled out. The temporal pattern of the first appearance of primitive haematopoietic progenitors was unchanged in exposed and control EBs further supporting the need for appropriate microenvironment to effect differentiation. Since a feeder free culture system was used and no exogenous cytokines or conditioned medium was added the influencing effects on haematopoietic commitment are most likely be solely attributable to DMSO and RA. Monitoring temporal ß-gal activity during a time course of *in vitro* differentiation and after induction with DMSO and RA respectively was performed by using a simple ß-gal assay. Potentially interesting gene trap integrations for haematopoietic development were expected to show onset of ß-gal activity or increase of low level ß-gal activity at around day 4-6 of differentiation which would coincides with the first appearance of primitive haematopoietic progenitors as defined by CFU-A analysis. Further indication for their involvement in haematopoietic development would be a significant increase in ß-gal activity after exposure to DMSO and suppression after exposure to RA respectively during this time period. Although the gene trap lines used for these studies were selected on the basis of their spatial *in vivo* expression pattern and two lines were thought to be potential positive controls for involvement in haematopoiesis none of the 'gene trap' lines showed the anticipated ß-gal

activity expression pattern for haematopoietic gene trap integrations. In fact both lines (I114 and ST598) which display to YS and FL restricted reporter gene expression *in vivo* have been identified as not being involved in haematopoiesis (38, 43, Skarnes, W., personal communication). Therefore the indication obtained from *in vitro* analysis that gene trap integrations I114 and ST598 are not involved in haematopoietic development are consistent with results from *in vivo* studies. Clearly an identified haematopoietic gene trap integration would be the most convincing proof for the applicability of this *in vitro* screening strategy but the evidence presented in this study gives every reason to believe that this screening strategy will enable us to enrich for gene trap integrations involved in early haematopoietic commitment. Temporal and spatial reporter gene expression can be easily monitored by two simple assays, the ß-gal activity and the X-gal *in situ* staining assay. However, neither assay allows the identification of cells expressing the reporter gene. Although isolation of *lacZ* expressing cells by FACS sorting would probably allow such cells to be identified the EB disaggregation required for such analyses precludes any spatial information. Results from CFU-A analyses and transplantation studies using this ES cell system (28) strongly suggested that interactions between haematopoietic progenitors and an appropriate microenvironment provided by the EB structure is essential for haematopoietic commitment.

Current work involves a preliminary study to explore co-ordinate expression of trapped genes with emerging haematopoietic structures over the time course of EB differentiation. RT-PCR analyses of differentiating EBs (28) have identified haematopoietic candidate genes the expression of which may be localised to well defined areas within the EB. Whole mount *in situ* hybridisation can be used to examine the cell structures surrounding foci of haematopoiesis within the developing EB. Co-localisation of the *lacZ* reporter gene expression with the candidate gene over the time course of EB differentiation will provide a powerful strategy for identication of genes involved in haematopoietic development. Furthermore this approach will also provide the rationale for investigating the interaction between haematopoietic progenitors and their stromal microenvironment. Data presented in this manuscript and preliminary experiments with whole mount *in situ* hybridisation suggest that a large-scale *in vitro* prescreen for gene trap events identifying genes involved in early haematopoiesis is indicated.

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FIGURE LEGENDS

Figure 1: Time course analysis of the emergence of CFU-A cells within differentiating EBs. The number of EBs forming CFU-A colonies is expressed as a percentage of plated EBs (cloning efficiency). Time is expressed as a number of days EBs were allowed to differentiate in suspension culture in the absence of LIF. (A) Comparison of the parental ES cell line CGR8 and derived 'gene trap' lines ST598 and Zin40 with the control ES cell line EFC-1. (B) Comparison of the parental ES cell line R1 and derived 'gene trap' lines I114 and R68 with the control ES cell line EFC-1.

Figure 2.1: Time course analysis of the appearance of primitive haematopoietic progenitors within EBs differentiated from control ES cell line EFC-1, and parental ES cell lines CGR8, and R1 as detected by CFU-A assay after induction for 48 in DMSO or RA. Controls are untreated EBs.

Figure 2.2: Time course analysis of the appearance of primitive haematopoietic progenitors within EBs differentiated from 'gene trap' lines I114, R68, ST598, and Zin40 induction for 48 hours in DMSO or RA. Controls are untreated EBs.

Figure 3: ß-galactosidase activity assay of the control ES cell line (EFC-1), parental ES cell lines (CGR8, R1) and derived 'gene trap' lines (ST598, Zin40, I114, R68). Results are expressed as OD 405nm from standardised amount of protein used for assay. Time is expressed as a number of days EBs were allowed to differentiate in suspension culture in the absence of LIF.

Figure 4: ß-galactosidase activity assay of 'gene trap' lines after induction with DMSO or RA, respectively; I114, R68, ST598, Zin40.

TABLES

Table 1: Summary of the effect of morphogenic factors on ES cell lines and their subclones containing gene trap insertions.

Cell line	*in vivo* Expression	Effect of morphogens on CFU-A profile		Effect of morphogens (ß-gal activity (OD 405n	
		DMSO	RA	DMSO	RA
EFC-1 (1)		+++	---	nr	nr
CGR8 (2)		+++	---	nr	nr
R1 (3)		+++	---	nr	nr
I114	YS, FL	+++	---	=	=
R68	fetal heart	+	---	*	+
ST598	YS, FL	*	-	-	*
Zin40	ubiquitous	*	---	*	+

(1) Control ES cell line; (2) Parental ES cell line for ST598 and Zin40; (3) Parental ES cell line for R68 and I114;

nr, Not relevant; +++ Enhanced; + Enhanced; - Reduced; --- Inhibited; = No effect; * Changed, see text;

TABLE 1

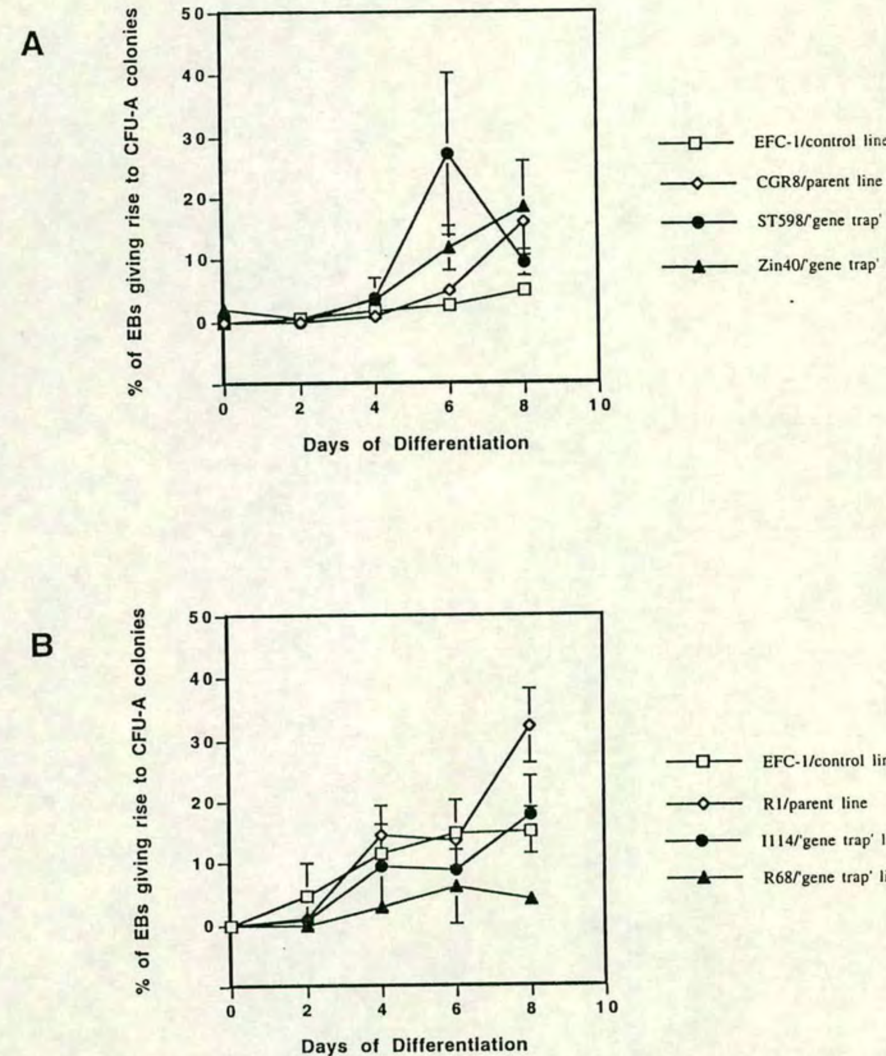
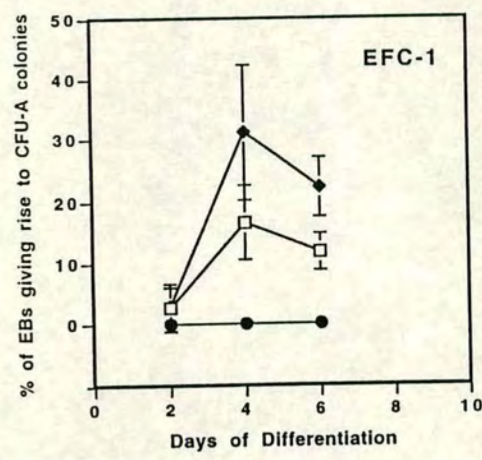
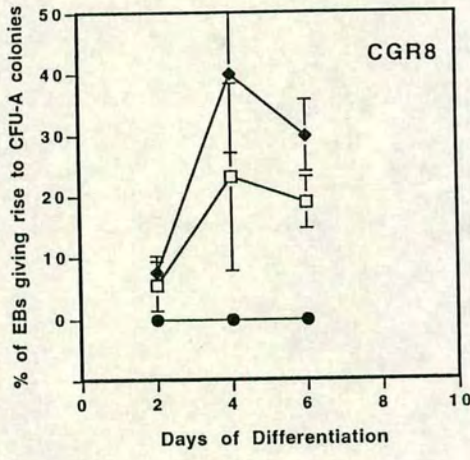


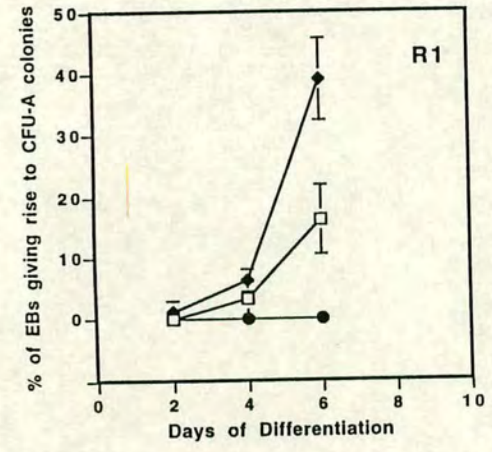
FIGURE: 1



EFC-1/control
EFC-1/DMSO
EFC-1/RA

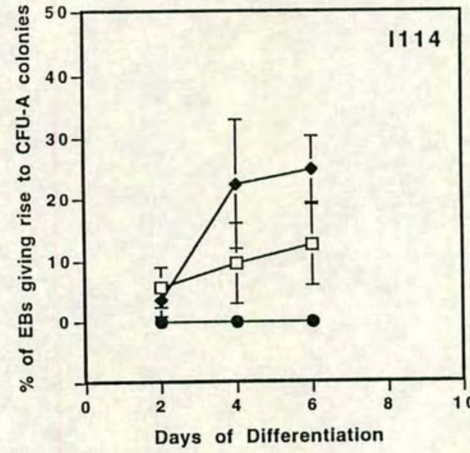


CGR8/control
CGR8/DMSO
CGR8/RA

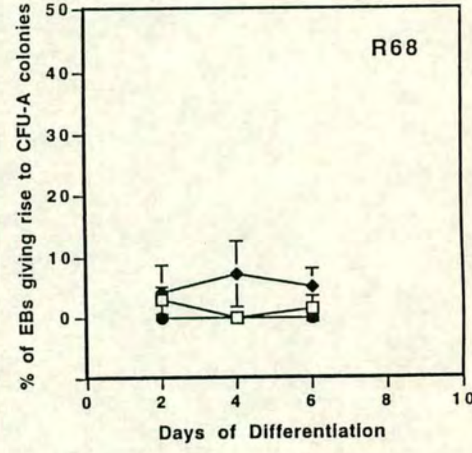


R1/control
R1/DMSO
R1/RA

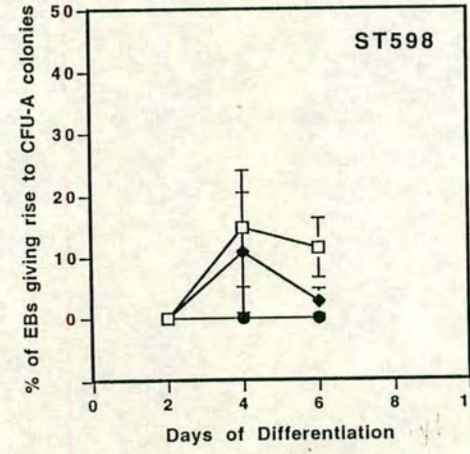
FIGURE: 2.1



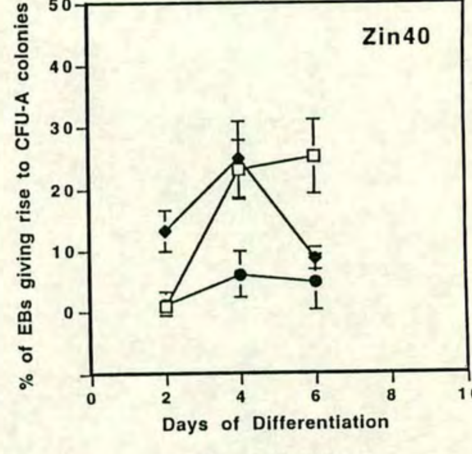
I114/control
I114/DMSO
I114/RA



R68/control
R68/DMSO
R68/RA



ST598/control
ST598/DMSO
ST598/RA



Zin40/control
Zin40/DMSO
Zin40/RA

FIGURE: 2.2

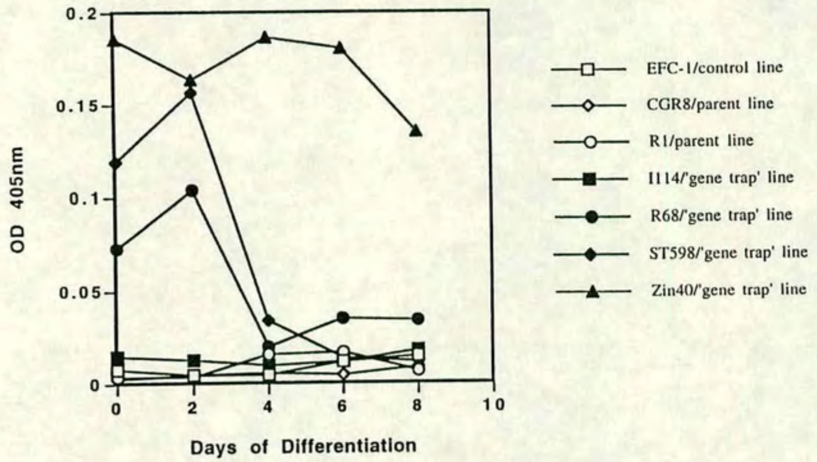


FIGURE: 3

I114

OD 405nm

Days of Differentiation

I114/control

I114/DMSO

I114/RA

R68

OD 405nm

Days of Differentiation

R68/control

R68/DMSO

R68/RA

ST598

OD 405nm

Days of Differentiation

ST598/control

ST598/DMSO

ST598/RA

Zin40

OD 405nm

Days of Differentiation

Zin40/control

Zin40/DMSO

Zin40/RA

Figure: 4

A Limited Temporal Window for the Derivation of Multilineage Repopulating Hematopoietic Progenitors During Embryonal Stem Cell Differentiation In Vitro

By N. Hole, G.J. Graham, U. Menzel, and J.D. Ansell

Embryonal stem cells have been shown to differentiate in vitro into all hematopoietic lineages. This has been used successfully as one approach to the study of genetic events occurring during haematopoiesis. However, studies on the commitment of mesodermal precursors to the hematopoietic lineage have been limited due to the inability to define a system in which embryonal stem (ES) cells will give rise to primitive hematopoietic stem cells in vitro. Using a colony forming assay (CFU-A), we determined that the earliest time point at which primitive multilineage hematopoietic precursors can be detected during ES cell differentiation in vitro in the absence of exogenous conditioned medium or stromal cell culture is 4 days. Lethally irradiated adult recipient mice that received differentiated ES cells from this time point survived for more than 3 weeks; and in two out three experiments, peripheral blood from these animals contained ES-derived progeny. Fluorescence activated cell sorting (FACS) found ES-derived CD45⁺ hematopoietic cells in both lymphoid and myeloid compartments at 12 weeks post-transplantation, suggesting that the population of day 4 differentiated ES cells contains primitive hematopoietic precursors. A preliminary RT-PCR analysis of gene expression around this time point suggests that there are very few hematopoietic cells present. This approach should prove useful in studies of genetic control of commitment to and maintenance of hematopoietic lineages in vitro and in vivo.
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THE HEMATOPOIETIC system is a complex developmental system consisting of lymphoid, myeloid, and erythroid elements, which can be shown experimentally to be derived from a single multipotent stem cell.[1] Cells within this system are regulated by a range of growth factors[2] that control both proliferative and differentiative processes. Little is known of the precise nature of the inter- and intra-cellular signals involved in control of hematopoietic differentiation of the earliest hematopoietic stem cells.[3] These cells remain difficult to analyze because of their low frequency in hematopoietic tissues and their paucity of specific markers. For example, in the mouse long-term repopulating cells exist in the marrow at a frequency of 1 or 2×10^5 cells[4] and are a small fraction of the progenitor population that is defined by the absence of lineage markers and expression of Thy-1 Sca-1 and lectin binding receptors.[5] In the human, putative long-term repopulating stem cells are similarly defined (but not exclusively) by the absence of lineage markers and the specific phenotype, $CD34^+$ $CD38^-$ $HLADr^-$.[6] Highly pluripotent human hematopoietic stem cells (HSC) can be defined biologically by their ability to repopulate bone marrow ablated recipients over the long term, but their presence is only inferred. In mice, groups of stem cells that have self-renewing and more transient repopulation ability can be assayed in vivo by the spleen colony assay or by a variety of equivalent in vitro clonal assays, which rely on addition of various growth factors and cytokines.

The differentiation of hematopoietic stem cells from embryonal stem (ES) cells in vitro may hold the key to the problem of generating sufficient and accessible numbers of highly pluripotent HSC to analyze the genetic control of their differentiation. ES cells are totipotent cells derived from the inner cell mass of 3.5 day murine blastocysts[7] and maintained in their totipotent state in vitro in the presence of the cytokine DIA/LIF.[8] The capacity of ES cells to contribute to all embryologic lineages in the developing mouse[9,10] is mimicked in vitro after removal of DIA/LIF, which stimulates ES cell differentiation into a range of embryonic cell types including those of myogenic, neuronal, and hematopoietic lineages. When ES cells are cultured as aggregates or embryoid bodies (EBs) and then allowed to differentiate, hematopoiesis is routinely observed as islands of hemoglobinized erythroid cells, or blood islands, within the developing EBs.[11] These structures contain progenitor cells capable of giving rise to cell types from myeloid, erythroid, and lymphoid lineages.[12-15] PCR studies have also demonstrated the expression of a range of hematoregulatory cytokines and their receptors within the developing EBs.[15-17] More recently a broad spectrum of mouse hematopoietic differentiation has been achieved by growth of ES cells on a feeder layer derived from bone marrow stroma of the op/op (osteoperotic) mutant.[18] It is reasonable to assume that during EB differentiation, ES cells transit through an HSC phenotype, identification and amplification of which may provide a target for genetic and physiologic analysis and modification. A more precise phenotypic examination of such manipulated stem cells would be made possible by their efficacy in the reconstitution of the hematopoietic system of, for example, sublethally irradiated mice. Several workers have reported ES-derived repopulation in restricted lineages.[14,19] A recent report has suggested that HSC can be isolated from differentiating ES cells.[20] These workers demonstrated multilineage reconstitution at a relatively high level, which appeared to be dependent on stromal cell co-culture of differentiating

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ES cells.[20] However, the use of this co-culture system has two possible problems: identification of the time points critical to HSC commitment was not described, and the use of exogenous cytokines/stromal cell layers may or may not be more likely to perturb the normal differentiation program of the totipotential ES cells. If it were possible to more closely define the time course of hematopoietic commitment and to rely on endogenous ES cell driven differentiation, then this may be a more suitable system for identifying genes and gene products that could regulate the commitment to and differentiation from HSC.

We report here evidence that ES cell differentiation in vitro in the absence of conditioned medium or stromal cell culture can give rise to multilineage repopulating hematopoietic progenitors. Using an in vitro assay for the detection of primitive hematopoietic progenitors (the CFU-A assay) we have characterized the earliest time points at which hematopoietic commitment can be detected. On the basis of this information, a critical and transient time point was identified at which cells capable of multilineage hematopoietic reconstitution of lethally irradiated adult recipient mice were present. Rescue of lethally irradiated mice could be uncoupled from reconstitution by injection of ES-derived progeny from different stages of differentiation. Analysis of gene expression at this time point has identified candidate genes that could be used to further characterize the molecular and cellular events occurring in early hematopoietic commitment.

MATERIALS AND METHODS

ES Cell Culture and Embryoid Body Formation

The ES cell line EFC-1[21] was routinely passaged and maintained in an undifferentiated state as described.[22] All experiments described used EFC-1 cells of less than 30 passages showing no evidence of aneuploidy. EBs were formed as described.[23] In brief, ES cells were cultured in hanging drops (10 μL) at a concentration of 3×10^4 cells/mL in the presence of leukemia inhibitory factor (LIF) for 48 hours in a humidified 5% CO_2 atmosphere. ES cell aggregates were harvested into a petri dish (10^2 aggregates/10 mL ES cell culture medium lacking LIF) and allowed to differentiate for varying periods of time (up to 35 days). Medium was replaced every 2 days.

The CFU-A Assay

The in vitro CFU-A assay was set up as described previously.[24,25] Briefly, a feeder layer consisting of 0.6% agar in α-modified Eagle's medium (α-MEM) with conditioned medium from two cell lines (AF1-19T; a source of granulocyte macrophage colony-stimulating factor [GM-CSF]; and L929: a source of CSF-1) was poured into 3 cm diameter tissue culture grade dishes (1 mL per layer). Embryoid bodies (intact or homogenized) were added to 0.3% agar in α-MEM and added to form an upper layer. The dishes were incubated for 11 days at 37°C in a humidified atmosphere with 5% O_2/10% CO_2. EBs were plated out either intact (50 EB/plate) or following disruption with trypsin (0.05%) in PBS/EDTA (1 mmol/L) for 30 minutes at 37°C (10^4 cells/plate).

Clonal analysis of CFU-A from the bone marrow of transplanted mice was carried out by plating into 96 well dishes at 400 cells/well, using 50 μL/well of feeder and upper layers. Agar concentrations were reduced to 0.3% and 0.15% for the feeder and upper layers, respectively.

The responsiveness of the cells giving rise to hematopoiesis in the CFU-A assay to SCI/MIP-1α, a previously described stem cell inhibitor,[26] was tested by its direct addition to the underlay in the CFU-A assay plates. Inhibition was assessed by the ability of SCI/MIP-1α to block formation of hematopoietic colonies.[27]

Replating Studies

Colonies forming from EBs in the CFU-A assay were picked at day 7 and disaggregated by vigorous pipetting in 100 mL of α-MEM. Single cell suspensions from both EB-derived hematopoietic colonies and intact EBs were replated under the same CFU-A culture conditions. One colony was replated in a single dish and the extent of disaggregation of the colonies assessed microscopically to ensure that no cell clumps could have mistakenly been scored as secondary colonies. Secondary colonies generated in this way were scored at day 11 as macroscopic colonies (>1 mm diameter) and clusters consisting of >50 cells but with a diameter of <1 mm.

Reconstitution of Irradiated Recipient Mice

For reconstitution, EBs were harvested and disaggregated with dispase (1 U/mL; Boehringer Mannheim, Lewes, UK) in phosphate-buffered saline (PBS), washed and injected intravenously (10^6 cells in 0.4 mL PBS; approx 200 EB equivalents) into lethally irradiated (10.5 Gy) female 129/Ola or CBA/129 F1 mice after mixing with a limiting dose of autologous (female) spleen carrier cells (5×10^5). Mice were maintained on Neomycin sulphate (Sigma, Poole, UK) drinking water to control for opportunistic infection. After 3 weeks, retro-orbital blood samples were collected at intervals. Selected samples were sorted into lymphoid and myeloid components on the basis of their forward and right angle scatter and/or their expression of lymphoid and myeloid markers on a Becton Dickinson FACstar Plus fluorescence activated cell sorter as described.[28] The proportion of peripheral blood leukocytes from whole blood and after sorting that were derived from male ES cells was determined by PCR essentially as described,[29] with the exception that ZFY and Myogenin reactions were carried out in separate tubes for 25 cycles and that the PCR temperature profile for myogenin was as follows: 94°C for 5 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. PCR products were run on 2% agarose gels, Southern blotted, and probed with ZFY or myogenin ^{32}P-labeled probes as appropriate. ^{32}P label associated with bands on the blots was quantitated either directly using a phosphoimager (Molecular Dynamics, Chesham, UK) or bands were excised from the blot and label counted on a Packard β-counter. Results are expressed as ^{32}P CPM ZFY/myogenin ratios. In experiment 3, half of the recipient mice were CBA/129 F1 and half 129/Ola. There was no obvious difference between these groups; results shown are pooled from both sets.

PCR

One microgram of total RNA, isolated using RNAzol B (Biotecx), was reverse transcribed using AMV reverse transcriptase (Promega) according to the manufacturer's instructions. Random oligo hexamers (Boehringer) were used as primers. PCR reactions were then carried out essentially as described[16] except for the variations listed in Table 1. PCR products were run on 2% agarose gels and stained with ethidium bromide. Results were assessed on the presence or absence of the appropriately sized PCR products. RNA controls were included to monitor genomic contamination.

RESULTS

Intact EB-Derived Colonies

The ability of intact EBs to form colonies in the CFU-A assay was followed over a 2-week time course (Fig 1). These

Table 1. PCR Primer Pairs

Gene	5' Primer	3' Primer	Anneal Temp (°C)	Product Size
BCL-2	AGAATTCAACCAGACATGCACCTACC	TAAGCTTATGGCGCAAGCCGGGAGAACAG	57	597
CD2	GAGACAATGAGACCATCTGGGG	GAATCTGTCTGCCTGATGGAGC	63	850
Ly-6	CCCCTACCCTGATGGAGTCTGT	GGATTAGAGCACCTACCTACCC	63	450
TNF	TCTGTCCCTTTCACTCACTGGC	GATAGCAAATCGGCTGACGGTG	63	544
TNF-R (p55)	CTCCTCCGCTTGCAAATGTCAC	GGCGGAGTCTTCCCATTTCTGA	63	448
LIF	TTATATGTCGACGAGATGAGATGCAGG	AAAATTGTCGACGCTCCAGTATATAAATC	60	180
CD5	CACAGGAGTGAACCAGAACA	TATAGTCGACGTCCTTGTAAGTACCCAC	50	640
GP130	CTCGTCGACGTCCCTGCAAGAT	GCTGGACAACTGGAAATTCAGG	60	130
MIP1α	CGCCATATGGAGCTGACACCCCGACTGCC	TCAGGCAATCAGTTCCAGGTCAGT	65	299
LIFR	ACGACATCACAGTAGAGC	GCCAGTTCTTCTACGTATCC	60	282

Other primer pairs and conditions are as described elsewhere.[16]

studies showed that CFU-A–like hematopoietic colonies started to appear around days 4 to 6 after removal from DIA/LIF. No substantial hematopoiesis as defined by colony formation was detectable before the 4 day time point and this temporal pattern of emergence of hematopoietic progenitors within the differentiating EBs was precise and reproducible. In these experiments the cloning efficiency as defined by the percentage of plated EBs forming robust CFU-A–like hematopoietic colonies was often as high as 40% to 50% and in subsequent experiments has been noted as being as high as 70% to 80%. Colony size varied but the majority of colonies were macroscopic with a diameter of 2 mm or greater. These large colonies appeared to radiate out from the embryoid bodies and morphologic analysis revealed them to be essentially macrophage in composition, typical of the progenitor-derived colonies detected by this assay.

Undifferentiated ES cells, either as aggregates or as cell suspensions, were unable to form hematopoietic colonies in the CFU-A assay, indicating both the need for differentiation within the EB before CFU-A formation and the inability of the cytokines in the CFU-A assay to induce hematopoietic

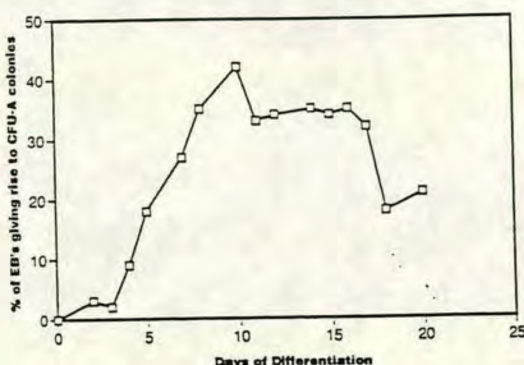

Fig 1. Time course analysis of the emergence of CFU-A cells within differentiating EBs. The number of EBs forming CFU-A colonies is expressed as a percentage of plated EBs (cloning efficiency). Time is expressed as number of days EBs were in suspension culture in the absence of DIA/LIF.

commitment in EBs where there were no pre-existing hematopoietic progenitors. EBs cultured in semisolid agar in the absence of added cytokines failed to produce any hematopoietic colonies. Similarly, normal bone marrow cells could not autonomously produce CFU-A–like colonies.[25]

CFU-A Colony Formation by Isolated EB-Derived Hematopoietic Cells

CFU-A colony formation from intact EBs does not give any qualitative information about the size of colonies produced by individual clonogenic cells within the aggregates, nor does it indicate how many clonogenic cells were present within each EB. In order to address this, EBs were prepared as described above, enzymatically disrupted into single cell suspensions and plated out in the CFU-A assay (Fig 2). Up to day 4 of differentiation, small tight colonies of residual undifferentiated ES cells were detected (data not shown). These colonies never went on to produce hematopoietic cells. Hematopoietic colonies were detected at 4 days of differentiation, a result consistent with data from intact EBs (Fig 1). The diameter of colonies produced in the CFU-A assay is a measure of the multipotency of the clonogenic cell: small (<2 mm) colonies arise from committed progenitors. We found that large (>2 mm) CFU-A colonies were typically detected early on in the differentiation process, although in some experiments, CFU-A colonies could be detected as late as 15 days of differentiation.

The number of colonies produced was relatively small in relation to the number of EBs plated out. When individual EBs were disrupted and plated out in CFU-A assay, those EBs that contained hematopoietic progenitors were shown to possess only one or two such cells (day 4 EB: $n = 1.3 \pm 0.4$; day 5 EB $n = 1.5 \pm 0.5$; day 6 EB $n = 1.6 \pm 0.5$).

EB-Derived Hematopoietic Colonies Are the Progeny of CFU-A Like Cells

To investigate the provenance of the cells within the EBs that were responsible for the development of macroscopic colonies in the CFU-A assay, a number of characteristics of these cells, which are known hallmarks of bone marrow derived CFU-A progenitors, were investigated. Days 4 to 6 EBs were used for these studies.

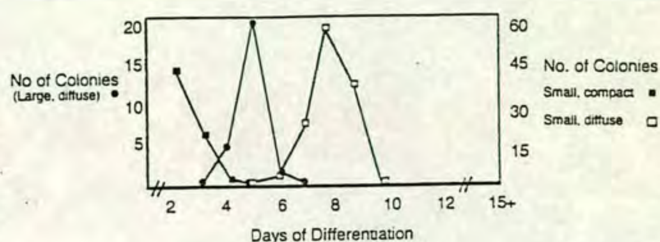

Fig 2. Time course of emergence of clonogenic hematopoietic cells during EB differentiation as detected by CFU-A assay. EBs are disrupted and plated out at 10^6 cells/plate. Results are expressed as number of colonies/plate of ES cell (small, compact) colonies and large and small hematopoietic (diffuse) colonies.

Replating efficiency. The ability of cells within colonies derived from primitive hematopoietic cells to produce secondary colonies on replating is well documented. Results from studies on the replating efficiency of the EB-derived colonies are outlined in Table 2. These results show that the majority (78%) of primary colonies formed from the EBs were capable of forming secondary colonies following replating under CFU-A assay conditions and that 30% to 40% of these secondary colonies were macroscopic in nature and resembled the primary colonies.

Responsiveness to a stem sell specific regulator. Results from a titration of SCI/MIP-1α (a potent inhibitor of stem cell proliferation/differentiation)[26] in the CFU-A assay are shown in Table 3 and indicate that the majority of EB-derived macroscopic colonies (70%) were inhibited from developing in the presence of this negative regulator, with a half maximal inhibition being achieved at approximately 12.5 ng/mL, a value that is identical to that required to inhibit normal bone marrow derived CFU-A stem cells in vitro.[26] There appears to be a resistant subpopulation of EB-derived colonies that presumably represent more mature progenitor cells and do not respond to concentrations of SCI/MIP-1α as high as 100 ng/mL. SCI/MIP-1α has no effect on the growth of nonhematopoietic EBs in the CFU-A assay and appears therefore to be specific for primitive hematopoietic cells within the differentiating EBs.

Repopulation of Lethally Irradiated Mice with ES-Derived Hematopoietic Precursors

Having used the CFU-A assay as a simple detection system to define the time at which EBs contain primitive self-renewing hematopoietic progenitors, we have investigated the ability of EB cells just before this time point (ie, d4 EBs) to reconstitute the hematopoietic system of lethally irradiated adult mice.

EBs were collected following 4 days of suspension culture after removal from DIA/LIF (d4 EB), dissaggregated, and injected intravenously into lethally irradiated (10.5 Gy) female mice after mixing with limiting doses of carrier female spleen cells. ES progeny in the reconstituted mice were detected by a polymerase chain reaction (PCR) based assay for detection of the male ZFY gene (see Materials and Methods). A PCR assay to detect reconstitution has the advantage of speed and the small quantities needed for priming DNA. However, it can be poorly quantitative. Using 25 cycle PCR, we found that increasing the percentage of male cells in a female control sample increased the ZFY/Myo ratio in a broadly proportional manner (Fig 3) and the quantity of priming DNA over a 100-fold range had little effect on this ratio (<10% variation; data not shown). These data demonstrate that this assay provides a semiquantitative measure of small proportions of male ES cells derived in female blood, which is sufficient to follow reconstitution with confidence.

In each of a series of three reconstitution experiments (Table 4) lethally irradiated mice that received both d4 EB cells and carrier spleen cells showed a substantial improvement in post-irradiation survival over those receiving spleen cells alone. Peripheral blood from mice that received d4 EB cells were found to contain ES-derived leukocytes (Table 4 and Fig 4A and B) although in one of the experiments (expt 2), this was restricted to a single time point in one mouse. There was variation in the level of reconstitution between mice and over time within individual mice (Fig 4C) such that interpretation of any trends in level of hematopoietic reconstitution has to be circumspect. However, such reconstitution may have increased from a very low level at 1 month posttransplantation to as much as 20% to 30% of peripheral blood leukocytes of surviving mice at 6 months in experiment 1. In experiment 3, although ES-derived progeny

Table 2. Replating Efficiency of ES-Derived CFU-A Colonies

	Days of EB Differentiation	
	5	6
Colonies plucked	18	18
Original colonies forming 2° colonies	14	14
Cloning efficiency	78%	78%
2° Colonies <1 mm	24	22
2° Colonies >1 mm (CFU-A like)	14	10

Table 3. Sensitivity of ES-Derived CFU-A Colonies to MIP-1α Inhibition

MIP1α (ng/mL)	Percent of Embryoid Bodies Giving Rise to CFU-A Colonies (±SD)
0	24.5 ± 3.5
2.5	15.8 ± 4.6
12.5	14.0 ± 4.0
25	6 ± 2.0
50	7.5 ± 4.5
100	8.2 ± 4.25

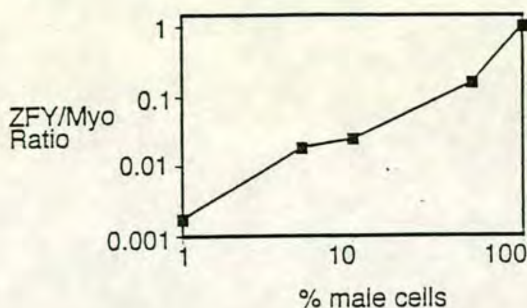

Fig 3. Relationship between the proportion of male cells in priming DNA and ZFY/Myo ratio. PCR products (25 cycle) for ZFY and Myo were run on 2% agarose gels, blotted, and hybridized with ^{32}P-labeled specific probes. Results were expressed as ratios of the signal from male ZFY PCR products to Myo PCR products (loading control). The ZFY/Myo ratio shows a good relationship with the proportion of male cell DNA.

were detected over a 5-month period, the level of repopulation was no more than 5% (Fig 4D). One interpretation of this data could be that ES-derived repopulating progenitors may be analogous to "fetal" HSC in their ability to compete out "adult" type HSC over time.[30] Due to the greater propensity of fetal HSC to remain in quiescence,[31] they can be present in greater numbers in bone marrow than the proportion of their progeny in the periphery would indicate. In order to investigate this possibility, bone marrow from ES transplanted mice was plated out at limiting dose in a 96 well CFU-A assay such that colonies were present in 39% of the wells. When DNA from colonies was tested with ZFY PCR, 6 of 80 (8.5%) were positive for male ZFY. Interestingly, the ZFY/MYO ratio for peripheral blood of that recipient suggested that ES-derived peripheral blood leukocytes were present at a level of 1% to 2% (data not shown).

HSC have the properties of both long-term reconstitution and multilineage repopulation. In order to determine in what lineages ES-derived progeny might be found, peripheral blood from transplanted animals was sorted into lymphoid and granulocyte populations by fluorescence activated cell sorting (FACS). In the first experiment this was on the basis of their forward and right-angle scatter characteristics[28] and the ES cell contribution to these populations was assessed by PCR. Contamination of one population by the other was <5% (data not shown). Peripheral blood from four of six recipient mice at 12 weeks posttransplantation contained both lymphocytes and granulocytes that were ES cell derived (Fig 5). ES cell derived leukocytes were restricted to the lymphoid compartment in two of the animals. The possibilities that male ES-derived, but nonhematopoietic, cells were present in these gated populations or that gross contamination of one population by the other were giving artifactual results was addressed by FACS sorting of peripheral blood from mice in experiment 3. In two animals, sorting was on the basis of cell surface expression of CD45 (expressed on all circulating, nucleated bone marrow-derived cells) and the presence or absence of granulocyte markers (Fig 6). Figure 6C and D shows that sorted cells that were either positive or negative for granulocyte markers were all CD45$^-$ and their forward and side scatter profiles were similar to those for granulocytes (high scattering) or lymphocytes, respectively (Fig 6E and F) with between 98% and 99% purity. ES-derived hematopoietic progeny were detected in both these populations (myeloid and lymphoid lineage, respectively) at 9 weeks posttransplantation (Fig 7).

PCR Analysis

It appeared that some EBs at day 4 of differentiation contained cells that were capable of reconstituting at least two discrete hematopoietic lineages. In order to make a preliminary determination of the genetic events that may control commitment at or around this time point, qualitative PCR analysis was carried out on reverse transcribed mRNA extracted from EBs at varying stages of differentiation (Table 5). Given that our results for the reconstitution of animals differed from those published by Muller and Dzierzak,[19] this RT-PCR analysis would further serve to highlight any differences in the differentiation processes described above and described elsewhere.[16,32] Data in Table 5 show that markers of hematopoietic commitment tended to appear by day 8 of differentiation, with two notable exceptions: Thy-1 and Ly-6 were detected in ES cells and globin: although undetectable at day 2, they began strong and persistent expression by day 4. Expression of several cytokines (IL-3, IL-6, G-CSF, GM-CSF, and M-CSF) was undetectable at the level of analysis adopted, but in contrast, the receptors for at least M-CSF,

Table 4. Transplantation of EB-Derived Cells to Lethally Irradiated Recipient Mice

	Expt 1		Expt 2		Expt 3	
	Transplanted Mice	Control Mice	Transplanted Mice	Control Mice	Transplanted Mice	Control Mice
No. of mice irradiated	16	10	10	10	20	10
No. of mice surviving >3 wk	10	3	8	4	13	2
No. of mice surviving >3 mo	10	3	ND	ND	7	2
No. of mice PCR positive for ES-derived progeny	10	0	1*	0	6	0
Blood FACS sorted?	Yes		No		Yes	

Abbreviation: ND, not determined.

* Transient detection. No PCR signal detected from 5 weeks onward.

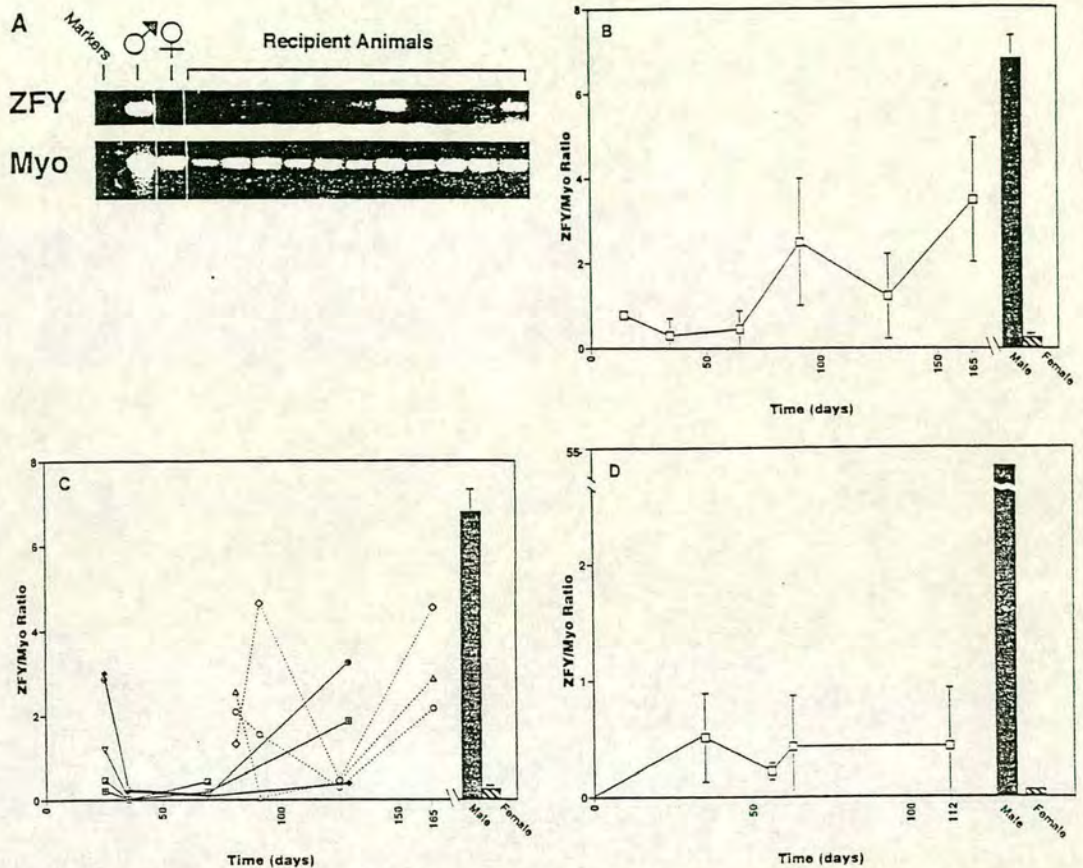

Fig 4. Analysis of the extent of ES-derived hematopoietic reconstitution in day 4 EB recipient mice. (A) PCR analysis of the peripheral blood of day 4 EB recipient mice; ethidium bromide staining of PCR products run on 2% agarose gels. For this example, PCR was carried out for 30 rather than 25 cycles in order to visualize the bands. (B) Time course for PCR analysis of peripheral blood of day 4 EB recipient mice (expt 1). (C) Time course for PCR analysis of peripheral blood of individual day 4 EB recipient mice (expt 1). (D) Time course for PCR analysis of peripheral blood of day 4 EB recipient mice (expt 3).

IL-3, and G-CSF were present. The inhibitory cytokines LIF and MIP-1α showed a reciprocal pattern of expression, with LIF (but not MIP-1α) being expressed in undifferentiated EBs, and early EBs with MIP-1α (but not LIF) only detectable in late (d12) EBs.

DISCUSSION

We have used the CFU-A assay to define the point at which ES cells enter a hematopoietic stem cell like phase in vitro. Our results indicate that between 4 and 6 days after release from the differentiation inhibiting activity of DIA/LIF, EBs contain within them cells capable of forming macroscopic colonies in the CFU-A assay. Forty to sixty percent of differentiating EBs are capable of forming such colonies. Those that do not demonstrate hematopoietic activity in primary CFU-A cultures can be shown to frequently contain hematopoietic colony-forming cells after disruption and replating into secondary CFU-A assays. Intriguingly we have found that once the EBs are disrupted, the component cell's ability to form colonies in primary CFU-A assays is very limited and in general, when colonies were formed they tended to be very small and diffuse. This is especially true of EBs that are disaggregated after 7 or more days of differentiation. These tended to produce smaller hematopoietic colonies in the CFU-A assay more typical of those derived from more committed progenitors, whereas the few colonies produced from d4-d6 EBs were predominantly of the large CFU-A type. More robust colonies tended to be associated with EB fragments that had escaped the homogenization process. Taken together these data suggest that both differentiation inhibiting and hematopoietic stimulating cytokines and/or niches[33] may be essential within the EB for the main-

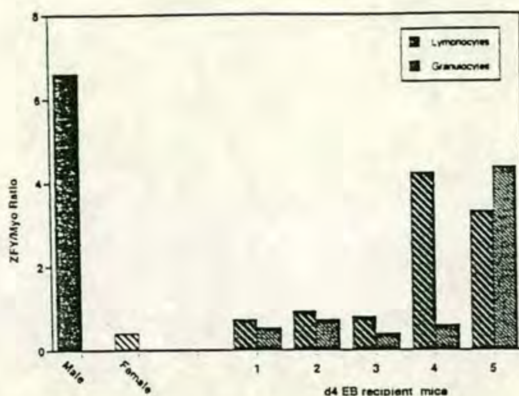

Fig 5. FACS sorting of lymphocytes and granulocytes from peripheral blood of animals from expt 1 on the basis of forward and side scatter properties: PCR analysis of lymphoid and myeloid populations of peripheral blood from experimental animals, FACS sorted on the basis of their forward and side scatter properties. Contamination of one population by the other was <5% (data not shown).

tenance and differentiation of primitive hematopoietic cells. These niches may include endogenous stromal or 'nurse' cell types analogous to those provided exogenously in previous studies that have reported the derivation of multilineage hematopoiesis[18] or HSC[20] from ES cells in vitro. An alternative explanation for this phenomenon is that compaction of the ES cells during EB formation allows the EB to act as a coordinate unit and that disruption of this unit impairs "normal" developmental processes. There is abundant evidence from compaction deficient ES and teratocarcinoma cell line studies that such mutations severely impair normal differentiation.[34]

Our EB replating experiments and the demonstration that the colony-forming elements within EBs are sensitive to the stem cell specific inhibitory cytokine (SCI/MIP-1α) provide further evidence that colonies derived from EBs are relatively primitive self-renewing hematopoietic progenitors with considerable self-renewing potential. They are likely to be analogues of the CFU-A/CFU-S colony-forming cells identified by similar assays of mouse bone marrow.

We reasoned that since the CFU-A/CFU-S cell is likely to have transient engrafting potential[35] and that in our hands CFU-A were typically maximal in EBs by day 5, day 4 EBs were likely to be a source of hematopoietic progenitors that would have long-term repopulating ability. Repopulation studies were therefore concentrated on cells derived from EBs at around this time point, and they show that although control animals largely failed to survive the irradiation, substantial numbers of mice receiving the d4 EB cells were rescued from the lethal effects of the radiation treatment, regardless of the detectable presence or absence of ES-derived progeny. This rescue suggests potentiation of either endogenous stem cells or those present in the carrier hematopoietic cells, normally capable of only transient engraftment.

Few ES cell derived hematopoietic progeny were detected in the peripheral blood for 3 to 6 weeks after repopulation. The reasons why ES-derived hematopoietic reconstitution was not observed in the second experiment were unclear. However, CFU-A analysis of a sample of the embryoid bodies used for this repopulation revealed a paucity of CFU-A compared with other experiments (data not shown). This, along with the observation that the number of hematopoietic precursors within each committed EB is likely to be low (probably between 1 and 2), suggest that too small a number of repopulation-competent cells may have been transplanted. Irradiation survival of the mice in experiment 2, however, was similar to that seen in other experiments, even in the absence of ES-derived reconstitution. Uncoupling of rescue from reconstitution in this way is reinforced by other experiments. Although we report here only data from transplantation of day 4 EBs, cells from day 6 EBs, although showing increased levels of hematopoiesis in vitro (Fig 1), similarly rescued mice from lethal irradiation but failed to contribute to detectable levels of hematopoiesis. The mechanisms underlying these provocative observations are currently under investigation. The initial results examining numbers of clonogenic progenitors in the bone marrow would suggest that peripheral blood reconstitution may not be the most appropriate measure of the numbers of hematopoietic progenitors derived from ES cells in vitro.

These data indicate the presence of long-term repopulating cells within day 4 EBs. Our detection of ES markers in both lymphocytes and granulocytes in peripheral blood further underlines the pluripotent nature of the EB-derived hematopoietic progenitors. Although the absence of clonal markers in these experiments do not rule out independent repopulation with granulocytic progenitors or lymphocytic progenitors, the short half-life of granulocytic precursors coupled with the increase in ES cell contribution over the time course of the experiment make it unlikely that two lineage restricted progenitors were co-injected.

Evidence from RT-PCR analysis of gene expression during EB differentiation suggests that there are no gross differences between the program of hematopoietic differentiation outlined here and that reported elsewhere.[15,16] Markers of lymphoid commitment (CD2, CD5) were detected consistently from day 8 onward. This is supported by additional experiments that detected RAG-1 expression from day 6 onward,[36] and is consistent with the detection of lymphoid cells at later time points of EB differentiation by other investigators. The sharp onset of globin expression at day 5 is remarkable, in part because erythroid commitment as measured by red blood cell formation is detected much later on (>10 days differentiation). This phenomenon has been confirmed by Northern blotting of mRNA (data not shown) and a similar observation has been reported by Schmidt et al.[16] The pattern of expression of these transcripts within embryoid bodies is currently under study by whole mount in situ hybridization. Hematopoietic stem cells have a characteristic surface antigen phenotype, expressing both Thy-1 and Ly-6.[5] However, these antigens are also expressed by a variety of other cell types. In the RT-PCR analysis, Thy-1

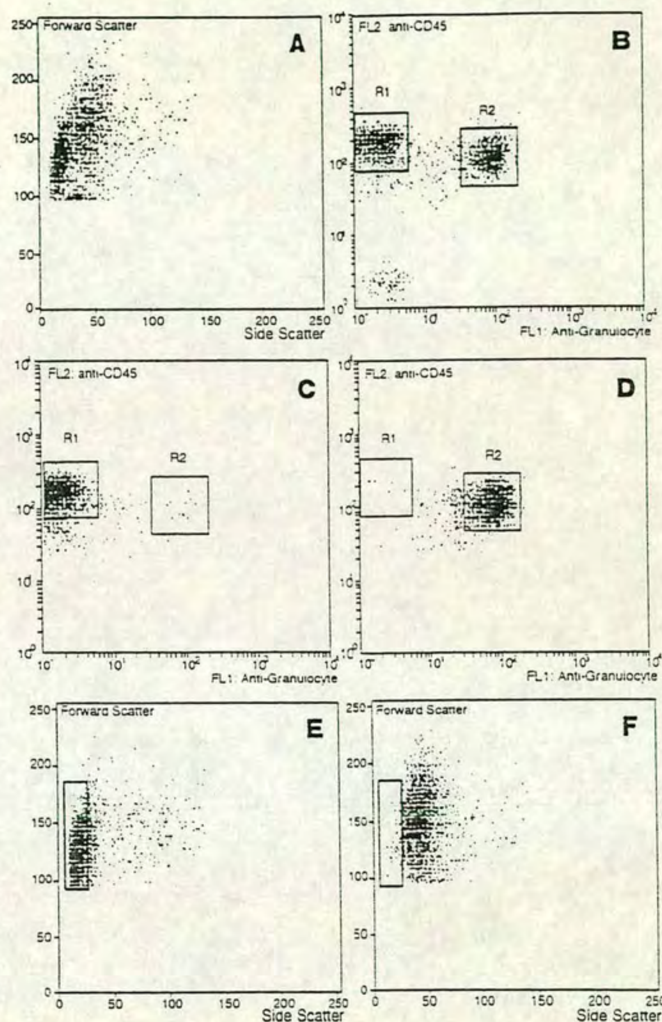

Fig 6. FACS sorting of lymphocytes and granulocytes from peripheral blood of recipient animals on the basis of expression of CD45 and granulocyte markers. (A) Scatter profile of unsorted peripheral blood. (B) CD45/granulocyte marker expression in peripheral blood. (C) Lymphocyte sort. (D) Granulocyte sort. Forward and side scatter properties of (E) lymphocytes and (F) granulocytes.

was expressed throughout the time course of differentiation, a result also seen by Schmidt et al.[16] The relative level of Thy-1 expression diminished from a peak in undifferentiated ES cells to a minimum at day 4, before increasing progressively to day 12, consistent with the clearance of undifferentiated ES cells by day 5 and the progressive appearance of Thy-1+ progeny (such as T cells) from then onward. Ly-6 transcripts were not so readily found, being present in undifferentiated ES cells, but only otherwise detectable at this level of analysis on day 4 of differentiation. The presence of both Thy-1 and Ly-6 transcripts as markers of hematopoietic precursor cells in the EBs remains an interesting possibility. Initial attempts to isolate such cells by FACS sorting of disrupted day 5 embryoid bodies proved unsuccessful (data not shown), probably due in part to the presence of nonhematopoietic Ly-6 or Thy-1+ contaminating cells and the small numbers of clonogenic cells present in each EB.

Both LIF and MIP-1α have been shown to influence maintenance of hematopoietic stem cells,[37,38] and were found in EBs in our program of differentiation. The possibility that these cytokines might influence the time course of hematopoietic commitment and/or numbers of hematopoietic progenitors derived from ES cells in vitro could be addressed by differentiation of double "knock out" ES cells. Given reports that expression of M-CSF can directly influence the hematopoietic commitment of ES cells in vitro,[18] it is interesting that although transcripts for M-CSF receptor were found, transcripts for ligand were undetected, although we cannot exclude the possibility of very low level expression.

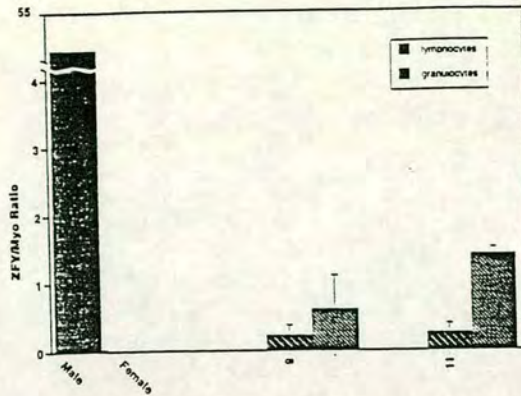

Fig 7. ZFY/Myo ratios of peripheral blood samples taken from recipient mice in expt 3 sorted for expression of CD45 and the presence or absence of granulocyte markers as shown in Fig 8.

In conclusion, therefore, the data presented here suggest that multipotent, long-term, repopulating hematopoietic progenitors may be transient within the EBs and developing at or around day 4 postdifferentiation initiation. This observation may suggest one explanation for the limited lineage reconstitution seen in other studies where ES cells were allowed to differentiate for extended periods before transplantation[14,19] and tends to negate the conclusions of Muller and Dzierzack[19] that ES cell derived hematopoietic precursors are qualitatively different from those found in the adult, having the capacity to generate only the lymphoid lineage in transplanted adult mice. These workers used different ES cell lines to the one described here. Although we have seen reconstitution with other ES cell lines (E14TG2a and ZIN40, data not shown) we cannot exclude the possibility that the differentiation of long-term repopulating cells is cell line and/or passage number dependent. Our work supports the observations of others that have reported the derivation of multilineage repopulating hematopoietic progenitors from ES cells in vitro, with substantial ES-derived reconstitution in a hematopoiesis-defective mouse strain.[20] These workers reported the use of stromal cell lines and conditioned medium to enhance ES-derived hematopoiesis. Our results, along with the work of others, indicate that in the absence of exogenous stromal cells, intact embryoid body formation is a prerequisite for appropriate hematopoietic commitment by ES cells. It is entirely possible that the conditions of differentiation that we have used have allowed the differentiation of appropriate stromal or support cell types within the embryoid body. One approach to confirming this is to use whole mount in situ hybridization to examine the cell structures surrounding foci of hematopoiesis within the developing EB.

By relying on the endogenous differentiation machinery provided by the EB itself, we may have more clearly identi-

Table 5. Gene Expression During EB Differentiation

	Days of EB Differentiation							
	0	2	4	6	8	10	12	Thymus
Gene								
β-actin	+--	+--	--+	---	+-+	---	+--	---
BCL-2	+	=	=	+	+	--	+-	-
Hematopoietic markers								
CD45	-	-	-	-	+	-	+	+
CD2	-	±	-	-	=	=	=	-
CD5	-	-	-	-	-	-	=	+
Ly-6	+	-	±	-	-	-	-	-
Thy-1	+-	++	=	+	+	--	+-	+-
β-globin	-	-	--+	+-+	+++	---	+-+	-
Cytokines								
MIP-1α	-	-	-	-	-	-	±	-
LIF	+	=	-	=	-	-	-	-
TNF-α	-	-	-	=	±	-	=	-
Epo	±	-	-	-	-	=	-	-
SCF	+	=	+	+	++	--	--	-
Cytokine receptors								
c-kit	-	-	-	-	+-	--	+	--
M-CSF-R	-	+	-	+	+	--	+	-
IL-3-R β	-	-	-	+-	+	--	+	-
TNF-R	=	++	++	=	++	+	+-	-
G-CSF-R	-	-	-	-	+	+	+	-
Epo-R	+	+	+	+-	+	--	++	-
LIF-R	+	±	+	=	+	-	+	-
gp130	+	-	+	=	+	+	+	-

Results expressed as a qualitative measure of RT-PCR product as measured by ethidium bromide staining of agarose gels.

fied the time point at which the earliest hematopoietic progenitors may be detected, at the price of relatively poor reconstitution. We are currently exploring the use of defined cytokines as a means for expanding precommitted hematopoietic progenitors, an approach that has proved useful for repopulation with limiting numbers of conventional hematopoietic stem cells.[37]

We have identified a time point during the differentiation of EBs at which long-term multilineage repopulating hematopoietic cells can be detected and we have reported preliminary data on some of the concomitant genetic events. Although repopulation was not observed in all experiments, the identification of this apparently critical time point for hematopoiesis during ES cell differentiation should facilitate further investigation of the associated molecular events and the role of their perturbation on subsequent hematopoietic pathology. Although this work reports data with murine ES cells, the isolation of ES cell types from other species, including primates,[39] should allow investigation of de novo derivation of potentially therapeutic hematopoietic progenitors in vitro.

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Hematopoietic Differentiation of Embryonal Stem Cells *in vitro.*

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Introduction

The hematopoietic system can be considered as a spectrum of differentiation and self renewal with at one extreme terminally differentiated cells such as erythrocytes or macrophages, and at the other, pluripotential hematopoietic stem cells (HSC) , which can give rise to all the different lineages of the hematopoietic system, and due to their capacity for self renewal, persist throughout adult life (Spangrude et al. 1991). Introduction of limiting numbers of these cells into recipients whose own hematopoietic systems have been ablated (by, for example, irradiation) results in long-term reconstitution by the donor cells. These properties of HSC have make them tempting targets for gene manipulation, both for investigating the molecular and cellular biology of genes that are expressed in different lineages of the hematopoietic system and in somatic gene therapy. In the former case, gene manipulation of HSC would have particular benefit where the mutations, if transmitted through the germ line, would result in embryonic lethality. Gene manipulation in HSC has proved difficult, at least in part due to an inadequate understanding of the biology of these cells; addition of genes (transgenesis) is dependent on retroviral infection (Fairbairn and Spooncer 1993). Gene targeting by homologous recombination in HSC has not yet been shown to be practical. In contrast, there is considerable experience in specific and facile manipulation of the mouse genome using embryonal stem (ES) cells, which as totipotential cells, can give rise to all the different cellular lineages in the organism (Evans and Kaufman 1981, Robertson 1986) . In particular, ES cells have proved to be amenable to gene targeting by homologous recombination. Early work demonstrated the appearance of erythrocytes following differentiation of ES cell under specific conditions *in vitro* (Doetschman et al. 1985). Under conventional circumstances, these cells could only have arisen from differentiation of pluripotent hematopoietic precursors, themselves derived from the ES cells. ES cells are competent to produce HSC; using HSC isolated from fetal livers of mice derived in their entirety from ES cells are capable of rescuing and reconstituting secondary recipient mice (Forrester et al. 1991). The question of whether HSC can be derived from ES cell differentiation *in vitro* is currently an active area of research (Muller and Dzierzak 1993, Hole et al. 1994). However, most mature hematopoietic lineages can be detected following *in vitro* differentiation of ES cells (Gutierrez-Ramos and Palacios 1992, Keller et al. 1993, Wiles 1993, Nakano et al. 1994, Chen et al. 1993). As part of our study of the hematopoietic potential of ES cells, we have examined the lymphoid commitment of ES cells differentiated *in vitro*. The ability to isolate T lymphocytes from ES cells *in vitro* may offer an alternative strategy in the study of thymocyte development and positive and negative T cell selection.

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Materials and Methods

ES cell differentiation.

This was carried out essentially as described (Hole and Smith 1994). In brief, ES cells were cultured as hanging drops in the presence of LIF for 48 hours prior to harvesting (day 0 of differentiation) and suspension culture. During this suspension culture, the ES cell aggregates differentiate into cystic structures known as embryoid bodies (Doetschman, et al. 1985).

Flow cytometry

Embryoid bodies of varying periods of differentiation were disaggregated into single cell suspension by digestion with Dispase (1u/ml). Primary rat monoclonal antibody (Pharmingen) binding was detected with either goat anti-rat FITC conjugated antibody (TAGO tissue culture services) or streptavidin-FITC/PE (Sigma) as appropriate. Fluorescence was determined on FACScan flow cytometer (Becton Dickenson).

RT-PCR.

Embryoid bodies from various time points were harvested and washed. mRNA was isolated using RNAzol (Biotecs Lab) and cDNA reverse transcribed by AMV reverse transcriptase (Promega) according to the respective manufacturers

instructions. PCR reactions were as described (Schmidt et al. 1991). In brief, denaturation (95°C), anneal and extension (72°C) times were 20, 60 and 60 seconds respectively for thirty cycles. Anneal temperatures and primers were as noted below.

Gene	5' Primer	3' Primer	Product size	Anneal Temp. (°C)
FLK2	GCTGCTGCTTGTTGTTTTGTCA	TTCATACTCTTCTTGCGAGCTG	1060	62
FLK2 ligand	CTGTTACTTCAGCCACAGTCCC	CTTCTTGCCCTTTGCCAGCGAA	560	62
RAG1	GATGGGAAGTCAAGCGACAAAG	GCAGTTGGAGATCTTCTTCAGG	520	62
Ly-6E	CCCCTACCCTGATGGAGTCTGT	GGATTAGAGCACCTACCTACCC	450	62
CD2	GAGACAATGAGACCATCTGGGG	GAATCTGTGTGCCTGATGGAGC	850	62
β-globin	AACCCTCATCAATGGCCTGTGG	TCAGTGGTACTTGTGGGACAGC	415	62
CD5	CACAGGAGTGAACCAGAACA	TATAGTGGAGGTCCTTGTAAGTAC CAC	640	50
Thy-1	ACAGCCTGCCTGGTGAACCAAA	GGCCCAACCAGTCACAGAGAAA	423	68
Actin	GTGACGAGGCCCAGAGCAAGAG	AGGGGCCGGACTCATCGTACTC	934	62
CD45	GATATGAGTCACTTCTCTTCCA	GATGAAAAGCAGCTGATGGA	520	50

PCR products from both reverse transcribed and mock-transcribed reactions were run on agarose gels and detected by ethidium bromide staining.

Results

Flow cytometry

The expression of surface antigens by single cells liberated from the differentiating embryoid bodies is shown in figure 1a-c. HSC from mice are believed to have a characteristic phenotype, namely Ly-6E^{+ve}, Thy-1^{+ve} (Spangrude, et al. 1991). Expression of these antigens was assessed individually (fig.1a and b) and together (data not shown). Thy-1 was found on undifferentiated ES cells, the numbers of cells expressing the antigen decreasing over the first 5 days of differentiation and apparently increasing thereafter to approximately 30% of embryoid body cells over the time course studied. The numbers of cells expressing both antigens were less than 0.1% of total cells at any time point examined. Thy-1 and Ly6E are not HSC-restricted, and are expressed on other cell types, including T cells. In order to examine the total hematopoietic commitment and T-cell commitment, expression of CD45, CD3 and αβTCR were examined (Figs 1b and 1c). CD45+ve hematopoietic cells increase in number over the time course, from about 1% of cells at day 7 to approximately 7% of cells after 20 days of differentiation. However, a relatively small number of these cells are likely to be T cells; CD3 or αβ TCR +ve cells are first detected around day 10, again increasing over the time course to about 10% of the numbers of CD45+ve hematopoietic population. The small numbers of TCR +ve cells recovered precluded two-colour flow cytometry. However, CD4+ve and CD2+ve cells were also found around the time point when these T cell markers could be detected.

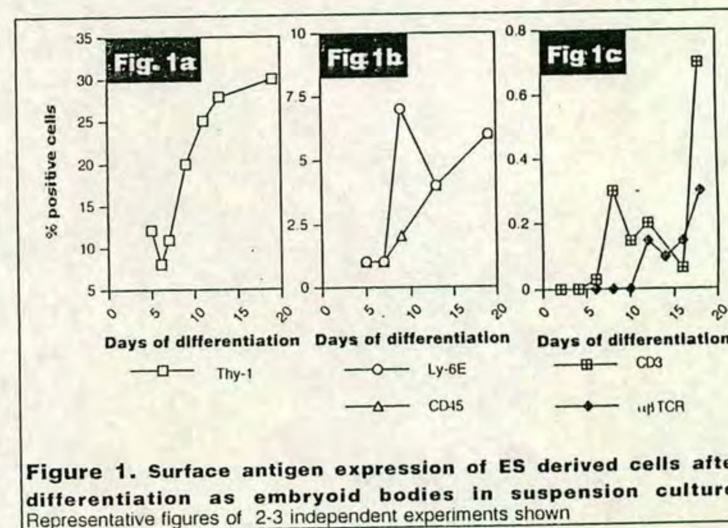

Figure 1. Surface antigen expression of ES derived cells after differentiation as embryoid bodies in suspension culture. Representative figures of 2-3 independent experiments shown

These data suggested that in common with several other studies, we were able to derive lymphoid cells from ES cells by *in vitro* differentiation (in a separate study, B cell development was indicated by detection of IgM+ve, IgD+ve and/or B220+ve cells in differentiation at around 12 days (data not shown). To confirm these results and examine the transcription of developmentally regulated genes, we carried out a selected series of RT-PCR reactions.

RT-PCR

A schematic of the results obtained is shown in table 1. The integrity of the cDNA for PCR was confirmed by a positive result for actin transcripts in all of the samples. In brief, the results support the observation that lymphoblastoid cells are appearing

later in the differentiation pathway. RAG-1 expression was detected as early as day 6 after initiation of differentiation, coincident with detection of CD2 transcripts. CD45 was detected by day 8. Thy-1 and Ly-6E transcripts were detected early in differentiation; in ES cells or day 2 differentiated cells respectively. Of interest was the relatively early appearance of a marker of another hematopoietic lineage, β-globin. However, no red blood cells were observed in embryoid bodies during this series of experiments. Detection of FLK2 ligand transcripts in differentiating ES cells was in contrast to its receptor, whose transcripts were not detected at the level of analysis adopted.

	Days of differentiation						
Transcript	**0**	**2**	**4**	**6**	**8**	**10**	**16**
Rag-1	-	-	-	+	+	+	+
CD2	-	-	-	+	+	-	+
CD5	-	-	-	-	-	-	+
Thy-1	+	+	+	+	+	+	+
CD45	-	-	-	-	+	-	±
β-globin	-	-	-	++	++	++	++
FLK2 ligand	-	+	-	+	-	+	+
FLK2	-	-	-	-	-	-	-
Ly-6E	-	+	+	+	+	+	+
Actin	++	++	++	++	++	++	++

Table 1. RT-PCR detection of mRNA transcripts in differentiating ES cells Results expressed as either absence of detectable PCR product (-) or a subjective assessment of the intensity of the appropriately sized PCR product (+, +, ++).

Discussion

This works suggests that lymphoid commitment of ES cells can be demonstrated *in vitro*. Detectable numbers of cells bearing lymphoblastoid characteristic markers can be detected at or around 10 days of differentiation *in vitro*, albeit at a low level. The presence of markers for CD5, CD2, CD3 all indicate that T cell development is detected. Productive expression of αβTCR is dependent on the presence of the product of RAG-1 gene (Wayne et al. 1994). It is not surprising therefore to find that RAG-1 expression in these differentiating cells precedes the appearance of cells bearing αβTCR (fig1). The numbers of cells expressing the T cell markers Thy-1 and Ly6E are dramatically higher than those expressing the markers listed above. This is indicative of one of the problems in this system; embryoid bodies contain cells of many different lineages, not just hematopoietic. Indeed undifferentiated ES cells contain already contain transcripts for Thy-1. Embryonal stem cells are clearly competent to produce the entire range of hematopoietic lineages; ES-derived fetal livers contain pluripotent repopulating HSC capable of repopulating the entire hematopoietic system of recipient mice (Forrester, et al. 1991). However, inducing ES cells to progress down all hematopoietic lineages *in vitro* has proved more elusive. Myeloid lineage progenitors can be detected following *in vitro* differentiation of ES cells (Wiles 1993), yet reconstitution of the myeloid lineages in lethally irradiated mice by adoptive transfer of ES-derived progeny has proved more elusive. In contrast, in at least one report the lymphoid lineages of mice have been reconstituted with the *in vitro* differentiated progeny of ES cells (Muller and Dzierzak 1993). Part of the problem with this system may the relatively low numbers of hematopoietic cells arising as a result of *in vitro* differentiation. In this study, although percentages varied between experiments, the numbers of αβTCR+ve cells were typically less than 5% at best. One approach to enhance the numbers produced may be to exploit cytokines to enhance hematopoietic development. One cytokine that is thought to play a role in early hematopoietic development is FLK2 ligand (Zeigler et al. 1994). At least in this system, transcripts for FLK2 ligand can be detected within the developing embryoid bodies, allowing the possibly of paracrine regulation of hematopoiesis. However, the failure to detect its receptor at this level of analysis suggests that the numbers of cells that may express the receptor may be very low. An alternative, less well defined system would be to use stromal cell lines to support hematopoietic development. This approach has been used by others (Gutierrez-Ramos and Palacios 1992, Nakano, et al. 1994). The presence of certain cytokines may interfere with hematopoietic differentiation *in vitro*, as has been suggested for M-CSF (Nakano, et al. 1994). Of interest is that transcripts for M-CSF cannot be detected in the early differentiating embryoid bodies in our system (data not shown). The approaches of cytokine enhancement and stromal cell support are currently under study.

The detection of cells expressing productive αβTCR suggests that these cells may be capable of positive and negative selection. The relatively facile nature of transgenesis of ES cells may allow us the opportunity to address issues not just of the control of primitive hematopoietic commitment, but of T cell development. The development of these transgenic cells endogenously in embryoid bodies, *in vitro* in

fetal thymus organ culture and *in vivo* in rescued and heamtopoietically reconstituted mice is currently under study.

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REAGENTS AND SUPPLIERS

Water: Milli-Q purified	Millipore
10x Glasgow's Modified Eagle's Medium	GIBCO-BRL
Sodium bicarbonate	GIBCO-BRL
Non-essential amino acids	GIBCO-BRL
Glutamine/Pyruvate	GIBCO-BRL
2-Mercaptoethanol	Sigma
Fetal calf serum (culture medium)	GlobePharm
Fetal calf serum (differentiation medium)	Advanced Protein Products
Phosphate Buffered Saline tablets	Oxid
EDTA	BDH
Trypsin	GIBCO-BRL
Chicken serum	GIBCO-BRL
Gelatine	Sigma
Tryptose Phosphate Broth	GIBCO-BRL
Cellform^{TM} Polymer	
(poly (2-hydroxyethylmethacrylate))	ICN Biomedicals
Retinoic acid	Sigma
DMSO	Sigma
Ethanol	BDH