Haematopoietic differentiation of embryonic stem cells by aorta-gonad-mesonephros region co-culture

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Declaration

I declare that the work presented in this thesis is my own,
unless otherwise stated

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Abstract

The pluripotential nature of embryonic stem (ES) cells *in vitro* has been used to develop model systems for the differentiation of tissue-specific lineages during organogenesis. The differentiation of ES cells into haematopoietic lineages *in vitro* has been particularly well characterised, but the successful generation of substantial numbers of haematopoietic stem cells (HSCs) able to engraft lethally irradiated mice *in vivo* has been limited. It is possible that the *in vitro* differentiation protocols used to date do not provide a suitable microenvironment for HSC development from ES cells. This thesis describes the effect of co-culturing differentiating ES cells in the presence of embryonic tissue that was predicted to provide a more appropriate microenvironment for haematopoietic commitment.

The aorta-gonad-mesonephros (AGM) region is the earliest site in the embryo proper that is able to generate definitive, adult-type HSCs autonomously. Clusters of HSCs appear in the AGM region at day 10 of gestation in the mouse, where they proliferate before colonising the foetal liver to continue haematopoietic development. Long term repopulating HSC activity has been found to increase within AGM region explant cultures indicating that elements of the supporting microenvironment for definitive HSC expansion can be captured *in vitro*.

In this study an explant culture system was developed to examine the inductive properties of the AGM region on differentiating ES cells. A highly significant increase in the number of primitive haematopoietic progenitors, as measured by *in vitro* colony assays, was observed after co-culture of ES cells with the AGM region from a 10.5 day embryo. However, engraftment *in vivo* of ES-derived progenitors after transplantation was not achieved. The effect of three stromal cell lines derived from the AGM region and foetal liver on the differentiation of ES cells in co-culture was also examined. Although the cell lines were similar to each other in terms of the expression of surface markers, they exhibited diverse effects on the differentiation of ES cells. Preliminary data also suggest that the AGM region-derived factor(s) responsible for the increase in haematopoietic differentiation of ES cells is dependent on direct cell-cell contact.

The AGM region culture system was also used as a novel investigative tool in the analysis of a putative haematopoietic phenotype in a mouse mutant deficient in the

murine homologue of the ubiquitin conjugating enzyme, Ubc7, generated previously by a gene-trapping technique. The numbers of haematopoietic progenitors in AGM regions from wildtype, heterozygous and homozygous embryos after culture were compared, but in this case no measurable haematopoietic defect was observed.

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Chapter One:

Introduction

1.1 Introduction

The establishment of totipotent embryonic stem (ES) cell lines from blastocysts nearly 25 years ago (Evans and Kaufman, 1981; Martin, 1981) made new experimental approaches possible in the study of mammalian developmental biology. ES cells can chimerise with all host tissues including the germ line when introduced into blastocysts and have the capacity to differentiate into a large number of lineages *in vitro*. These properties made it possible to study the function of genes during development by introducing mutations into the genome of ES cells by homologous recombination, which could be transmitted through the germ line to generate mice with specific genetic deletions ("knock-outs"). ES cells can also differentiate into multiple cell types *in vitro* and thus have provided an accessible system to study the commitment of cells to particular lineages. Such an *in vitro* system is the focus of this thesis. The differentiation of ES cells into haematopoietic cells *in vitro* has so far been a powerful tool for studying developmental haematopoiesis but there are also some limitations. For example, only a small number of cells from a particular lineage (and this applies to all tissues) are obtained from a large number of differentiating ES cells and the populations are very heterogenous. Improving the directed differentiation of ES cells is important not only for optimising the experimental system but also if ES cells are ever to be useful for tissue therapy in a clinical setting.

The experiments described in this thesis test whether the differentiation of ES cells into early haematopoietic progenitors can be enhanced by a novel embryonic organ rudiment co-culture system. Therefore, this chapter will:

- Describe the haematopoietic system in adult bone marrow.
- Outline the markers and functional *in vivo* and *in vitro* assays used to identify haematopoietic stem cells and their progeny.
- Summarise the experimental evidence that lead to the current model for the development of the haematopoietic system in the embryo.
- Outline the molecular control of haematopoietic stem cell induction and proliferation.

- Discuss haematopoietic differentiation of embryonic stem cells *in vitro* and the relationship to haematopoiesis *in vivo*.
- Explain the aim of the experiments in this thesis.

The work was performed using the murine system and therefore the studies cited here refer mainly to the mouse.

1.2 Haematopoiesis in the adult and the haematopoietic stem cell (HSC)

1.2.1 Bone marrow haematopoiesis

Adult blood is composed of many distinct mature cells including erythrocytes, platelet producing megakaryocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, natural killer (NK) cells, B- and T-lymphocytes, each with specific functions such as oxygen transport, wound clotting and immune protection. All of these mature cells have a finite life span and are therefore continually being replaced from a population of self-renewing, pluripotent haematopoietic stem cells (HSCs) resident in the bone marrow. This process of haematopoiesis by which the HSCs give rise to terminally differentiated blood cells occurs through a descending hierarchy of progenitor cells with increasing levels of commitment to a specific lineage accompanied by a decreased capacity for self-renewal (**Figure 1.1**).

1.2.2 HSCs in the adult

At the top of the hierarchy two types of stem cells have been broadly defined as long term and short term repopulating. Long term repopulating (LTR) HSCs represent a very small number (approximately 1 per 100,000) of the nucleated cells in adult bone marrow (Micklem et al., 1987), which can generate all the blood cells of an individual for its entire life and self-renew to produce progeny that demonstrate this capacity after transplantation into another individual (Morrison and Weissman, 1994). A single LTR-HSC can proliferate and differentiate to reconstitute the haematopoietic system of a lethally irradiated recipient as demonstrated by retroviral marking studies (Lemischka et al., 1986; Snodgrass and Keller, 1987). The majority

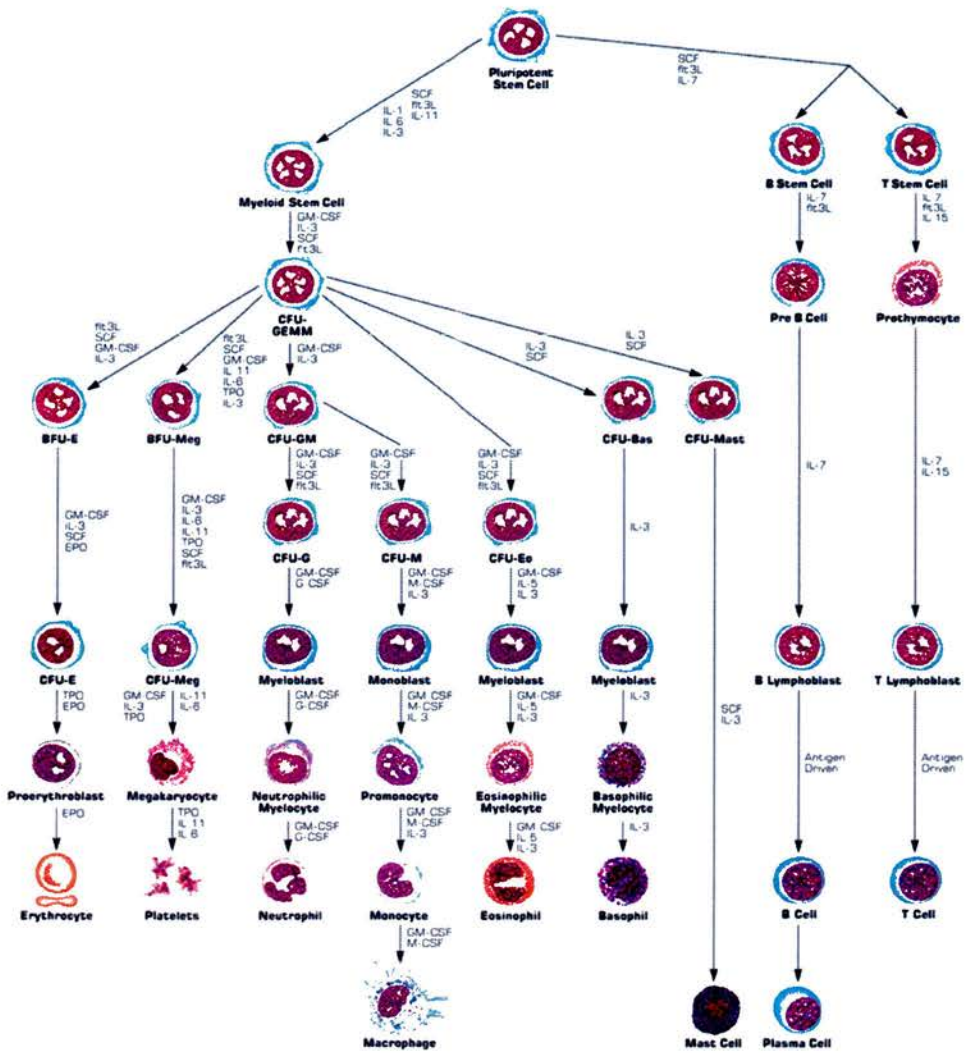


Figure 1.1 The haematopoietic hierarchy. The diagram illustrates all the cell types of the haematopoietic system in the adult. The progenitors have been defined in terms of the cytokine combinations to which they are responsive.

of LTR-HSCs in the bone marrow are quiescent at any one time despite their massive self-renewal capacity (Cheshier et al., 1999; Jordan and Lemischka, 1990). So it is through the expansion of the LTR-HSC progeny, the short term repopulating (STR) HSCs capable only of transient multulineage reconstitution (Morrison et al., 1997), that the large numbers of mature cells in the blood are generated. It is thought that perhaps this mechanism safeguards against mutations in the most potent stem cells, which could arise during DNA replication.

The balance between self-renewal of a HSC and differentiation into the various haematopoietic lineages is crucial to maintain a steady state but must also be flexible to respond to any changes in the environment, such as blood loss through injury or low oxygen at high altitudes (Morrison and Weissman, 1994).

The gold standard assay for LTR-HSCs is their capacity for long term reconstitution of all haematopoietic lineages after transplantation into lethally irradiated adults including secondary and even tertiary recipients. Till and McCulloch (Till and Mc, 1961) identified macroscopic colonies on irradiated hosts' spleens shortly after injection of bone marrow cells. It was originally thought that these "colony forming units in the spleen" (CFU-S) indicated the presence of HSCs, but they have since been found to be multipotent progenitors of erythroid and myeloid lineages with self-renewal potential and are responsible for haematopoietic reconstitution in the short term (Jones et al., 1989). CFU-S progenitors are a heterogenous population and activity is assessed 8-12 days post transplantation with the most primitive progenitors giving rise to colonies at later time points (Hodgson and Bradley, 1984).

Long term reconstitution and the CFU-S are functional assays and only indicate retrospectively the presence of HSCs and progenitors in a given population of cells. With monoclonal antibodies to cell surface antigens and the development of sophisticated fluorescence activated cell sorting (FACS) technology, HSCs have been extensively characterised. While the prospective isolation of pure HSCs is not yet possible, due to the absence of exclusive markers and the heterogenous nature of the stem cell compartment, populations highly enriched for LTR-HSC activity can be

achieved. With no single marker exclusive to HSCs yet identified, the presence and absence of specific combinations of surface antigens are used.

HSCs in the bone marrow can be identified by the expression of c-kit, stem cell antigen-1 (Sca-1), low levels of Thy1.1 and the absence of lineage associated markers (Lin^-) such as B220, Gr1, Mac1, Ter119, NK1.1, CD2, CD3, CD4, CD5 and CD8, i.e. $c\text{-}kit^+Thy1.1^{lo}Lin^-Sca\text{-}1^+$ (KTLS) (Okada et al., 1991; Spangrude et al., 1988). CD34, a cell surface glycoprotein discovered on human bone marrow (Civin et al., 1984) is also associated with murine HSCs and is lost from stem and progenitor cells as they mature (Krause et al., 1994). Much debate surrounds the status of CD34 expression on HSCs (Goodell, 1999). Some studies have identified the $CD34^{-/lo}c\text{-}kit^+Sca\text{-}1^+lin^-$ population as the long term repopulating cells (Osawa et al., 1996) and others have found that mobilised HSCs are $CD34^+$ (Sato et al., 1999), suggesting that these differences in levels of CD34 expression may be related to the stage of stem cell activation.

It must be noted that the markers associated with HSCs are not exclusive to these cells but are also expressed by other non-haematopoietic lineages. For example, CD34 is expressed by vascular endothelial cells (Fina et al., 1990; Young et al., 1995). c-kit is expressed by several cell types including germ cells (Manova et al., 1990), melanocytes (Manova and Bachvarova, 1991), neurons (Motro et al., 1991) and stem cells in the heart (Beltrami et al., 2003) as is Sca-1, which is expressed, for example, on non-haematopoietic stem cells in mammary gland (Welm et al., 2002).

Certain functional properties have also been used to identify and purify HSCs. The resistance of quiescent HSCs to cytotoxic drugs, such as 5-Fluorouracil (5-FU), has been exploited to enrich HSC by elimination of the other more mature cells (Hodgson and Bradley, 1979). The ability of HSCs to exclude fluorescent dyes can also be used in their purification. Levels of Rhodamine-123 (Rho) uptake have been used to separate LTR-HSCs (Rho^{lo}) from more mature short term repopulating progeny ($Rho^{med/hi}$) (Zijlmans et al., 1995). A population of cells termed "side population" (SP) cells, based on their low uptake of Hoechst 33342 as determined by dual wavelength analysis of Hoechst dye fluorescence, are highly enriched for quiescent repopulating cells (Goodell et al., 1996). Low uptake of Hoechst 33342 in

HSCs is thought to be attributed to the ABCG2 transporter that actively effluxes the dye from the cells (Zhou et al., 2001). Dye efflux properties/SP cells have also been found in other stem cell populations such as neural stem cells (NSCs) (Kim and Morshead, 2003), muscle (Jackson et al., 1999), mammary gland stem cells (Welm et al., 2002), spermatogonial stem cells (Lassalle et al., 2004) and in a subpopulation of ES cells (Zhou et al., 2001). Therefore this is not a specific marker of HSCs, but perhaps a marker of "stemness" a concept that has raised much interest recently (Cai et al., 2004).

Over the last few years there have been numerous reports that HSCs can also contribute to other, non-haematopoietic lineages and *vice versa*, but these findings remain somewhat controversial (Grove et al., 2004; Wagers and Weissman, 2004). These studies are bound by technical constraints in the purification and precise characterisation of both donor and target cell types and the absence in many cases of unequivocal proof of function in the target tissue, often because of low levels of engraftment.

1.2.3 Haematopoietic progenitors

Much of what is understood about the haematopoietic hierarchy (**Figure 1.1**) in the adult arose from the culture of progenitors in defined conditions in semisolid medium. Haematopoietic progenitors can, in response to specific cytokines or combinations of cytokines, generate colonies *in vitro*, which consist of the mature cells for which they are a precursor. As differentiation proceeds the precursors (colony forming units/cells; CFU/CFC) are increasingly lineage restricted in terms of the cells generated in the colonies.

The most primitive progenitors measurable by colony assays (see section 1.6.4.1), are multipotent myeloid stem cells with limited self-renewal potential *in vitro*, which correspond to the *in vivo* CFU-S assays and are the colony forming unit-type A (CFU-A) (Pragnell et al., 1988) and the high proliferative potential-colony forming cell (HPP-CFC) (Bradley and Hodgson, 1979). These progenitors have some self-renewal potential *in vitro*, whereas the more mature CFU/CFCs do not.

1.3 Haematopoiesis during embryonic development

Two temporally, spatially and functionally distinct waves of haematopoiesis have been identified during embryonic development. The first wave, referred to as primitive haematopoiesis, occurs in the extraembryonic tissues, is transient and satisfies the developing embryos' immediate requirements for oxygen and perhaps clearance of cell debris. The term "primitive" was originally used to describe the first haematopoietic cells because the morphology of erythrocytes in early mammalian embryos resembled more those found in lower vertebrates (nucleated) than in midgestation embryos or adult animals (enucleated). The second wave, termed definitive or adult type haematopoiesis, occurs mainly at intraembryonic sites and generates the stem cells, which expand in numbers and then differentiate.

1.3.1 Extraembryonic haematopoiesis

The yolk sac consists of adjacent layers of extraembryonic mesoderm and visceral (primitive) endoderm. Aggregates of mesodermal cells form the blood islands during the neural plate stage between E7 and E7.5. From E8.0 to E9.5 the outer cell layer of the hitherto apparently homogenous blood islands differentiate into endothelial cells and the inner cells progressively lose their cell-cell connections and differentiate into erythrocytes (Haar and Ackerman, 1971).

The intimate developmental relationship between endothelial and haematopoietic cells has led to the concept of a common bipotential "haemangioblast" precursor to both lineages, although there is as yet no direct *in vivo* proof. *In vitro* studies with chick embryos (Eichmann et al., 1997) and murine ES cells (Choi et al., 1998) together with the absence of both lineages in the receptor tyrosine kinase, Flk-1, deficient mouse embryos (Shalaby et al., 1997; Shalaby et al., 1995) support the existence of a haemangioblast.

Once the vascular connections between the yolk sac and embryo proper are established by E8.5, the primitive erythroid cells enter the circulation where they continue to divide and differentiate until E13 (Palis and Yoder, 2001). The primitive nucleated erythrocytes express embryonic globins (ζ, βH1, and ε) as opposed to the adult globins (β-major and α) expressed by definitve enucleated erythrocytes (Barker, 1968). It was originally thought that primitive erythroid cells were absent

from E16, but a more recent study shows the presence of enucleated erythrocytes expressing βH1 suggesting that primitive erythroid cells enucleate between E12.5 and E16.5 and continue to circulate until just after birth (Kingsley et al., 2004).

As with the haematopoietic hierarchy in the bone marrow, *in vitro* colony assays have been useful in examining the dynamics and potential of the blood cells produced by the yolk sac. A primitive erythroid colony forming cell (EryP-CFC) was identified which generates colonies of large, nucleated red cells with a distinct timing of differentiation in culture compared with BFU-E and CFU-E (Kennedy et al., 1997; Wong et al., 1986a; Wong et al., 1986b) and express embryonic and adult globins in contrast to BFU-E and CFU-E, which express only adult globins (Palis et al., 1999). The EryP-CFC emerge in the yolk sac at E7, where they expand in numbers peaking at E8.25 and are no longer detected by E9 (Palis et al., 1999).

Primitive myeloid cells also emerge and expand in the yolk sac between E7 and 10 (Palis et al., 1999). Since only cells with a macrophage morphology are typically observed by histology, while multiple myeloid lineages are not, it is through the use of colony assays that the timing of the appearance of myeloid progenitors have been determined. The first and most numerous myeloid cells in the yolk sac are primitive macrophage progenitors (Mac-CFC) (Moore and Metcalf, 1970; Palis et al., 1999). In contrast to mature macrophages found in the tissues of adult animals which are derived from monocytes, yolk sac derived macrophages have an accelerated maturation bypassing the monocyte stage. Mast cell progenitors (Mast-CFC) appear slightly later and a very small number of bipotential granulocyte-macrophage progenitors (GM-CFC) are detected last (Palis et al., 1999). There is also evidence of primitive yolk sac megakaryocytes, which as in the case of the primitive macrophages, show an accelerated maturation and production of platelets compared to their adult counterparts.

Definitive haematopoietic progenitors are also present in the yolk sac (Huang and Auerbach, 1993), with definitive precursors overlapping temporally with the primitive cells described above. Definitive erythroid precursors (BFU-E) arise in the yolk sac at E8.25, increase in numbers until E9.5, enter the circulation and differentiate, indicating that the yolk sac plays a more significant haematopoietic role than merely the contribution of primitive erythrocytes (Palis et al., 1999). In support

of this, HPP-CFCs have been found to be restricted to the yolk sac in the pre-circulation embryo (Palis et al., 2001). HSCs able to engraft and contribute to haematopoiesis in adult recipients are detected at E11 after vascular connections have been established (Muller et al., 1994).

However, multilineage long term repopulation can be achieved from E9-10 yolk sac cells if injected into newborn pups (Yoder and Hiatt, 1997; Yoder et al., 1997b), suggesting that an immature pre-HSC population already exists in the yolk sac at this time and questions the criteria used to define LTR-HSCs. More importantly, LTR-HSC activity in the yolk sac at E12 was found to increase in organ culture showing that the mature yolk sac is capable of expanding HSCs and that they perhaps may also be generated in this tissue (Kumaravelu et al., 2002).

1.3.2 Intraembryonic haematopoiesis

It was initially thought that HSCs originated in the yolk sac blood islands and migrated to the foetal liver and subsequently to the bone marrow (Moore and Metcalf, 1970). However, this idea was challenged by evidence of an intraembryonic haematogenic site after orthotopic embryo grafting experiments in avian and amphibian model systems (Zon, 1995). In interspecies chimeras between chick and quail embryos generated by grafting extraembryonic tissues from one species to the embryo body explants of the other taken before circulation is established, the definitive haematopoiesis was found to be of intraembryonic origin and the extraembryonic yolk sac cells showed no long term self-renewal capacity (Dieterlen-Lievre, 1975). The mesodermal region containing the dorsal aorta was later found to harbour the haematopoietic activity (Cormier and Dieterlen-Lievre, 1988; Dieterlen-Lievre and Martin, 1981). Similarly, grafts between the amphibian (*Rana pipiens*) yolk sac equivalent (ventral blood islands) and the cytologenetically marked intraembryonic mesoderm containing the dorsal aorta, pronephros and liver (dorsal lateral plate), demonstrated that the intraembryonic site is the major source of adult haematopoiesis (Turpen et al., 1981). Haematopoietic cells have since been identified in the floor of the dorsal aorta in *Xenopus* (Ciau-Uitz et al., 2000), chick (Jaffredo et al., 1998) and zebrafish (Burns et al., 2002) embryos.

The analogous intraembryonic region in mammals is the para-aortic splanchnopleura (P-Sp), which develops into the aorta, gonad and mesonephros (AGM) region. The P-Sp/AGM region extends between the fore- and hind limb buds of the mouse embryo. The term P-Sp is used to refer to this tissue at early embryonic stages (E7.5-9.0) when it includes the splanchnic mesoderm (mesoderm associated with the endodermal germ layer), paired dorsal aorta, omphalomesenteric artery and endoderm of the developing gut (Godin et al., 1995; Godin et al., 1993). During organogenesis from E9.0, the urogenital system (pro-, mesonephros and genital ridge) develops next to the dorsal aorta, hence the term AGM region (Medvinsky et al., 1993).

Many studies have examined the haematopoietic activity of the P-Sp/AGM region. CFU-S (day 8 and 11) activity was detected in embryonic AGM region at the 31-33 somite stage (early E10) and at a higher frequency than in yolk sac but was not detected in foetal liver until 38-40 somites (late E10/early E11). The fall in the CFU-S activity of the AGM region at E11 coincides with a sharp increase in the foetal liver, suggesting the migration of haematopoietic progenitors (Medvinsky et al., 1993). Grafting P-Sp from E8.5-9 embryos under the kidney capsule of adult immunodeficient SCID mice resulted in the donor derived B-cell progenitors in the host, showing lymphoid potential of the P-Sp (Godin et al., 1993). Transplantation of AGM region cells from late E10 embryos into irradiated adult recipients gave rise to long term reconstitution of all haematopoietic lineages in serial recipients, demonstrating a full HSC potential. LTR-HSC activity was found in the AGM region before the appearance of stem cell activity in the yolk sac and foetal liver (Muller et al., 1994).

While these experiments suggested that the AGM region was the most potent site of definitive HSC activity prior to the foetal liver, they did not identify the primary source of HSCs due to the possibility of cell migration. Since intricate grafting experiments, as those performed in the non-mammalian vertebrates, were not possible in mammalian embryos, Medvinsky and colleagues (Medvinsky and Dzierzak, 1996) developed an *in vitro* organ culture approach to isolate yolk sac, AGM region and foetal liver thus preventing cell migration. The study showed that at E10 only the AGM region was able to initiate and expand LTR-HSC activity. By

E11 some HSC activity was detected in cultured yolk sac, but at a much lower frequency than cultured in AGM region. This finding is in strong support of autonomous *de novo* generation and expansion of HSCs in the intraembryonic AGM region, which subsequently colonise the foetal liver. Furthermore, Cumano and others (Cumano et al., 1996) showed that cultured E7.5 P-Sp isolated prior to circulation had both lymphoid and myeloid potential *in vitro*, while cultured yolk sac at the same gestational stage had a reduced myeloid and no lymphoid capacity.

While no *in vivo* reconstitution by pre-circulation P-Sp cells had previously been achieved (Cumano et al., 1996; Muller et al., 1994), LTR-HSC activity was found more recently in cultured pre-circulation P-Sp when transplanted into adult Rag2$\gamma c^{-/-}$ (NK cell deficient) mice, but only short term erythromyeloid reconstitution was achieved from cultured yolk sac (Cumano et al., 2001). Mice lacking NK activity were used as recipients based on the rationale that levels of MHC class I expression are low on embryonic cells from before E10.5 and are therefore killed by NK cells (Ozato et al., 1985). These data provide further evidence for the P-Sp/AGM region as the first site of definitive LTR-HSC emergence because the tissues were explanted before circulation is established and before reconstituting activity is detected in these tissues *in vivo*. Of course the possibility that the P-Sp is more suitable for this type of organ culture compared to the yolk sac cannot be ruled out.

In morphological studies clusters of haematopoietic cells have been found along the ventral wall of the dorsal aorta in mouse (Garcia-Porrero et al., 1995; Wood et al., 1997) and human embryos (Tavian et al., 1999) supporting the data provided by transplantation experiments that HSCs emerge at this site.

HSC and progenitor activity peaks in the AGM region at E11-12 (Godin et al., 1999; Kumaravelu et al., 2002; Muller et al., 1994) and then decreases as HSC activity increases in the foetal liver (Ema and Nakauchi, 2000; Kumaravelu et al., 2002). It has often been postulated that HSCs colonise the liver rudiment via the circulation but adult repopulating HSC activity had never been detected in embryonic blood until recently (Kumaravelu et al., 2002). HSC activity in the foetal liver peaks at E16 (Ema and Nakauchi, 2000). HSC seed the spleen at E15 (Godin et al., 1999) and finally the bone marrow around birth.

1.3.3 HSCs in the embryo

The surface phenotype of the HSCs that emerge during ontogeny has been extensively characterised in an attempt to gain an insight into their developmental origin. There are some significant differences in the markers used to discriminate LTR-HSCs in the adult bone marrow (see section 1.2.2) compared to the those expressed by repopulating cells in the embryo. They perhaps relate to or reflect the contrasts between embryonic HSCs and their adult counterparts in properties such as cycling status.

As in the adult bone marrow, all LTR-HSCs in the AGM region and foetal liver express c-kit, but there are also c-kit$^+$ cells (pre-HSCs) in early E10 AGM region that do not yet have the capacity to repopulate (Sanchez et al., 1996). The majority of c-kit$^+$ HSCs from the AGM region and foetal liver are also express CD34 (Sanchez et al., 1996) in contrast to adult bone marrow, which are CD34$^-$ (Osawa et al., 1996). The authors do not quantify the numbers of CD34$^+$c-kit$^+$ HSCs but base their conclusion on the fact that high numbers of CD34$^-$ cells from AGM region and foetal liver did reconstitute a small number of recipients, but also suggest this could be due to the purity of the sort. In addition, E9 yolk sac and P-Sp cells that can repopulate newborn but not adult recipients express CD34 (and c-kit), while the CD34$^-$ population did not have that ability (CD34$^-$ P-Sp cells were not tested) (Yoder et al., 1997a). Nevertheless, this difference in CD34 expression by AGM region, foetal liver and bone marrow HSCs might be related to the cell cycle status in embryonic and adult HSCs. While repopulating cells in the embryo are predominantly in cycle, their adult bone marrow counterparts are mostly quiescent. CD34 expression on bone marrow HSCs has been associated with proliferation (Sato et al., 1999). Recent work has found that bone marrow HSCs in the mouse are CD34$^+$ until 7-10 weeks, after which the numbers are dramatically reduced (Ito et al., 2000).

Sca-1, another marker used to purify HSCs from adult bone marrow is also found on repopulating cells from the foetal liver (Jordan et al., 1995). HSCs from the AGM region, on the other hand, are contained within both the positive and negative cell populations sorted on their surface antigen expression of Sca-1 (de

Bruijn et al., 2002). However, in transgenic mice generated to express a GFP reporter under the control of regulatory elements of the *Ly-6A* gene (which encodes Sca-1), only GFP^+ cells gave rise to long term repopulating activity from the AGM region (de Bruijn et al., 2002). The authors say this indicates that the Sca-1 antigen is expressed at low levels on HSCs from the AGM region since there were eight copies of the GFP transgene compared to the usual two alleles of the *Ly-6A/E* gene. However, it may also reflect the additional post translational modifications for surface membrane expression (Sca-1) compared to a cytoplasmic protein (GFP), or the loss of specific enhancers or repressors in the transgene. No GFP^+ cells were observed in the yolk sacs of the transgenic embryos (de Bruijn et al., 2002) and *in vivo* reconstituting cells from the yolk sac have not been found to express significant levels of Sca-1 surface antigen (Huang and Auerbach, 1993).

Foetal liver LTR-HSCs are highly enriched in the $AA4.1^+$ fraction (Jordan et al., 1995; Jordan et al., 1990). AA4.1 expression can be used to enrich for yolk sac HSCs (Huang and Auerbach, 1993) and has been detected on multipotent progenitors in P-Sp (Godin et al., 1995). In contrast, while AA4.1 is detected on adult bone marrow cells, it is not a marker of the stem cell population in this tissue (Rebel et al., 1996; Trevisan and Iscove, 1995).

Interestingly a macrophage lineage marker used to deplete bone marrow of mature cells, Mac-1, is expressed on all the LTR-HSCs in foetal liver (Morrison et al., 1995). Mac-1 is also detected on cells from the AGM region, but both positive and negative populations contained long term repopulating activity at similar frequencies (Sanchez et al., 1996).

Dye efflux properties differ on embryonic HSCs compared to those in the adult. Foetal liver HSCs appear to retain Rhodamine-123 and are $Sca\text{-}1^+Mac\text{-}1^+Rho^{bright}$ (Rebel et al., 1996). SP cells have been isolated from yolk sac and embryo proper from E7.5, but before circulation is established they show no haematopoietic activity *in vitro* and express endothelial markers (VE-cadherin and Flk-1). From E9.5 some SP cells had begun to express c-kit and acquired haematopoietic potential. SP cells from the yolk sac and embryo proper, especially, had low sensitivity to verapamil compared to bone marrow SP cells, indicative of a difference in the efflux pumps used by the two cell populations and again highlighting the differences

between embryonic and adult HSCs (Nadin et al., 2003). These experiments have not been performed on dissected AGM regions (Dr Karen Hirschi, personal communication).

1.3.4 Model of the origins of haematopoiesis in the embryo

In summary, the experimental evidence to date supports a complex model of haematopoiesis in the embryo. The yolk sac supplies all the early primitive erythrocytes from E7 and then also early definitive erythroid and myeloid progenitors from E8.5. There are no haematopoietic precursors detected in the embryo proper before this time. The progenitors enter the embryo proper via the circulation, which has been established by E8.5. Since little haematopoietic maturation occurs in the yolk sac and precursors are present in the circulation and increasingly in the developing liver rudiment, it seemed likely that cells were being produced in the yolk sac for export to other sites. Therefore the yolk sac provides the developing embryo with primitive functional erythrocytes and definitive progenitors.

The first adult repopulating HSCs emerge in the intraembryonic AGM region between E10 and 12, where they proliferate before colonising the foetal liver, which takes over as the main site of haematopoiesis. HSCs are present in the yolk sac immediately after appearing in the AGM region and there is ongoing controversy as to whether they arise here *de novo* at this stage or are seeded from the AGM region. The main reason for this controversy is that the assay for HSCs is the full reconstitution of haematopoiesis in irradiated adult animals, which relies on the homing of HSCs to the bone marrow. It has been argued that it is perhaps unreasonable to expect embryonic cells to be able to home to a place that does not yet exist in its embryonic origin. This is supported by the engraftment of early yolk sac or P-Sp cells in foetal and newborn environments or in NK cells deficient hosts but not in adult bone marrow. However it appears that HSCs can expand (and are perhaps generated) in yolk sac and colonise the foetal liver between E11 and 13. Expansion and maturation of the HSCs then takes place in the foetal liver before migrating to the spleen and bone marrow around birth.

1.4 Emergence of HSCs in the embryo and the adult stem cell niche

1.4.1 Haematopoietic clusters in the embryonic vessels

Transplantation of subdissected AGM regions lead to the conclusion that LTR-HSCs first emerge in the dorsal aorta and surrounding mesenchyme as well as the vitelline and umbilical arteries of the E11 mouse embryo (de Bruijn et al., 2000). The LTR-HSC activity coincides with the appearance of clusters of presumptive haematopoietic progenitors on the ventral wall of these arteries (Garcia-Porrero et al., 1995), which also appear in other species including humans (Tavian et al., 1999). These clusters express surface antigens and transcription factors that have been shown to be associated with HSCs, such as c-kit (Bernex et al., 1996; North et al., 2002), AA4.1, CD45, Lmo2 (Manaia et al., 2000), CD34 (Garcia-Porrero et al., 1998; Wood et al., 1997), Aml1/Runx1 (North et al., 2002), endomucin (Brachtendorf et al., 2001) and Tie2/TEK (Takakura et al., 1998) (see section 1.3.3 and below).

These presumptive haematopoietic clusters have been documented since early last century in various species and the idea that they arise from the endothelium of the vessels has contributed to the speculation about the existence of a haemangioblast or a haematogenic endothelium. Lineage tracing analyses in chick embryos suggested that the haematopoietic clusters are derived from endothelial cells (Jaffredo et al., 1998) and in mice that circulating haematopoietic cells are generated from endothelial cells in the E10 embryo (Sugiyama et al., 2003). Similarly, candidate cells from the AGM region sorted by flow cytometry for expression of endothelial markers, for example VE-cadherin (Nishikawa et al., 1998a) or Tie2/TEK (Hamaguchi et al., 1999) can generate haematopoietic progeny *in vitro*. In the latter study, single Tie2/TEK$^+$ cells could, very infrequently, give rise to both haematopoietic and endothelial colonies, which the authors point out supports the haemangioblast hypothesis in the AGM region. However the possibility that the HSCs develop from a multipotent mesodermal precursor cannot be ruled out.

More recently, mesodermal precursors have been identified in the mesenchyme beneath the floor of the dorsal aorta. Before E8.5 the tissue underlying

the ventral aorta is endoderm, but as the mesodermal cells accumulate and the splanchopleura becomes the P-Sp/AGM region, GATA-3 is expressed. At E10 a few of the cells beneath the aortic clusters express GATA-3 more strongly, and by E11 the transcript is exclusively detected there. These regions have been defined as the "sub-aortic patches" and are present for the duration of HSC emergence, disappearing when production ceases (Manaia et al., 2000). The precise developmental relationship between the HSCs and the sub-artic patches has not been determined, but it is likely that HSCs are generated from mesodermal precursors, which migrate through the vessels, proliferate and enter the circulation.

1.4.2 Molecular determinants of HSC induction in the embryo

Several transcription factors were discovered to have important roles in haematopoiesis by their identification in chromosomal translocations found in leukaemias. Transgenic mouse models with targeted deletions of these genes have enabled some analyses of their function in haematopoietic commitment. Some of the genes are involved in primitive and definitive haematopoiesis (e.g. SCL/tal, Lmo2), while others are only involved in definitive haematopoiesis (e.g. AML-1/Runx1, c-Myb), highlighting both the similarities and differences in their development. Here, the focus is on genes involved in the initial development of adult-type HSCs.

The disruption of SCL, a basic helix-loop-helix transcription factor, in embryos was found to result in failure of yolk sac erythropoiesis, although the endothelium appeared to form normally, resulting in embryonic death at E9-10.5 (Robb et al., 1995; Shivdasani et al., 1995). To circumvent the embryonic lethality and assess the function of SCL later in development, chimeric mice were made using *Scl*$^{-/-}$ ES cells and no contribution to haematopoietic lineages was found (Porcher et al., 1996). Furthermore, long term repopulation from transplanted bone marrow cells where *Scl* was conditionally deleted demonstrated that SCL is not required for self-renewal or differentiation (except erythroid and megakaryocyte lineages) of HSCs but is necessary for their initial specification (Mikkola et al., 2003).

Similarly, the LIM finger protein LMO2, is essential for yolk sac erythropoiesis and null mutant mice die at about E9-10 (Warren et al., 1994). As was found with SCL, *LMO2*$^{-/-}$ ES cells did not contribute to any haematopoietic

lineages in chimeras (Yamada et al., 1998). Expression of LMO2 is maintained in P-Sp/AGM region during the whole phase of HSC generation and has been postulated to play a part in the early specification of mesodermal precursors to HSCs (Manaia et al., 2000). LMO2 protein directly interacts with SCL in erythroid cells as part of a complex and has been proposed as a bridging molecule, which brings together DNA binding proteins.

AML1/Runx1, which encodes the DNA binding subunit of a core-binding factor (CBF), is required for the establishment of definitive but not primitive haematopoiesis. *Runx1*$^{-/-}$ embryos develop normal blood islands, which generate primitive progenitors that seed the foetal liver rudiment, but die at E11-12.5. Haemorrhage and necrosis of the CNS suggests additional vascular defects (Okuda et al., 1996; Wang et al., 1996a). AML1/Runx1 is expressed in the endothelial cells of the ventral wall of the dorsal aorta before the emergence of the haematopoietic clusters, in cells of the para-aortic mesenchyme and later in CD45^{+} intra-aortic clusters, which are absent in AML1/Runx1 deficient embryos (North et al., 1999; North et al., 2002), suggesting a role in specification of HSCs from the endothelium. *In vitro* culture of wild type P-Sp/AGM region generates both endothelial and haematopoietic cells, but *AML1/Runx1*$^{-/-}$ P-Sp gave rise to only endothelial cells in support of this. Interestingly, haematopoietic potential was rescued by introduction of a retroviral vector containing *AML1/Runx1* cDNA but only in cultures of early (E9.5) P-Sp, (Mukouyama et al., 2000), suggesting that haematogenic precursors are specified independently of AML1/Runx1 at E9.5 but it is required for their maintenance and haematopoietic commitment.

The proto-oncogene, *c-Myb*, encodes a transcription factor required for definitive haematopoiesis, since lack of c-Myb causes death in mice around E15 purportedly from severe haematopoietic defects in the foetal liver (Mucenski et al., 1991), although cultures of *c-Myb*$^{-/-}$ P-Sp/AGM regions were devoid of haematopoietic progenitors, implicating an earlier defect in HSC generation (Mukouyama et al., 1999).

Other transcription factors, such as and GATA-2 are involved in the proliferation of HSCs and early progenitors, rather than their generation (Tsai et al., 1994).

Studies in *Xenopus* embryos have shown that LMO2, SCL, GATA-2 and possibly Xaml (the orthologue of AML1/Runx1) are inducible by BMP-4 (Sadlon et al., 2004). In mammals there is no direct evidence for such induction, but BMP-4 is thought to act as a downstream mediator of Indian hedgehog signalling cascade, which has been shown by *in vitro* explant co-culture assays to be the diffusible factor secreted by visceral endoderm necessary for the development of primitive haematopoiesis in extraembryonic mesoderm (Belaoussoff et al., 1998; Dyer et al., 2001). There is also evidence suggestive of a definitive haematopoiesis-inducing role in the AGM region. In human embryos, when the haematopoietic clusters of the dorsal aorta are forming, BMP-4 is expressed in the underlying mesenchyme (sub-aortic patches) consistent with an anterior-posterior developmental progression (Marshall et al., 2000). Once the clusters disappear, the strong BMP-4 expression is gone. Furthermore, BMP-4 is present in a highly polarised gradient along the dorsal-ventral axis of the AGM region mesoderm, with highest levels in the ventral side, beneath the aortic clusters.

1.4.3 The HSC niche in adult bone marrow

The niche in which HSCs reside has remained relatively uncharacterised compared to those of other adult somatic tissues such as the gut and skin (Alonso and Fuchs, 2003; Marshman et al., 2002). The self-renewal and differentiation of the stem cell is regulated through the interaction with its microenvironment. For the HSC, the microenvironment that provides the structural scaffolding for haematopoiesis is the stromal component of the bone marrow. The non-random spatial distribution of primitive haematopoietic cells and progenitors in the bone marrow has been defined, with the primitive cells (CFU-S) found in the endosteal marrow and more mature cells found approaching the central longitudinal axis of the bone (Gong, 1978); (Lord, 1990). It was postulated that the most primitive HSC was essentially a "fixed tissue cell" because it remained in such close association with other cells (Schofield, 1978). A more recent study has shown that after intravenous injection bone marrow subpopulations localise within the bone marrow according to their phenotype, with the HSC-enriched fractions lodging in the endosteal region, in contrast to the lineage committed fractions, which were found in the central marrow

region. This redistribution occurred within 5-15 hours after transplant (Nilsson et al., 2001).

The close association of and the interactions between HSCs and the cells that line the endosteal surfaces (osteoblasts) has lead to speculations about their role in haematopoiesis (Taichman and Emerson, 1998). Recently, two groups have demonstrated that osteoblasts are a regulatory component of the HSC niche (Calvi et al., 2003; Zhang et al., 2003). Both studies found that by using genetic strategies to increase the number of osteoblasts an increased number of HSCs resulted. One study found evidence to suggest that this increase occurred through Notch activation (Calvi et al., 2003), while the other found evidence that BMP signalling regulated the number of HSCs indirectly by controlling the niche size in terms of the number of supporting osteoblasts (spindle-shaped N-cadherin^{+}CD45^{-}) or SNO cells, which possibly interact through N-cadherin and β-catenin (Zhang et al., 2003). An increase in bone marrow HSCs is observed after conditional deletion of *c-myc*, which has also been postulated may be due to increased adherence to SNO cells via N-cadherin (Anne Wilson, personal communication). Notch1 activation has been previously found to enhance HSC self-renewal and expand the stem cell pool (Stier et al., 2002). Wnt signalling via β-catenin has been shown to have a similar role in promoting stem cell proliferation and may act upstream of Notch. Activated β-catenin or exogenous Wnt3a resulted in expansion of HSCs *in vitro* (Reya et al., 2003; Willert et al., 2003). Interestingly, inducible inactivation of *β-catenin* in bone marrow had no adverse effect on HSC self-renewal or multilineage reconstitution (Cobas et al., 2004), which suggests functional redundancy. Transduction of primary bone marrow cells with *HoxB4* resulted in *ex vivo* expansion of transplantable HSCs (Antonchuk et al., 2002) and enhanced repopulating activity *in vivo* (Thorsteinsdottir et al., 1999). Wnt signalling may also regulate expression of *HoxB4* (Reya et al., 2003).

There have now been several reports on the role played by bone marrow osteoblasts on HSC function. Membrane bound SCF (tm-SCF) may also have a role in the adherence of HSC to osteoblasts. Few donor cells were evident in the endosteal region of Sl/Sl^{d} recipients (lacking tm-SCF) compared to $Sl^{d/+}$ mice following transplant of $Sl^{d/+}$ HSCs. Homing to the bone marrow was not thought to be impaired because equivalent numbers of transplanted HSCs were found in the

central femoral section of the bone marrow up to 15 hours post transplant of cells to $Sl^{d/+}$ or Sl/Sl^{d} mice. These findings were mimicked by neutralising antibodies to c-kit, the receptor for SCF (Driessen et al., 2003). Another study demonstrated that $Tie2^{+}$ HSCs adhere to osteoblasts by interaction with Angiopoietin-1. It was suggested that Tie2/Ang-1 signalling acted to maintain the repopulating activity of HSCs by inhibiting cell division (Arai et al., 2004).

All the data discussed so far highlight the important role the microenvironment plays in the control of HSC development and function, from the first induction during embryogenesis to haematopoiesis in the foetus and adult animal.

1.5 Generating haematopoiesis from embryonic stem cells

Embryonic stem (ES) cell differentiation has been used as an *in vitro* model of haematopoiesis to, for example, identify novel haematopoietic genes (Baird et al., 2001; Stanford et al., 1998).

Since the derivation of human embryonic stem (ES) cells several years ago (Thomson et al., 1998) and their subsequent differentiation *in vitro* down various lineages including haematopoietic cells (Kaufman et al., 2001), much speculation has surrounded the possibility of obtaining transplantable HSCs from differentiating ES cells as an alternative source to bone marrow. The transplantation of HSCs is used to treat certain haematological disorders including leukaemias, anaemias and autoimmune disease. Unfortunately it is often difficult to find suitable donors because of the high level of tissue matching required for a successful transplant. Furthermore although advances have been made in the identification and molecular characterisation of HSCs, it has so far proven difficult to reliably maintain and expand these stem cells in culture for prolonged periods. For these reasons a source of HSCs other than donor bone marrow would be useful. Although there are still limitations and technical and ethical hurdles to overcome, ES-derived HSCs theoretically have several advantages over bone marrow HSCs. ES cells can be expanded indefinitely *in vitro*, so they are potentially an unlimited supply of cells.

Embryonic HSCs are more competitive in repopulating than their adult counterparts (Harrison et al., 1997; Jordan et al., 1995; Rebel et al., 1996) improving engraftment efficiency. ES-derived HSCs would be "younger" than donor bone marrow and aged bone marrow accumulates mutations (Sudo et al., 2000) and is associated with decreased patient survival and increased graft versus host disease (Kollman et al., 2001). Although it is unknown whether these problems would also arise if the ES cells were derived from "reprogrammed" blastocysts generated by nuclear transfer using a mature donor cell.

However, arguably the more interesting reason for acquiring long term repopulating (LTR) HSCs from ES derived cells is for studying the development of mammalian HSCs in an accessible system. For the differentiation of ES cells *in vitro* to be a representative tool to investigate the factors controlling HSC induction, proliferation and differentiation, it is important that HSCs can be produced efficiently and reproducibly and that they can be identified and isolated.

1.5.1 Embryonic stem (ES) cells

Embryonic stem cells were first isolated over twenty years ago from the inner cell mass (ICM) of preimplantation E3.5 mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). Cells of the ICM are pluripotent with the capacity to form all the cells of the embryo proper and although this pluripotency is only transient *in vivo*, ES cell lines can be maintained indefinitely in an undifferentiated state while retaining pluripotency when cultured on a feeder layer of murine embryonic fibroblasts (MEFs) and/or in the presence of leukaemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988).

Pluripotency is demonstrated by their ability to contribute to all the lineages of the embryo proper *in vivo*, including the germ line, when introduced into the early embryo by either injection into blastocysts (Hogan et al., 1994) or aggregation to morulae (Nagy et al., 1990) forming chimaeras with the host cells. Indeed completely ES cell derived embryos can be produced by aggregation with tetraploid 4-cell stage embryos, because the ES cells form the embryo proper and the tetraploid cells contribute to the extraembryonic tissues (Nagy et al., 1990; Nagy et al., 1993).

Since the developmental capacities of the diploid and tetraploid components complement each other the process is known as tetraploid complementation.

Differentiation of ES cells into various cell lineages occurs spontaneously *in vitro* by the withdrawal of LIF. Derivatives of all three germ layers (ectoderm, mesoderm and endoderm) of the embryo develop when teratoma-like structures of semi-organised tissues called embryoid bodies (EBs) are allowed to form (Doetschman et al., 1985). There are several methods used to generate EBs: culture of ES cells either in suspension at high density (Doetschman et al., 1985), in semisolid media containing methylcellulose (Wiles and Keller, 1991) or in "hanging drops" of medium to form more uniform aggregates of cells (Wobus et al., 1991) (see Chapter 2: Materials and Methods, section 2.2.1.3.1). By such methods many cell types have been obtained from differentiating ES cells *in vitro*, including muscle, cardiomyocytes, neuronal, adipocytes, chondrocytes, osteoblasts, endothelial, lung, hepatocytes, pancreatic islet (Rippon and Bishop, 2004) and cells of the haematopoietic lineages (see section below).

1.5.2 Haematopoietic differentiation of ES cells in vitro

It is well established that most mature haematopoietic cells can be obtained from populations of differentiating ES cells, including of the erythroid (Wiles and Keller, 1991), myeloid (Burkert et al., 1991; Fujimoto et al., 2003; Wiles and Keller, 1991) and lymphoid (Nakano et al., 1994; Nakayama et al., 1998; Potocnik et al., 1994) lineages. Haematopoietic development is initiated spontaneously within EBs without the addition of exogenous growth factors or cytokines, except for those present in the FCS (Hole et al., 1996; Keller et al., 1993), most likely because differentiating ES cells express many cytokines and their receptors endogenously (Croizat and Bouhassira, 1999; Hole et al., 1996; Keller et al., 1993; Schmitt et al., 1991).

However, changing the culture conditions, adding specific cytokines or culture on stromal cell lines, can enhance or skew *in vitro* haematopoietic differentiation of ES cells down certain lineages. For example, the addition of SCF, Il-3 and M-CSF to increases the yield of macrophages (Lieschke and Dunn, 1995), reduced oxygen conditions increase and prolong erythroid development (Potocnik et

al., 1994) enhance mesoderm and early haematopoietic/endothelial commitment (Ramirez-Bergeron et al., 2004) and increase the number of early myeloid progenitors (A.K. and H.T., unpublished) in EBs. Perhaps most dramatically is a stromal cell (OP9) based co-culture system that reproducibly induces the differentiation of ES cells (without the prior formation of EBs) through a defined mesoderm stage to first primitive and then more mature haematopoietic lineages including lymphoid cells, which are not frequently observed in EBs (Nakano et al., 1994). The crucial element to this induction system is the use of the OP9 cell line, which was derived from the bone marrow stroma of newborn osteoporotic mice lacking functional M-CSF, because ES cells have a tendency for preferential differentiation into the macrophage lineage. It is generally believed that in the absence of exogenous stromal cells, EB formation is required for significant haematopoietic development during ES cell differentiation *in vitro*.

In a detailed time course analysis of ES cell differentiation, the appearance of haematopoietic lineages within differentiating EBs, as determined by colony assays, responsiveness to cytokines and gene expression, was found to be similar to that observed in the yolk sac (see section 1.3.1) indicating that the early stages of haematopoietic commitment in the EB parallels that found in the embryo (Keller et al., 1993) making a suitable *in vitro* model system of haematopoietic development. In this study no haematopoietic progenitors were detected before day 3 of differentiation. Primitive erythroid progenitors (EryP) appeared transiently from day 3.5 EBs, increased in numbers dramatically and were undetected by day 10. Definitive precursors emerge shortly after primitive precursors in the EBs, starting with erythroid progenitors (EryD) from day 5, macrophage precursors from day 6 and mast cell progenitors from day 10. Bipotential (GM-CFC) and multipotential (CFC-mix) were also detected within differentiating EBs. It is unclear whether EryP and EryD in the embryo are derived from a common precursor (Wong et al., 1986a; Wong et al., 1986b), but studies using OP9 induction show ES derived EryP and EryD appear in two distinct waves and that they have different growth factor requirements. These data taken together with limiting dilution analysis provide evidence for distinct precursors for EryP and EryD in developing EBs (Nakano et al., 1996).

The model system provided by ES cell differentiation has also been used to investigate the development of pre-haematopoietic precursors. *In vitro* colony assays in combination with gene targeting studies have lead to a possible model of early haematopoietic commitment.

A common bipotential precursor to the haematopoietic and endothelial lineages (the haemangioblast) has been identified during ES cell differentiation by Gordon Keller and his colleagues (Choi et al., 1998; Kennedy et al., 1997). Blast-colony forming cells (BL-CFC), which give rise to blast colonies in response to VEGF when replated in methylcellulose cultures, emerge from EBs before the appearance of haematopoietic progenitors. The blast cells within the colonies expressed genes common to both haematopoietic and endothelial lineages, such as *Scl*, *CD34* and *Flk-1*. More importantly, they contain cells with both endothelial and haematopoietic (primitive and definitive) progenitors as demonstrated by replating in medium containing specific growth factors. These data suggest that the BL-CFC is the *in vitro* equivalent to the putative haemangioblast and provide the strongest evidence to date in support of such a bipotential precursor. Another study demonstrated that EB derived Flk-1$^+$ cells contained precursors with both haematopoietic and endothelial potential (Nishikawa et al., 1998b). BL-CFC have since been shown to be Flk-1$^+$ (Faloon et al., 2000), which is not surprising since they have been shown to grow in response to VEGF (Kennedy et al., 1997), although not all Flk-1$^+$ cells are BL-CFC (Faloon et al., 2000). It is likely that the progenitors identified by Nishikawa *et al,* and the BL-CFC are identical on the basis of their *in vitro* potential and the expression of *Flk-1*.

Two studies investigating the genetic regulation of BL-CFC commitment by *Flk-1* and *Scl* during EB differentiation identified a more primitive cell type emerging one day before BL-CFCs, which gave rise to so-called "transitional" (Robertson et al., 2000) or "blast-like" (Faloon et al., 2000) colonies. The co-expression of the early mesodermal marker, *Brachyury*, and markers for haematopoietic and endothelial lineages in these "transitional" colonies, and the generation of blast colonies on replating, suggested that the "transitional" colony forming cell (Trans-CFC) represents an earlier mesodermal developmental stage than the BL-CFC (Robertson et al., 2000).

The role of SCL in haematopoiesis during mouse development has to some extent been determined by targeted deletion (see section 1.4), but due to its early embryonic lethal phenotype, the *in vitro* model for haematopoiesis provided by ES cells has provided further insight into its function. SCL is required for the formation of haematopoietic lineages *in vitro* and in chimeras (Porcher et al., 1996; Robb et al., 1995). *Scl*$^{-/-}$ EBs do not express haematopoietic-specific genes, but do express genes that are associated with mesoderm or co-expressed by haematopoietic and endothelial lineages (Elefanty et al., 1997). In addition SCL-deficient EBs do not contain BL-CFC, but can generate "transitional" colonies (Faloon et al., 2000; Robertson et al., 2000). Taken together, these data provide support for a model in which SCL is essential for haematopoietic commitment at the putative haemangioblast stage.

Flk-1, a receptor for VEGF, is involved in haematopoietic and endothelial development during embryogenesis. Mice deficient in Flk1 do not develop blood vessels or yolk sac blood islands (Shalaby et al., 1995), which is also illustrated by the failure of Flk-1 deficient ES cells to contribute to vessels or haematopoiesis in chimeras (Shalaby et al., 1997). *In vitro Flk-1*$^{-/-}$ EBs show a significant reduction in the frequency of BL-CFC (Schuh et al., 1999) but are able to differentiate into haematopoietic and endothelial progenitors (Hidaka et al., 1999; Schuh et al., 1999). This supports the hypothesis that haematopoietic and endothelial progenitors can arise independently of Flk-1, and the phenotype of Flk-1 deficient mice is due to defective expansion and migration of those progenitors.

These and other studies using ES cells (Chung et al., 2002) have contributed to a model of early haematopoietic development whereby Flk-1^{+}SCL^{-} cells are mesodermal precursors (Trans-CFC) to the putative haemangioblast, which coexpress Flk-1 and SCL. As haematopoietic and endothelial commitment proceeds, Flk-1^{+}SCL^{+} cells eventually become positive for either SCL or Flk-1, respectively. Since Flk-1^{+} endothelial cells emerge in the absence of SCL some endothelial cells are thought to develop directly from Flk-1^{+} mesoderm bypassing the haemangioblast stage.

Runx1 is required for definitive haematopoiesis *in vivo* (see section 1.4) and a similar block in definitive haematopoietic potential has also been observed in mutant

Runx1$^{-/-}$ EBs (Miller et al., 2001; Okuda et al., 1996). More recently, by ES cell differentiation *in vitro* a role in early definitive haematopoietic commitment at the haemangioblast level was defined (Lacaud et al., 2002). This study showed that *Runx1* is expressed by EB-derived BL-CFC but not by Trans-CFC. Furthermore, Runx1 deficient EBs showed a dramatic reduction in BL-CFC, which on replating only gave rise to a endothelial cells and a few colonies of primitive erythroid cells but no definitive lineages, demonstrating that Runx1 is essential for definitive haematopoiesis acting at the putative haemangioblast stage. Interestingly *Runx1* haploinsufficiency results in accelerated commitment to mesodermal, haemangioblast and haematopoietic development in ES cells (Lacaud et al., 2004) as observed in the AGM region *in vivo* (Cai et al., 2000).

BMP-4 is essential for haematopoietic differentiation of ES cells in chemically defined serum free medium (Johansson and Wiles, 1995) and has more recently been found to be essential for ES cell self-renewal (Ying et al., 2003). Interestingly, the self-renewal capacity of early haematopoietic progenitors (CFU) derived from human ES cells was supported by BMP-4, independently of cytokines, as quantified by the number of secondary colonies generated after replating the primary CFC colonies (Chadwick et al., 2003).

Some of the factors implicated to have a role in HSC self-renewal in the bone marrow niche have been found to effect the haematopoietic differentiation of EBs. Transfection of ES cells with retroviral vectors expressing Wnt3 and Wnt5a resulted in a 2-fold increase in the proportion of EBs commited to a haematopoietic fate (Lako et al., 2001). Retroviral overexpression of *HoxB4* resulted in an increase in definitive, but not primitive (as determined by colony assays and gene expression) haematopoietic differentiation of ES cells *in vivo* (Helgason et al., 1996). Overexpression of HoxB4 has since been shown to have a much more dramatic effect on the development of HSCs in EBs (see section 1.5.3).

1.5.3 Deriving transplantable HSCs from ES cells

ES cells have the capacity to differentiate into all haematopoietic lineages *in vivo*, as demonstrated by their contribution to these tissues in chimaeras. ES cells must also be able to produce all the cells of the haematopoietic niche which support

HSC development, since foetal liver HSCs isolated from embryos derived entirely from ES cells generated by tetraploid complementation can reconstitute lethally irradiated recipients (Forrester et al., 1991). Interestingly, uneven contribution to haematopoietic development in chimeras by donor ES cells has been reported (Berger et al., 1995). This study showed a bias towards ES-derived B-lymphocytes, while ES-derived T-lymphocytes in the spleen were underrepresented, indicating that haematopoietic commitment of differentiated ES cells may be different from host ICM cells. Nevertheless progeny of ES cells show LTR-HSC potential in chimeric mice after differentiation *in vivo*.

Unfortunately there has been limited success in long term haematopoietic reconstitution by ES-derived cells after differentiation *in vitro*. Direct injection of undifferentiated ES cells leads to the formation of tumours (Muller and Dzierzak, 1993), so prior *in vitro* differentiation is essential. Most transplantation studies report low levels of engraftment and contribution restricted to certain lineages. The following section outlines most of the reported data on the long term repopulating ability of *in vitro* differentiated ES cell progeny.

Muller and Dzierzak (Muller and Dzierzak, 1993) injected cells intravenously (i.v.) from differentiated EBs ($0.8x10^5$-$3.6x10^6$ Percoll fractionated or unfractionated cells per animal) into newborn immunodeficient SCID or W^v/ W^v anaemic, mast cell deficient mice. They found 0.1-4% of the nucleated cells in the peripheral blood to be donor ES-derived after 6.5 months, but only of the lymphoid lineages. B-cells were demonstrated to be functional by the presence of circulating antibodies in the peripheral blood of SCID recipients. Macrophages and mast cells were reportedly absent. All days of EBs (between 5 and 22 days of differentiation) produced repopulating activity, but the greatest proportion of mice repopulated with EB cells were those given day 11-13 EBs, a later time point than perhaps expected from previous *in vitro* data (see section 1.5.2). No short term reconstitution ($CFU\text{-}S_{10}$) was achieved in this study. However, it is interesting to note that a small increase in CFU-S compared to the irradiation controls was observed, but the colonies were not donor ES derived.

Guterrez-Ramos and Palacios (Gutierrez-Ramos and Palacios, 1992) also reported B- and T-lymphoid repopulation 10-16 weeks after injection of

differentiated ES cells ($2x10^5$-$1x10^6$ mononuclear cells per mouse) i.v., or directly into the liver, spleen or peritoneal cavity of sublethally irradiated SCID or normal mice. The ES cells were differentiated for 25 days on a bone marrow stromal cell line (RP.0.10) in the presence of cytokines (IL-3, 6 and 7) and were found to include cells that expressed early lymphoid markers but only a few with myeloid markers. Palacios and colleagues (Palacios et al., 1995) subsequently reported ES derived myeloid, erythroid and lymphoid progenitors in the bone marrow of sublethally irradiated recipient SCID mice 12-18 weeks after transplantation of $4\text{-}6x10^4$ sorted PgP-1$(CD44)^+Lin^-$ ES-derived cells. Haematopoieitc reconstitution was also achieved when bone marrow from primary recipients was transplanted into secondary SCID recipients to demonstrate the presence of long term repopulating activity. This study showed a promising result with high donor contribution. A similar differentiation regime to their previous study was used here, with the exception of a replating step onto fresh stromal cells after 21 days for a further 6-8 days and the addition of supernatant from a foetal liver stromal cell line (FLS4.1) containing FLT3 ligand, steel factor and purportedly a mystery cytokine which supports the proliferation of HSCs.

Another group injected 10^4 sorted $AA4.1^+B220^+$ or $AA4.1^+B220^-$ cells from day 15 EBs i.v. into sublethally irradiated *Rag-1*$^{-/-}$ recipients (Potocnik et al., 1997). Transplantation of $AA4.1^+B220^+$ EB cells resulted in a single transient wave of B cells and no T cells. On the other hand, $AA4.1^+B220^-$ cells gave rise to both B-and T-lymphocytes for at least 24 weeks, but the level of donor contribution decreased over time, indicating limited self-renewal capacity of the transplanted cells. The repopulation efficiency of the $AA4.1^+B220^-$ EB cells was also much lower than the equivalent population isolated from E14.5 foetal liver.

The only study to assess the long term repopulating activity of cells derived from EBs at earlier time points during *in vitro* differentiation was by Hole and colleagues (Hole et al., 1996). $1x10^6$ unsorted cells from day 4 EBs were injected i.v. into lethally irradiated adult mice along with a limiting dose ($5x10^5$) of autologous spleen carrier cells. Haematopoietic progenitors with short term reconstituting activity, as measured by the CFU-A assay (see section 1.2.3 and 1.6.4.1) appear from day 5 of EB differentiation, therefore cells from day 4 EBs were

transplanted to potentially coincide with the emergence of LTR-HSCs. Analysis of peripheral blood 12 week after transplantation detected donor EB-derived granulocytes and lymphocytes, demonstrating that cells with long term repopulating activity were present in EBs at day 4.

More recently, Flk-1$^+$ cells from day 4 EBs generated leukocytes (granulocytes and lymphocytes) and thrombocytes after injection into sublethally irradiated SCID recipients. (Miyagi et al., 2002). However, recipient animals were only analysed up to 30 days post transplant, so long term reconstituting activity was not assessed. Interestingly but not surprisingly, given the reported presence of a bipotential cell from differentiating ES cells, neoangiogenesis by donor cells was observed in the host spleens.

A variety of methods have been used in an attempt to optimise the *in vitro* differentiation of ES cells to obtain haematopoietic progenitors with repopulating ability, but no specific population of cells within EBs has yet been identified as LTR-HSCs. It is possible that ES-HSCs are not produced in sufficient numbers or are too similar to early yolk sac progenitors without long term repopulating ability. Much of the *in vitro* data described above (see section 1.5.2) would point to this scenario. In addition, the transplantation studies described above have not formally addressed the efficiency with which the progenitors derived from EBs or differentiated ES cells can colonise the bone marrow and therefore whether defective homing explains, at least in part, the low level of contribution observed in the peripheral blood of recipient mice.

To address this question Burt and others (Burt et al., 2004) compared the repopulation ability of haematopoietic progenitors from EBs injected either intravenously or directly into the femoral bone marrow cavity (IBM). Enriched c-kit$^+$CD45$^+$ cells from day 7 EBs (grown in the presence of SCF, Il-3 and Il-6) were selected as the transplanted population based on their efficiency for haematopoietic colony formation *in vitro*. The authors stated that unsorted day 7 EB cells injected i.v. into lethally irradiated animals had lead to death within 8-13 days (also observed previously with day 4-6 EBs by Hole *et al*, 1996) and injected IBM had resulted in very low levels of haematopoietic contribution and in some cases teratomas formed at the site of injection. However, when 10^5 sorted progenitors were injected IBM,

donor chimerism of peripheral blood mononuclear cells (including T- and B-lymphocytes, monocytes and granulocytes) at 20 weeks was found to range from 45.7-91% compared to 7.9-18.6% after i.v. injection of 10^6 cells, strongly suggesting that homing to the bone marrow via the i.v. route of administration is inefficient and a reason for the low levels of contribution seen previously. However, creating "space" in the bone marrow was essential for engraftment since a sublethal dose of irradiation prior to IBM transplantation (compared to a lethal dose) resulted in lower levels of haematopoietic contribution. The c-kit$^+$CD45$^+$ cells were enriched for cells expressing Sca-1 and lacking lineage markers (lin$^-$), and thus immunophenotypically resemble KTLS cells (see section 1.2.2). The authors also reported that although donor and recipients were MHC mismatched, the was no clinical or histological evidence for graft-versus-host disease and no *in vitro* mixed lymphocyte response (MLR) of chimeric mice to donor or recipient antigen despite an intact response to a third party antigen and normal production of INF-γ. The data reported in this study are promising when considering the future possibility of using human ES cells for cell therapy in the clinic.

Using a different approach, ectopic expression of the homeobox gene *HoxB4* was shown to endow differentiated ES cells with LTR-HSC activity (Kyba et al., 2002). ES cells with a doxycycline inducible *HoxB4* transgene were differentiated as EBs for six days, which were then dissociated for further culture on OP9 stromal cells. Doxycycline was added between EB days 4 and 6 to induce *HoxB4* expression and maintained during OP9 co-culture. $2x10^6$ EB cells were injected i.v. into lethally irradiated adult mice, resulting in full, normal, multilineage reconstitution in primary and secondary recipients. This method was also used to partially repair the immune function of *Rag2*$^{-/-}$ immunodeficient mice (Rideout et al., 2002). A *Rag2*$^{-/-}$ ES cell line was generated by nuclear transfer/therapeutic cloning, one allele was repaired by homologous recombination resulting in *Rag2*$^{+/-}$ ntES cells, which were retrovirally transduced with *HoxB4*, differentiated as above and transplanted into the *Rag2*$^{-/-}$ immunodeficient mice, reconstituting their haematopoietic system and partially restoring their immune function. These experiments provide a "proof-of-principle" for the combined use of nuclear transfer and ES cell differentiation technology in the treatment of genetic disorders.

1.6 Thesis aim/experiments

1.6.1 Problem

There are still limitations in the ES cell differentiation system. Haematopoietic progenitors are generated in very low numbers within EBs. At day 6 of differentiation 0.5-1% of EB cells are CFCs (Keller et al., 1993), whereas only 0.002% are HPP-CFCs (Palis et al., 1999) and 0.1% are CFU-A (see Figure 3.5), which is approximately one cell per EB (Hole et al., 1996); Figure 5.2). These data are from differentiation in the absence of exogenous growth factors (apart from those in FCS) and advances are being made in identifying the cytokines and growth factors which can increase the number of haematopoietic cells generated from EBs (see section 1.5.2). The OP9 stromal cell differentiation system, although importantly lymphoid lineages can readily be obtained by this method, the frequency of early haematopoietic progenitors is low. When the system is working efficiently, 90% of colonies after the first 5 days of co-culture are mesodermal and by a further five days after replating onto fresh OP9 cells, between 1/500 and 1/1000 (0.1-0.2%) cells give rise to CFC-mix (Kitajima et al., 2003). A direct comparison between differentiation in EBs and in the OP9 system found that haematopoietic cells (Scl^+) developed more efficiently in EBs, while endothelial cell (VE-$cadherin^+$) maturation was better supported by OP9 cells (Zhang *et al*, Blood, in press).

Reliably and efficiently achieving long term multilineage repopulation by EB cells is limited. There are several possible explanations for this problem. ES-HSCs may be inefficient at homing to the bone marrow or developmentally too immature to function in an adult environment. It may also reflect the low numbers of progenitors generated or perhaps HSCs are generated but have a tendency for differentiation as opposed to self-renewal in EBs and hence are only present for a very short time.

1.6.2 Hypothesis

These limitations may partly be attributed to a sub-optimal microenvironment for full haematopoietic development within ES/EBs differentiating *in vitro*.

Elegant co-culture experiments between yolk sac and foetal liver rudiments demonstrate that diffusible factors from the foetal liver are able to induce differentiation of primitive yolk sac erythrocytes towards a foetal/adult type of erythropoiesis, and that in the absence of the yolk sac, foetal liver erythropoiesis is not initiated (Cudennec et al., 1981). In this set of experiments, E9.5 yolk sac cultured alone for seven days produced a single transient wave of primitive nucleated erythrocytes, which expressed embryonic globins, while E9.5 foetal liver cultured alone (before the emergence of haematopoietic cells) did not express any globins. When these organ rudiments were co-cultured to allow cell migration, a second wave of erythrocytes was observed in the yolk sac and both tissues expressed definitive, adult-type globins. However, when filters were used to separate the yolk sac and foetal liver such that cells could not pass between the organ rudiments, adult erythrocytes were found in the yolk sac but none in the foetal liver. Intriguingly, other parts of the embryo body had the same inductive effect on the yolk sac. These experiments were set up to demonstrate that foetal liver erythropoiesis is only initiated by colonising cells and that these cells are from the yolk sac, nevertheless they also illustrate the potential of an appropriate inductive microenvironment even *in vitro*. The optimal microenvironment, which provide the necessary signals for HSC development is found in the haematopoietic organ rudiments of the embryo during ontogeny. Therefore exposure of differentiating ES cells to haematopoietic organ rudiments may potentially enhance their haematopoietic development.

1.6.3 Experimental system

The experiments in this thesis test the effect of co-culturing differentiating ES cells in the presence of embryonic tissue that should provide a more appropriate microenvironment for haematopoietic commitment. The AGM region was chosen as the haematopoietic organ rudiment for the co-culture experiments for several reasons. First, the AGM region is the earliest site in the developing embryo able to generate definitive, adult type LTR-HSCs (see section 1.3.2) so it must be a potent

source of inductive signals for HSC commitment. The induction of LTR-HSC activity in E8.5 yolk sac after culture on a stromal cell line derived from the AGM region (Matsuoka et al., 2001) supports this idea. Second, the emerging HSC population expands in the AGM region without significant haematopoiesis. In addition to HSCs, primordial germ cells (PGCs) migrate through the AGM region from about E8.5 and arrive in the genital ridge around E10-11, where they continue to self-renew until E13.5 (Matsui et al., 1992; McLaren, 2003). It is the only site in the embryo where stem cell proliferation occurs in the absence of differentiation. For these reasons it would be logical to presume that there should be a strong drive promoting self-renewal in this environment. Third, LTR-HSC activity has been found to increase within AGM region explant cultures (Medvinsky and Dzierzak, 1996), indicating that the elements of the supporting microenvironment for expansion of definitive HSCs can be captured *in vitro*.

1.6.4 Readout for haematopoietic commitment

The differentiation of ES cells within EBs was quantitatively assessed by an *in vitro* colony assay (CFU-A), which detects early haematopoietic progenitors. A colony assay-based readout was chosen in favour of other methods, because it would be difficult to choose the most appropriate and specific system for identification. Colony assays have the additional advantage of measuring haematopoietic potential or function. A good illustration of this is by Burt and others (Burt et al., 2004), who achieved haematopoietic reconstitution with c-kit$^+$CD45$^+$ ES-derived cells injected IBM. The authors reported 3% of the EB cells had this phenotype, yet only 0.3 % of these cells had multilineage potential *in vitro* (4 CFU-mix colonies were produced per 1200 c-kit$^+$CD45$^+$ cells plated in methylcellulose).

1.6.4.1 The CFU-A assay

The CFU-A assay was chosen as the readout for early haematopoietic commitment in EBs in these experiments because this assay quantitatively measures one of the most primitive haematopoietic progenitors measurable *in vitro*. In the CFU-A assay, macroscopic multilineage colonies (see **Figure 2.4**) are generated from normal mouse bone marrow at a frequency of approximately 150 in 10^5 cells in

response to synergistic action by conditioned medium from a mouse lung fibroblast cell line (L929), a source of CSF-1, and a rat fibroblast cell line transformed with the malignant hystiocytosis sarcoma virus (AF1.19T), a source of GM-CSF (Pragnell et al., 1988). The CFU-A has been shown to be the *in vitro* correlate to the CFU-S_{12} based on several shared functional characteristics from investigations with normal and regenerating mouse bone marrow (Lorimore et al., 1990). A high proportion of both CFU-A and CFU-S_{12} are quiescent with only 10% of the clonogenic cells in S phase, compared to more mature, lineage-restricted progenitors (GM-CFC) where that figure was 30%. CFU-A displayed the same dose response to CFU-S_{12} proliferation regulators. The kinetics of CFU-A and CFU-S_{12} activity in recovering bone marrow after 5-Fu treatment are comparable as is their response to ionising radiation. CFU-A and CFU-S_{12} show similar radial distribution in bone marrow; both progenitors are detected with increasing frequency at distances further from the femoral axis and closer to the endosteal region.

With specific relevance here, the CFU-A detected in EBs has been shown to be functionally indistinguishable from its bone marrow equivalent in terms of sensitivity to the stem cell specific regulator SCI/MIP1α. However replating efficiency is lower in EB-CFU-A compared to those in bone marrow (Hole et al., 1996; Pragnell et al., 1988). Furthermore, a highly reproducible time course of emergence has been established for the CFU-A in differentiating ES cells (Hole et al., 1996).

The precise relationship between the CFU-A and the various HPP-CFC is not fully established, but it has been suggested that the CFU-A is more mature than the most primitive HPP-CFC (type 1) (Ivanovic et al., 1999; Pragnell et al., 1994). Here the CFU-A was compared to the HPP-CFC type 2 (McNiece and Briddell, 1994; McNiece et al., 1987).

Chapter Two:

Materials and Methods

2.1 Animals

Animals were bred and maintained at the Biomedical Research Facility (BRF) of the University of Edinburgh or purchased from Harlan Olac, Ltd. Mice were treated according to the provisions of the Animals (Scientific Procedures) Act (UK) 1986. The daily care of the animals and plug checking was done by the staff at the BRF.

C57Bl/6 mice were donors for all wild type embryo material used. Female 129/Sv mice were recipients in transplantation experiments. Female mutant mice heterozygous for the gene trap integration, GT411, into the endothelial cell aminopeptidase gene ($Ecap^{Gt411For/+}$) (Wallis, Axton and Forrester, unpublished) on a 129/Sv background were the source of carrier spleen cells in transplantation experiments (see section 2.9).

The mutant mice heterozygous for the gene trap integration, GT246, into the ubiquitin conjugating enzyme 7 gene ($Ubc7^{Gt246For/+}$) were either on an outbred MF1 background or 129/Sv backcrossed onto a CBA/Ca background. (For a note on the nomenclature of gene trap loci see section 2.10).

2.1.1 Embryo harvest

AGM regions were isolated from E10.5 and E11.5 embryos. Foetal liver was isolated from E13.5 embryos. Noon on the day the vaginal plug was found was considered day 0.5 of gestation, based on the assumption that mating took place around midnight. At the appropriate stage of gestation, female mice were killed by cervical dislocation. All subsequent manipulations were performed under sterile conditions in a horizontal laminar flow hood to facilitate embryonic organ rudiment culture. The mice were sprayed with 70% ethanol and all instruments used had been sterilised. Uteri from the pregnant mice were dissected as described in Hogan *et al* (Hogan et al., 1994). The skin on the lower abdomen was pinched away from the peritoneum and a small lateral nick made in the midline. The skin was pulled apart towards the head and tail to expose the peritoneum while ensuring the fur was

sufficiently out of the way to avoid contamination. A midline incision was made in the peritoneum. The intestines were moved out of the way and the uterus was cut at the level of the cervix. The two uterine horns containing the embryos were pulled out of the mouse, cutting away any attached mesenteric tissue and severed at the fallopian tubes.

The embryos were dissected out of the uterus in PBS in a 5cm bacteriological grade petri dish using a dissection microscope. With forceps, the muscular wall of the uterus was gently torn apart to release the embryos still attached to their placentas within the extraembryonic membranes. At this stage they were transferred to a fresh dish of PBS. The extraembryonic tissues were removed and the embryos were again transferred to fresh PBS.

2.1.1.1 AGM region dissection

Tungsten needles were used for AGM region dissections, the preparation and sharpening of which is described below. These dissections were carried out as quickly as possible from the time of killing the pregnant female to maintain cell viability for culture.

E10.5 or E11.5 embryos were bisected at the forelimb buds between the heart and liver. Using numerous small incisions, the somites were cut away as close to the dorsal aorta as possible, starting at the forelimb buds extending past the hindlimb buds. The remaining part of the embryo was turned dorsal side up with the limb buds splayed out. Now the dorsal aorta, genital ridges and mesonephri are clearly visible. The bilateral extraneous tissue was removed, followed by the gut and liver so that only the AGM region remained (**Figure 2.1**).

2.1.1.2 Preparation of tungsten needles by electrolysis

Tungsten needles used for dissection of the AGM region from the embryo were made and re-sharpened by exploiting an electrolysis reaction carried out in a fume cabinet (**Figure 2.2**) (Hogan et al., 1994). Tungsten wire (99.95% pure, 0.5mm diameter; Goodfellow) served as the anode, copper wire was the cathode and 1.0M NaOH solution was used as the electrolyte. In these conditions the tungsten metal was corroded into the electrolyte solution allowing a needle-like point to be shaped.

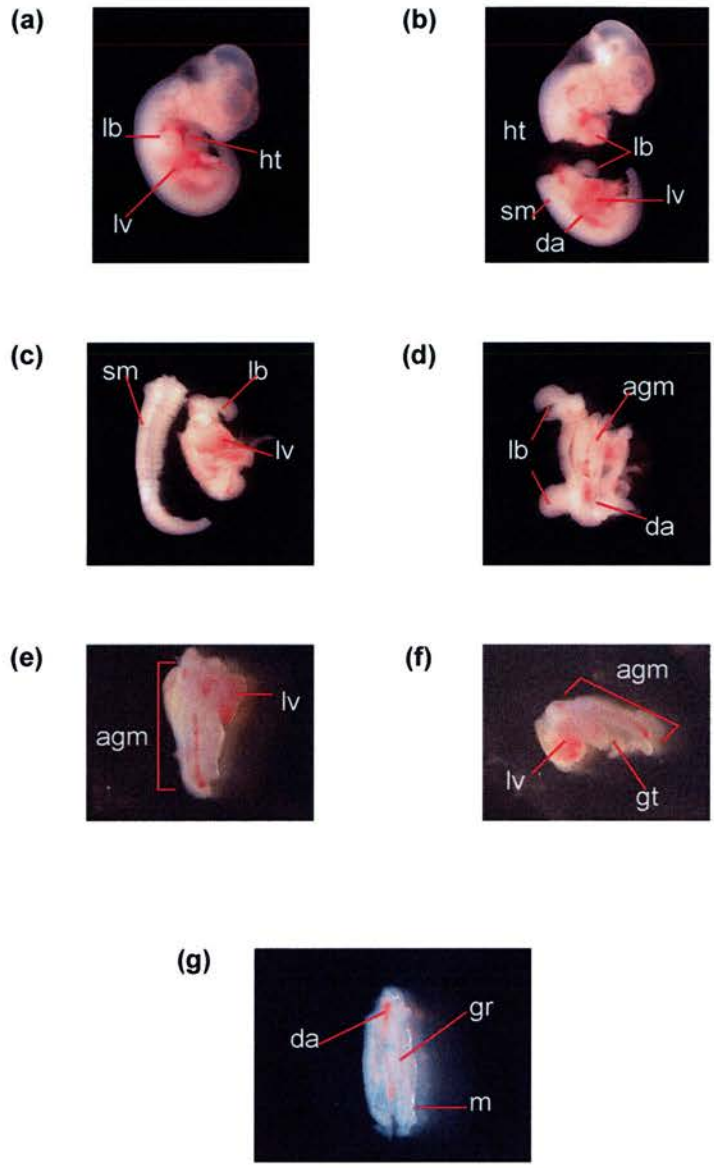


Figure 2.1 Dissection of the embryonic AGM region. (a) Late E10.5 embryo. (b) Step 1. The embryo is bisected at the forelimb buds between the heart and liver. (c) Step 2. Using numerous small incisions, the somites are cut away as close to the dorsal aorta as possible, starting at the forelimb buds extending past the hindlimb buds. (d-e) Step 3. The remaining part of the embryo is turned dorsal side up with the limb buds splayed out. Now the dorsal aorta, genital ridges and mesonephri are clearly visible. The bilateral extraneous tissue is removed. (f) Step 4. The remaining tissue is turned on its side and the liver and gut are removed so that only the AGM region remains. (g) A dissected AGM region. ht = heart, lb = limb bud, sm = somites, lv = liver, da = dorsal aorta, agm = AGM region, gt = gut, gr = genital ridge, m = mesonephros

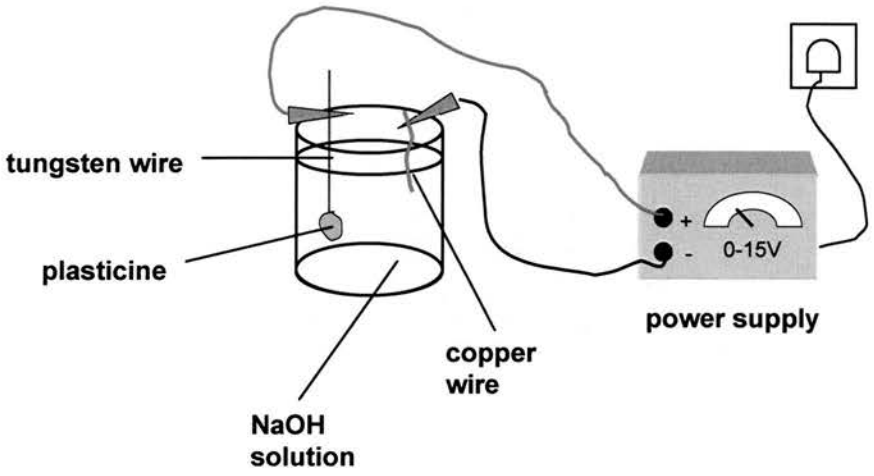


Figure 2.2 Preparation of tungsten needles for AGM region dissection by electrolysis. Metal is eroded from the tungsten wire when connected to a circuit with a piece of copper as the cathode and NaOH solution as the electrolyte.

The anode and cathode were connected by crocodile clips to wires leading to a transformer using mains power giving an output of up to 15V. To make a new needle, a suitable length of tungsten wire, with a piece of plasticine or BluTac™ attached to one end to act as a weight, was submerged in the NaOH electrolyte solution. For a smooth point, the tungsten wire was continuously moved a few millimetres up and down until the metal in the solution had been completely corroded leaving a needle-point. Needles were also sharpened regularly using this process.

2.2 Tissue culture

To avoid bacterial or fungal contamination, all cell and tissue culture including reagent preparation was carried out in a specialised tissue culture facility with Class II vertical laminar flow hoods, using sterile technique. The media were not routinely supplemented with antibiotics.

The stock solutions used for tissue culture, including the preparation and testing of supernatants containing cytokines (e.g. LIF) and batch testing of sera for haematopoietic differentiation, were routinely done by Helen Taylor, Julie Buchanan and Aileen Leask.

2.2.1 Embryonic stem (ES) cells

2.2.1.1 Maintenance of ES cells

The ES cell line GFP#7a (Gilchrist et al., 2003), which constitutively expresses GFP was used in co-culture experiments. ES lines EFC-1 (Nichols et al., 1990) and GFP#97a (Gilchrist et al., 2003) were also used in the initial optimisation experiments. GFP clones #7a and 97a were derived from parental ES line E14tg2a.

ES cells were maintained in an undifferentiated state by routine passage in feeder-free conditions on gelatin-coated tissue culture grade flasks with specialised medium (Glasgow Minimal Essential Medium containing 10% FCS, 0.25% sodium

bicarbonate, 1% non-essential amino acids, 4mM L-glutamine, 2mM sodium pyruvate and 0.1mM 2-mercaptoethanol) supplemented with 100U/ml Leukaemia Inhibitory Factor (LIF). LIF was obtained as culture supernatant from Cos-7 cells transiently transfected with a murine LIF expression plasmid (pCAGGSLIF-418). Serial dilutions of the supernatant were tested on CP1 ES cells (Bradley et al., 1984) and 100x the minimum concentration required to keep the cells undifferentiated was generally used as the working concentration. 1U of LIF is defined as the minimum concentration that keeps CP1 ES cells in a morphologically undifferentiated state.

ES cells were passaged when sub-confluent. This was usually every two days when seeded at a density of $6x10^4$ cells/cm^2 ($1.5x10^6$ cells/10 ml in a $25cm^2$ flask) giving a typical yield of $5\text{-}9x10^6$ cells. Flasks were coated with 0.1% gelatin in PBS for at least 5 minutes and removed before adding the cells. To passage, spent medium was aspirated from the flasks and the cells were washed briefly in PBS to remove any residual serum. To harvest cells, 1-2ml trypsin solution (0.025% trypsin, 0.1% chicken serum and 1.3mM EDTA in PBS) was added to the cells and incubated at 37°C for approximately two minutes before tapping the flask sharply in order to detach the cells and achieve a single cell suspension. Four times the volume of medium was added to neutralise the trypsin. The cell suspension was centrifuged at 100 x g for 5 minutes. The supernatant was aspirated off and the pellet resuspended in fresh medium. The cells were counted using a haemocytometer (Improved Nebauer) to seed the new flask at the appropriate density. Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

2.2.1.2 Thawing and Freezing of ES cells

Vials of frozen ES cells were taken directly from storage at -140°C and thawed quickly at 37°C in a waterbath. Cells were transferred to prewarmed ES medium and immediately centrifuged at 120 x g for 3 minutes. The medium was removed, the pellet resuspended with fresh ES medium and seeded in a gelatin-coated $25cm^2$ flask. The medium was replaced after about 8 hours to remove any dead cells and residual cryoprotectant.

To freeze, cells were harvested as during normal passage described above but the pellet resuspended in cold freezing medium (10% dimethyl sulphoxide [DMSO]

in ES medium) and aliquoted quickly into cryotubes. Cells were normally frozen in 2 x 1ml vials per confluent 25cm^2 flask. Cryotubes were put immediately at -80°C overnight to freeze slowly before being transferred to -140°C for long term storage. Exposure of ES cells to DMSO is kept to a minimum due to its toxicity to cells and its properties as an ES cell-differentiating agent.

2.2.1.3 Differentiation of ES cells

ES cells were differentiated as Embryoid Bodies (EBs) using the *hanging drop* method to make undifferentiated ES cell aggregates of as uniform a size as possible (Ansell and Hole, 2000).

2.2.1.3.1 The Hanging Drop method

ES cells were harvested as during normal passage described above. A suspension of single ES cells (3 x 10^4 cells/ml, typically 6x10^5 cells/20ml) in ES medium with LIF (100U/ml) was prepared. The cells were aliquoted with a multi-channelled pipette in 10µl drops (ca. 300 cells/drop) onto the underside of an upturned square-shaped bacteriological grade petri dish. Lids were replaced onto the dishes, which contained about 10ml of tissue culture grade water to humidify the hanging drops of cell suspension. Cells were cultured in this way at 37°C in a humidified 5% CO_2 atmosphere for 48 hours. After 2 days the hanging drops were harvested by tapping one side of the plate at an angle against the hood base allowing the medium to collect in the edge. The newly formed ES cell aggregates (day 0 EBs) were transferred to a universal tube and centrifuged at 80 x g for 3 minutes. The medium was removed and replaced with fresh ES differentiation medium, made up as ES medium without LIF and using a batch of FCS that had been tested for its potency for haematopoietic differentiation. Batches of sera were tested for their ability to promote haematopoietic differentiation in terms of the CFU-A assay (see below) in a time-course dependent manner.

EBs were cultured in suspension in bacteriological grade petri dishes. For the first 48 hours after harvest, penicillin/streptomycin was added to the cultures, as they are thought to be particularly vulnerable to bacterial contamination at this time.

EBs cultured in suspension were farmed every two days; EBs and medium were transferred to a universal tube where the EBs were allowed to settle with gravity to the bottom before the supernatant was removed, the EBs resuspended in fresh ES differentiation medium and moved to a new petri dish.

2.2.2 Stromal cell lines

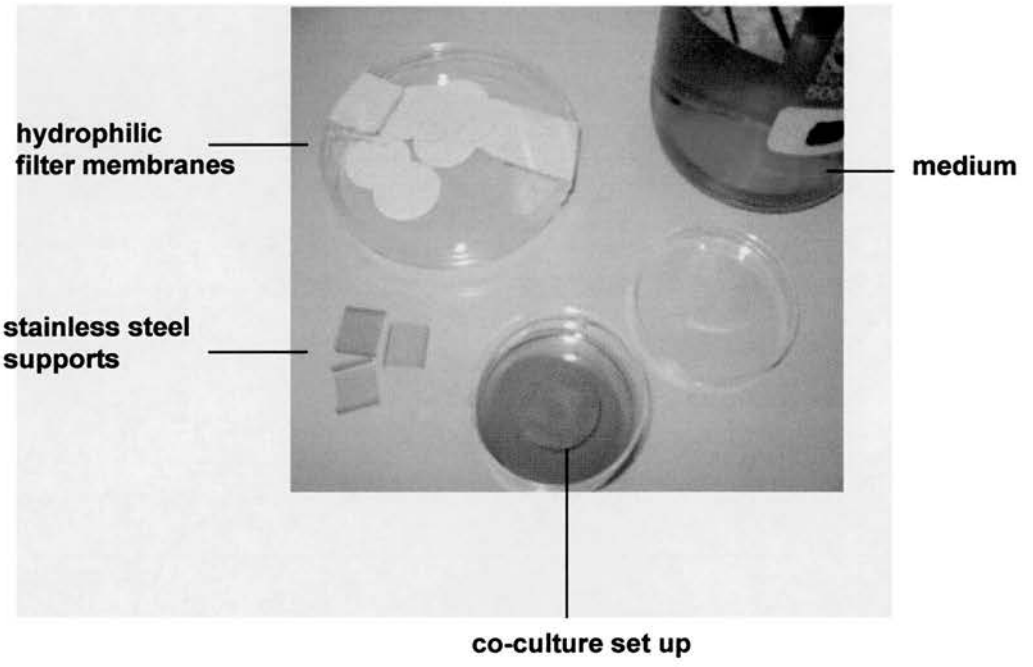
Stromal cell lines derived from the AGM region (AM20.1B4 and UG26.1B6) and foetal liver (EL08.1D2) as described previously (Oostendorp et al., 2002a; Oostendorp et al., 2002b), were a kind gift from Prof. Elaine Dzierzak at the Erasmus University, Rotterdam. Stromal cells were cultured in gelatin-coated tissue culture flasks in specialised stroma medium (50% MyeloCult long-term culture medium M5300 and 35% α-minimal essential medium containing 15% FCS, 4mM L-glutamine and 10μM 2-mercaptoethanol supplemented with 10-20% 0.2μm-filtered supernatant from the previous passage). Cells were passaged when sub-confluent, which was normally every 2-3 days if split in a 1:3 ratio. All stromal cultures were maintained at 33°C (due to the presence *tsA58* transgene encoding the temperature sensitive SV40 large T antigen, which immortalised the cell line) in a humid 5% CO_2 atmosphere.

Thawing and freezing of stromal cell lines was as described for ES cells with the exception that the freezing medium consisted of 10% DMSO in FCS instead of medium.

2.2.3 Organ culture of haematopoietic tissues

Haematopoietic organ rudiments dissected from embryos (AGM region and foetal liver) were cultured in 5cm bacteriological grade petri dishes at the air-medium interface on hydrophilic Durapore 0.56μm filter membranes supported by stainless steel mesh stands in ES medium (**Figure 2.3**). The filters were washed and sterilised in several changes of boiling tissue culture grade water before use in culture experiments. The mesh stands were made from expanded stainless steel minimesh (Expamet) which had been treated in concentrated nitric acid overnight,

(a) Co-culture materials



(b) Stainless steel support

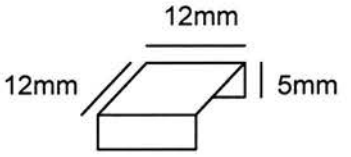


Figure 2.3 AGM region explant and EB co-culture materials. (a) Co-cultures are set up at the interface between the medium and the air on hydrophilic filter membranes supported by stainless steel grids. (b) Dimensions of the stainless steel supports which are hand made.

washed thoroughly several times in tissue culture grade water and autoclaved to sterilise before use in culture experiments. The stands could be washed in 7X®PF detergent, rinsed thoroughly in tissue culture grade water and autoclaved for re-use. Tissues were cultured in ES differentiation medium (with or without penicillin/streptomycin) at 37°C in a humid 5% CO_2 atmosphere.

2.2.4 Co-culture

2.2.4.1 Co-culture of ES cells and haematopoietic tissues

AGM region or foetal liver tissues and ES cells were co-cultured at the air-medium interface as described above. Cultures were in ES differentiation medium (see sections 2.2.1.3.1 and 2.2.1.1) without the addition of any cytokines. Day 0 or 1 EBs collected from the harvest of no more than 10ml *hanging drops* (giving rise to up to 1000 EBs) were cultured with three intact AGM regions or $1mm^3$ pieces of foetal liver. Co-culture took place over a time course lasting up to 6 days, at 37°C in a humidified 5% CO_2 atmosphere before harvesting to assess haematopoietic differentiation of the ES cells. Half the medium was replaced with fresh medium once if deemed necessary by a fall in the pH indicated by a change in colour of the medium from pink/orange to orange/yellow due to the presence of Phenol Red in 10x GMEM. Control cultures of EBs alone were cultured in the same way.

2.2.4.2 Co-culture of ES cells on primary cultures of dissociated AGM region derived cells

Dissected AGM regions were dissociated to a single cell suspension by digestion with dispase solution for 45 minutes at 37°C followed by mechanical disruption through a 23G syringe. Cells were plated onto gelatin-coated 24-well plates at a density of approximately 0.5 embryo equivalents per well in stromal medium (see section 2.2.2). Cells were allowed to grow for 7 days at 37°C in a humidified 5% CO_2 atmosphere, changing the medium on day 2 and 6. On day 7 the medium was exchanged for ES differentiation medium. Day 1 EBs were added 1 per

well either directly onto the AGM cell monolayer or into transwell inserts. Control EBs were cultured on gelatin.

2.2.4.3 Co-culture of ES cells and stromal cell lines

Stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2 were grown to confluence in stromal medium on gelatin-coated tissue culture grade 24-well, 6-well plates or 25cm^2 flasks and irradiated with 30 Gy from a Cs source. After irradiation cells were washed several times with PBS and fresh ES differentiation medium was added. Three methods of co-culture were employed: (i) approximately 20 day 0 EBs were cultured per well of a 6-well plate for up to 6 days on stromal cells and harvested to assess haematopoietic differentiation, (ii) single day 1 EB was cultured per well of a 24-well plate for up to 9 days on stromal cells and harvested to assess haematopoietic differentiation and (iii) 1.5 x 10^6 ES cells were plated onto stromal cells in 25cm^2 flasks and were passaged every two days, as described above for ES cells (see section 2.2.1.1) on gelatin, but seeded into fresh flasks of irradiated stroma. Cells were collected after every passage for 10 days for assessment of ES self-renewal potential. Control EBs or ES cells were cultured on gelatin-coated plastic.

2.2.4.4 Culture of EBs in conditioned medium

Stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2 were grown to confluence in stromal medium (see section 2.2.2). One day after reaching confluence, the supernatant was collected, centrifuged at 100 x g for 5 minutes to remove dead cells and debris, 0.2 μm filtered and stored at 4°C until needed. EBs were cultured in ES differentiation medium containing 50% conditioned medium from stromal cell lines. Control EBs were cultured in ES differentiation medium containing 50% fresh stromal medium.

2.3 Harvest of ES derived cells to assess haematopoietic differentiation.

All EBs and tissues were enzymatically dissociated by amino-endo peptidase treatment with dispase II (neutral protease) solution (1.2U/ml dispase containing

70μl/ml DNase I, added to prevent the cells from clumping) in PBS at 37°C before being pulled gently through a 23G syringe several time to achieve a single cell suspension.

2.3.1 Organ rudiment explant co-cultures

Durapore filter membranes with adhering cultures were removed from the metal stands with sterile forceps and transferred to a fresh, empty bacteriological grade petri dish. 1-1.5ml dispase solution was added carefully onto the filter membranes to cover its surface and incubated at 37°C for one hour. The dome-like drop of dispase solution is held in place on the membrane by surface tension. Cultures were not washed with PBS before adding dispase solution to minimise loss of cells. Instead, some of the excess medium could be removed by dabbing the back of the filter membrane on a sterile surface (e.g. the inside of the petri dish lid) before transfer to the fresh dish. After an hour the dispase solution was taken up by a syringe with a 23G needle and expelled over the culture to wash it off the membrane. The EBs and AGM regions were then pulled through the needle until released into a single cell suspension. The dispase was neutralised by transferring the cells back into the medium remaining in the petri dish where the culturing had taken place. This step was taken to avoid the loss of any single cells that may have floated off the membrane into the medium during culture. The cells were centrifuged at 120 x g for 5 minutes, the medium removed and the pellet resuspended in an appropriate volume (usually 10ml) of ES differentiation medium, counted with a haemocytometer and assessed for haematopoietic differentiation/activity (see section 2.4).

2.3.2 Stromal cell line or AGM region monolayer co-cultures

EBs co-cultured on stromal cells were washed gently with PBS after removing the medium. 1ml/well dispase solution was added to the 6-well plates and incubated for 1 hour at 37°C. After an hour a single cell suspension was also achieved here by pulling through a 23G needle. For EBs cultured in 24-well plates, individual EBs were picked off the stromal cells using a yellow tip and transferred to

separate wells of a 96-well plate containing 100µl/well dispase solution and incubated for 45 minutes at 37°C. Single cell suspensions were obtained by gentle pipetting with a yellow tip. The enzymes were neutralised with fresh medium.

2.3.3 Conditioned medium co-cultures

EBs cultured in suspension were harvested into universal tubes for dissociation as described in section 2.2.1.3.1. Once the EBs had settled at the bottom of the tubes, the medium was removed and the EBs were washed in PBS. 1ml dispase solution was added and incubated in an orbital incubator (Gallenkamp) at 15-20rev/min at 37°C. EBs cultured for more than 4 days were treated for 1 hour and EBs less than 4 days were treated for 45 minutes. Single cell suspensions were achieved by pulling through a 23G needle.

2.4 Harvest of embryonic tissues to assess haematopoietic differentiation

Cultured embryonic organ rudiments were dissociated as described in section 2.3.1 above. Freshly dissected organ rudiments were dissociated as in section 2.3.3 above. Single cells obtained were resuspended in an appropriate volume of medium, typically 0.5ml/organ rudiment (e.g. AGM).

2.5 Haematopoietic colony assays

Two agar based colony assays were used to quantify the primitive haematopoietic progenitor activity within the differentiating EBs: the Colony Forming Unit- Type A (CFU-A) and the High Proliferative Potential-Colony Forming Cell (HPP-CFC) assays (see Chapter 1: Introduction, section 1.2.3 and 1.6.4.1).

2.5.1 CFU-A assay

The CFU-A assay was set up as described previously (Pragnell et al., 1994; Pragnell et al., 1988). A feeder layer consisting of 0.6% agar in a modified Eagle's medium (25% α-MEM, 20% horse serum, 0.25% sodium bicarbonate and 4mM L-glutamine) supplemented with 10% conditioned medium from each of two cell lines (L929, a source of CSF-1 and AF1-19T, a source of GM-CSF) was poured into 3cm diameter tissue culture grade dishes (1ml per dish). Cells to be analysed were resuspended in 0.3% agar in Eagle's medium (as before) at a density of $3x10^4$ cells/ml and plated onto the set feeder layers in triplicate dishes. The dishes were incubated for 11 days at 37°C in a $5\%O_2$ and $10\%CO_2$ humidified atmosphere. Macroscopic colonies of neutrophils and macrophages formed after 11 days were scored as CFU-A colonies (**Figure 2.4**). For experiments involving differentiation of individual EBs in separate wells of a 24-well plate, dissociated EBs were not counted but all the cells were added to the top agar layer.

2.5.2 HPP-CFC assay

The HPP-CFC (McNiece and Briddell, 1994) assay was set up as the CFU-A assay, with the exception of the cytokines used. Conditioned medium from two cells lines were the source of the cytokines (L929, a source of CSF-1 and Wehi 3b, a source of IL3)(McNiece et al., 1986; McNiece et al., 1987).

2.6 ES cell self-renewal assay

$5x10^3$ cells were plated per well onto gelatin-coated 6-well plates containing standard ES medium supplemented with varying concentrations of LIF ranging from 0 to 100U/ml in duplicate wells. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 for 6 days. The resulting colonies were stained for alkaline phosphatase activity using the Leukocyte AP staining kit (Sigma Diagnostics) according to the manufacturer's instructions. Briefly, cells were fixed in citrate-

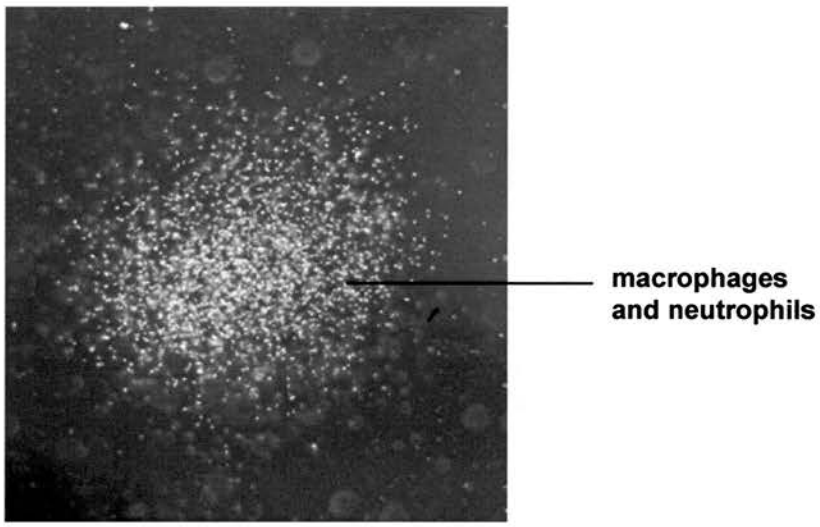


Figure 2.4 CFU-A colony. Macroscopic colonies consisting of macrophages and some neutrophils are generated by day 11 in reduced oxygen conditions from seeded progenitors which respond to M-CSF and GM-CSF. Colonies in the HPP-CFC assay look similar, but not as dense.

acetone-formaldehyde fixative (25ml citrate solution (18mM citric acid, 9mM sodium citrate, 12mM sodium chloride with surfactant, buffered at pH 3.6), 65ml acetone and 8ml 37% formaldehyde) for 30 seconds and washed gently in deionised water for 45 seconds. Cells were stained immediately with 1.5ml/well alkaline dye mixture (1ml 0.1M sodium nitrite solution added to 1ml FRV- Alkaline solution (5mg/ml fast red violet LB base in 0.4M hydrochloric acid, with stabiliser) in 45ml deionised water and 1ml Naphthol AS-BI Alkaline solution (4mg/ml naphthol AS-BI phosphate, in 2M AMPD buffer, pH 9.5)) for 15 minutes in the dark. Cells were washed thoroughly in tap water and air-dried. All incubations were carried out at room temperature.

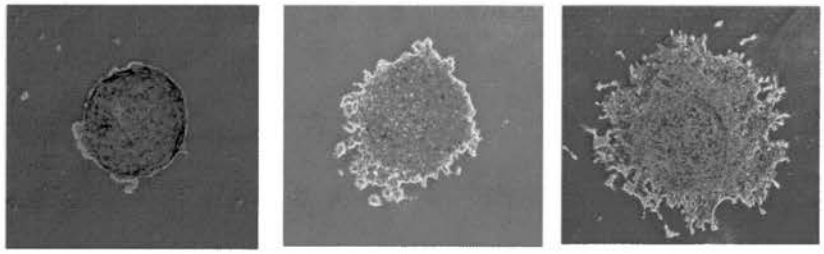
Cells were evaluated microscopically for the presence of undifferentiated stem cell, mixed or differentiated colonies as shown (**Figure 2.5**).

Duplicate wells were counterstained with 0.2% trypan blue for a couple of minutes to allow macroscopic visualisation of "white" differentiated colonies negative for AP staining.

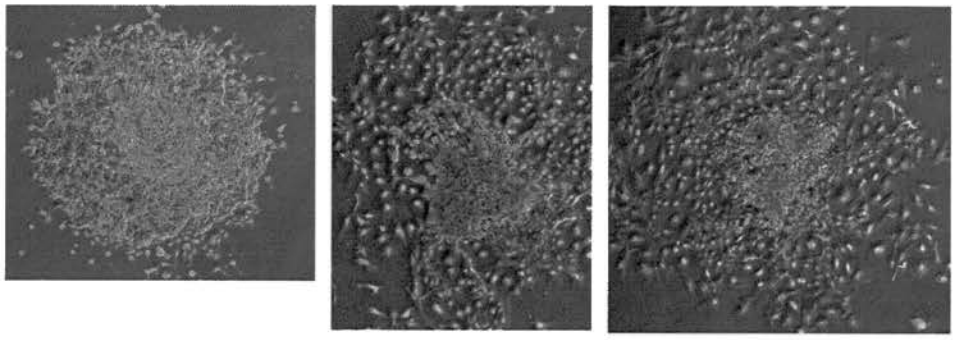
2.7 Cardiomyocyte assay

Day 1 EBs plated onto 24-well plates at one EB per well on the stromal cell lines AM20.1B4, UG26.1B6, EL08.1D2, a mixture of AM20.1B4 and UG26.1B6 cells or on gelatin (as section 2.2.4.3). EBs were co-cultured for a further 9 day. Medium was changed as necessary. At day 10 of differentiation, EBs were assessed microscopically. An EB was scored as positive for cardiac differentiation if areas of cells were observed to be twitching synchronously (Wobus et al., 1991). Cardiomyocyte differentiation was presented as a percentage of positive EBs out of the total number of EBs plated.

(a) Stem cell



(b) Mixed



(c) Differentiated

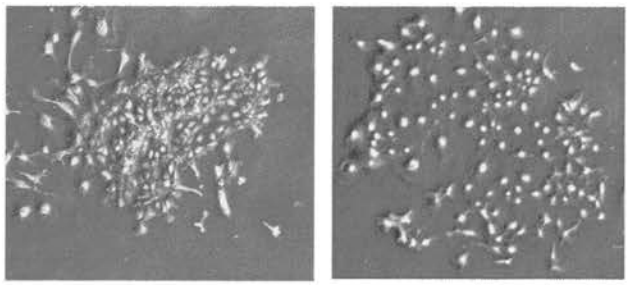


Figure 2.5 Classification of the colonies in the ES cell self-renewal assay in terms of morphology and alkaline phosphatase expression. (a) Stem cells have high self-renewal potential giving rise to tight colonies expressing AP. (b) As self-renewal potential decreases progenitors generate colonies with both AP positive stem cells and AP negative differentiated progeny. A range of morphology and AP expression is seen in the colonies, from mostly undifferentiated to mostly differentiated. (c) Progenitors with no self-renewal capacity generate diffuse colonies with cells which do not express AP.

2.8 Flow cytometry

2.8.1 Determining the percentage of GFP expressing cells in the co-cultures

Cells to be analysed were washed and resuspended in PBS (containing 0.1% sodium azide and 0.1% BSA) at a concentration of approximately $2x10^6$ cells/ml. Data for 40,000 live cells was acquired using a FACSCaliber equipped with a 488nm laser (Becton Dickinson). A sample of GFP^+ ES cells alone was used to define an electronic gate on a histogram of Fluorescence 1 (GFP) to exclude AGM derived GFP^- cells. Data was analysed with CellQuest software (Becton Dickinson) to determine the percentage of GFP-expressing (EB-derived) cells. The percentage of GFP^+ EB cells typically obtained from co-cultures of 3 intact AGM regions and up to 1000 EBs (see above) was between 75 and 90%. A small population (<1%) of GFP^- EB cells was found in the M1 gate of the GFP#7a EB cells cultured alone (**Figure 2.6**). Although most of the cells in this gate may be dead, the rare presence of GFP^- CFU-A colonies derived from GFP#7a EB cells (when visualised by fluorescence microscopy), and the detection of GFP^- cells reported in the original line (Gilchrist et al, 2003) indicate that viable cells are present in this population.

2.8.1.1 Normalisation of CFU-A and HPP-CFC scores to exclude AGM region derived colonies

These experiments depend on the ability to compare the number of haematopoietic progenitors, which arise spontaneously within populations of differentiating ES cells to their frequency after co-culture with the AGM region (or other tissues). The AGM region also contains cells, which form colonies when plated in the conditions of the haematopoietic colony assays. Therefore to compare only the haematopoietic activity within the EB-derived populations, the AGM region- and EB-derived cells must be distinguishable. The ES cell line used (GFP#7a) constitutively expressed the GFP reporter gene (Gilchrist et al., 2003). Dissociated pure EB- or co-cultures were plated into the haematopoietic colony assays at $3x10^4$ cells per dish (see section 2.5.1). The remaining cells were collected

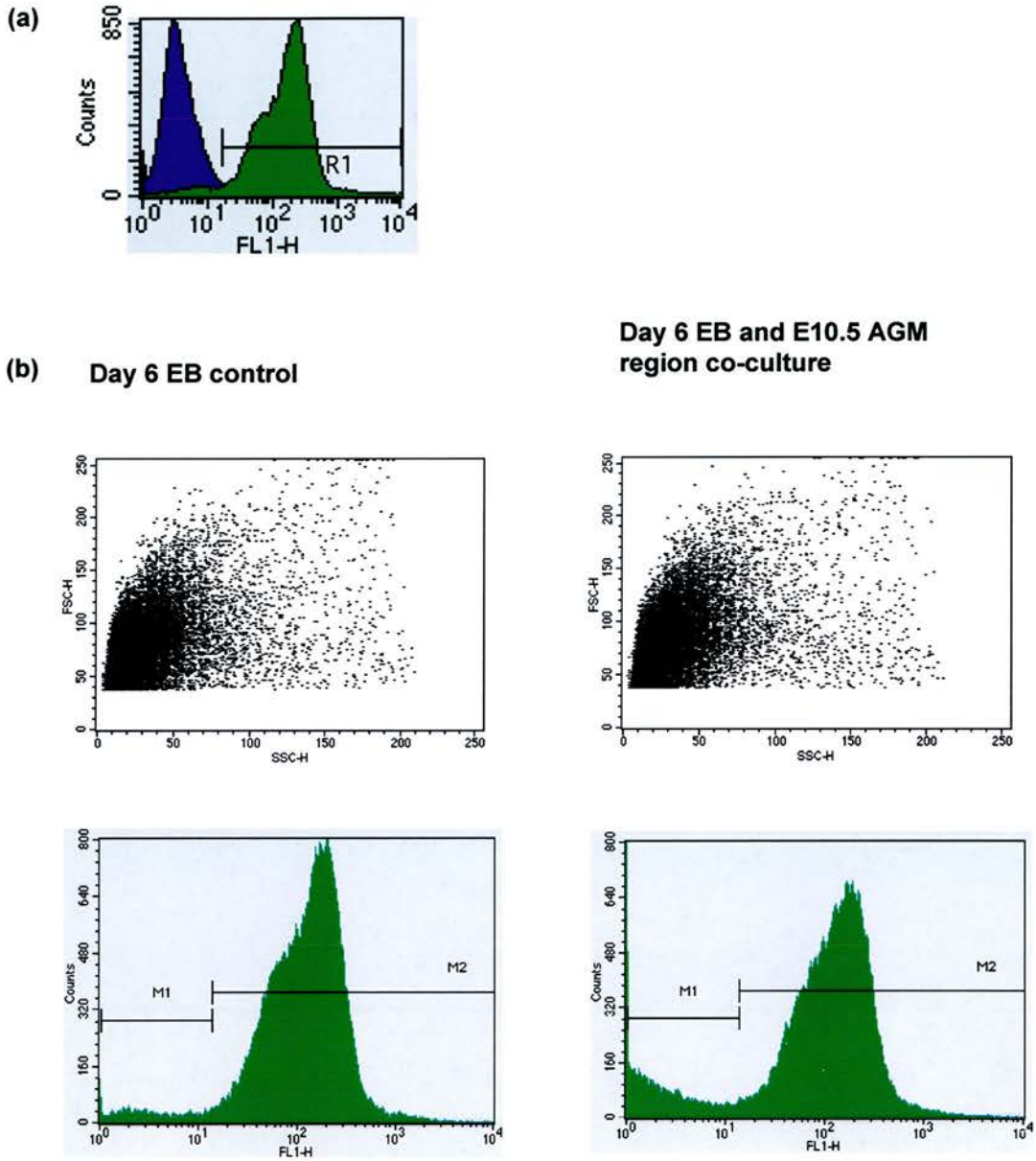


Figure 2.6 Flow cytometric analysis of day 6 GFP#7a EBs cultured alone or with E10.5 AGM region. (a) Histogram showing GFP expression of GFP#7a EB cells compared to EFC-1 EB cells. A sample of GFP^+ cells was used to define an electronic gate (R1) of Fluorescence 1 (GFP) to exclude GFP^- cells. (b) Top panels show the forward and side scatters. Lower panels show GFP expression in fluorescence channel 1 of GFP#7a EB cells and co-cultures of GFP#7a EB cells and AGM regions. The gates M1 and M2 denote GFP^- and GFP^+ populations respectively. A population of GFP^- AGM region derived cells is clearly seen in the M1 gate of the co-cultured cells. A small population (<1%) of GFP^- EB cells is also found in the M1 gate of the GFP#7a EB cells cultured alone as expected.

for analysis by flow cytometry to determine the percentage of GFP expressing (ES-derived) cells in the cultures (see section 2.7.1).

Haematopoietic colony assays were scored using a fluorescence microscope with *uv* through eGFP filters to count the proportion of green GFP-expressing colonies. Pure EB cultures produced haematopoietic colonies all of which were green as expected (see section 2.8.1). Co-cultures produced colonies of which a proportion was not green because they originated from the AGM region. The number of green EB-derived colonies from the co-cultures was then normalised to represent the number of colonies per $3x10^4$ GFP-expressing EB-derived cells, which excludes the AGM region cells. The following formula was used

$$\textbf{Normalised no. of EB-derived colonies} = \frac{\textbf{Number of green colonies counted}}{\textbf{Proportion of GFP expressing cells}}$$

For example, if there were a total of 120 CFU-A colonies in a dish in which $3x10^4$ co-culture cells were plated and 114 of them were green by *uv* microscopy and 79% of the cells plated express GFP by flow cytometry, then

$$\textbf{The normalised no. of EB-derived colonies} = \frac{114}{0.79} = 144$$

2.8.2 Surface phenotypic analysis of cells

2.8.2.1 Co-cultures

Co-cultures and EBs were dissociated (see section 2.3.1), washed in PBS (containing 0.1% BSA and 0.1% sodium azide), counted and finally re-suspended at $2x10^6$ cells/ml for use. Aliquots containing $2x10^5$ cells were incubated for 40 minutes at 4°C with optimum concentrations of appropriate monoclonal antibody (determined by titration), washed to remove unbound antibody and re-suspended in PBS for acquisition. Where a primary biotinylated antibody was used, a secondary avidin-phycoerythrin (PE) conjugate was used to detect binding and incubation before washing steps were repeated as above. Data for 40,000 live cells was acquired using a FACSCaliber equipped with a 488 nm laser (Becton Dickinson). A

sample of GFP^+ ES cells alone was used to define an electronic gate on a histogram of Fluorescence 1 (GFP) to exclude AGM derived GFP^- cells and analysed with CellQuest software (Becton Dickinson). Antibodies were all PE or tricolor (TC) conjugated rat anti-mouse monoclonal antibodies (Caltag and BD). Controls included were unstained cells and cells stained with avidin-PE alone. The antibodies used and the cell types to which they adhere are outlined in **Table 2.1**.

2.8.2.2 Stromal cell lines

Confluent stromal cells were harvested, washed and stained with antibodies (**Table 2.1**) as described above.

2.9 Transplantation experiment

Female 129/Sv mice (8-12 weeks) were irradiated with 10.5 Gy gamma (γ) rays from a Cs source in a single dose at a rate of 21.6 rad/minute. The mice received neomycin (500mg/l) in their drinking water for the first 10 days after transplantation to prevent infections that may result because of haemorrhaging from the gut caused by the high dose of irradiation. Co-cultures of AGM regions and EBs were harvested and dissociated after 5 days in culture as described above. 20 recipient mice were divided into four groups as listed below and cells were injected into the tail vein.

- irradiation controls
- 2×10^5 carrier spleen cells only
- 1×10^6 co-cultured EB and AGM region cells
- 1×10^6 co-cultured EB and AGM region cells with 2×10^5 carrier spleen cells

Carrier spleen cells were given as a source of short term repopulating HSCs to protect the recipients from the effects of irradiation before engraftment of donor test cells. The contribution of donor cells to the peripheral blood of surviving mice was

antigen	Conjugate	Other name
CD29	FITC	Integrin β_1 chain
CD31	PE	Pecam-1
CD34	PE	Mucosialin
CD44	FITC	Polymorphic glycoprotein 1
CD45	FITC	Common leukocyte antigen
CD49e	PE	Integrin α_5 chain
CD51	PE	Integrin α_v chain
CD61	FITC	Integrin β_3 chain
CD106	biotin	VCAM-1
CD117	TC	c-kit
Sca-1	PE	Ly6A/E
Flk-1	PE	VEGF-R2
Ly9.1		
Avidin	PE	

Table 2.1 of all antibodies and corresponding surface markers
FITC = Fluorescein, PE = Phycoerythrin, TC = TRI-COLOR® (PE-Cy5)

assessed 2 and 7 months after transplantation. Blood for analysis was collected from the tail vein of recipient animals at 2 months after injection. The animals were killed 7 months after injection to harvest the spleens and bone marrow along with peripheral blood. The markers used to distinguish between recipient, donor EB or AGM region cells and carrier spleen cells are outlined in **Table 4.1**.

Methods used to detect the various markers are described below. Briefly, the GPI-1 alloenzyme was identified by protein electrophoresis; GFP expression was detected by flow cytometry and fluorescence microscopy; Ly9.1 expression was detected by flow cytometry and the presence of the *YMT* and *LacZ* genes by PCR.

2.9.1 Analysis of haematopoietic tissues from surviving recipient mice transplanted with co-cultured AGM region and EB cells.

2.9.1.1 Glucose phosphate isomerase (GPI) electrophoresis of blood

The structural locus of the GPI-1 complex (*Gpi1*) which codes for the alloenzymes has four alleles (A, B, C and D). The expression of these alleles is found to vary among inbred mouse strains. Two of the alleles, A and B, are useful strain specific markers because the expression level and alloenzyme activities are similar allowing quantitative comparison and can be distinguished electrophoretically with a sensitivity that allows the detection of less than 1% contribution in small pieces of tissue. Electrophoretic separation of GPI alloenzymes was carried out using the Helena electrophoresis system (Helena Laboratories, Texas, USA) as described (Micklem et al., 1987).

Contribution of donor derived C57Bl/6 AGM region cells was assessed based on the presence of the GPI alloenzyme B in the 129/Sv host, which is A. One drop of blood from each animal was added to 50µl sample buffer (50mM triethanolamine hydrochloride pH 7.6 containing 0.3mg/ml dithioerythritol, 0.5mg/ml BSA, 2mg/ml digitonin) and stored at -80°C. Samples were thawed, vortexed and centrifuged in a microfuge to pellet cells. Using Super Z applicator tips 1-2µl of the supernatant of each sample was loaded onto Titan III cellulose acetate electrophoresis membranes that had been soaked in Supre heme electrophoresis buffer (Helena Laboratories) for

20 minutes and gently blotted dry. Cold Supra heme buffer was added to the anodal and cathodal chambers of the electrophoresis tank. Paper wicks soaked in the buffer were applied over the sides of the bridges. The membranes (2/tank) were placed, sample side down, onto the bridges of the tank. A small weight (5-10g e.g. a coin) was placed on each membrane to ensure maximum contact between the cellulose acetate and the wicks. Gels were run at 320V at 4°C for about 1 1/2 hours (or until the haemoglobin band reached the bottom). Gels were then stained with staining buffer (400µl 0.5M Tris-HCL pH 8.0, 1ml assay stock (1ml 1.0M Tris-HCL pH 8.0 containing 0.3mg NADP and 6.5mg fructose 6-phosphate), 15µl G6PD (300U/ml), 1.5ml $MgCl_2$:glycerol mix (50% 0.2% $MgCl_2$ and 50% glycerol) 500µl MTT (10mg/ml) and 500µl PMS (2.5mg/ml)) in the dark until bands appeared. When developed, gels were rinsed with water, fixed in 5% acetic acid for 5 minutes, washed in water for 15 minutes, dried and stored in the dark.

2.9.1.2 PCR analysis of genomic DNA from peripheral blood

2.9.1.2.1 Isolation of genomic DNA from blood

5-6 drops of blood were collected from each animal and stored at -20°C. Frozen samples were thawed and washed twice in 1ml SSC (8.8g/l NaCl and 4.4g/l sodium citrate in H_20, pH 7.0), vortexed to mix and centrifuged for one minute at 12,000rpm in a microfuge to pellet cells discarding all the supernatant after the second wash. To digest the cells, 375µl 0.2M NaOAC was added to each pellet and vortexed briefly, followed by 25µl 10% SDS and 5µl proteinase K (20mg/ml H_20), a brief vortex and incubated at 55°C for 1 hour. 120µl phenol:chloroform:isoamyl alcohol (phenol:chloroform 1:1 with isoamyl alcohol 25:1) was added to extract the DNA, the tubes inverted, vortexed for 30 seconds and centrifuged at 12,000rpm for 30 seconds. The aqueous layer was removed into a fresh 1.5ml tube and the DNA was precipitated with 1ml cold 100% EtOH incubated at -20°C for 15 minutes. Samples were centrifuged at 12,000rpm for 2 minutes, the supernatant decanted and the pellet air-dried. The DNA was resuspended in 50µl sterile distilled H_20 and stored at -20°C. This method yielded approximately 100ng/µl.

2.9.1.2.2 Polymerase chain reaction

PCR was used to determine the contribution of either donor ES or AGM region cells by the presence of the Y-chromosomal specific *YMT* gene or carrier spleen cells by the presence of the *LacZ* reporter gene from the gene trap vector.

500ng genomic DNA isolated from peripheral blood (see above) was used in each reaction. Oligonucleotides to as follows:

YMT	5'-CTGGAGCTCTACAGTGATGA-3'
	5'-CAGTTACCAATCAACACATCAC-3'
myogenin	5'-TTACGTCCATCGTGGACAGC-3'
	5'-TGGGCTGGGTGTTAGTCTTA-3'
LacZ	5'- TACAATCAGGTGGAGAGGTTCCTACTG-3'
	5'-GTTCTTCTTCTTTGGTTTTCGGGACCTGGGAC-3'

Myogenin was amplified in combination with *YMT* and as the internal PCR control. Primers for the amplification of *LacZ* were specific for the integration into *Ecap* by the gene trap vector. The reaction for *YMT/myogenin* was initial heating at 95°C for 5mins, followed by 30 cycles of 94°C for 10s, 60°C for 30s, 72°C for 35s and a final single cycle at 37°C for 10mins, producing amplicons for *YMT* at 342bp and *myogenin* at 245bp (Medvinsky and Dzierzak, 1996). The reaction for *LacZ* was initial heating at 94°C for 2mins, followed by 35 cycles of 94°C for 15s, 65°C for 30s, 72°C for 2mins producing an amplicon of approximately 1200bp. Since all the animals that received spleen cells died before blood samples were taken, the PCR reaction for the *LacZ* reporter was never performed. The products were separated on 2% agarose containing ethidium bromide.

2.9.1.3 Flow cytometric analysis of peripheral blood, spleen and bone marrow

Peripheral blood, spleens and femurs were harvested from killed recipient mice. Spleens were homogenised to obtain single cell suspensions. Bone marrow was flushed from the femurs with PBS using a 25G needle. The cells were washed with PBS counted and an aliquot of each was resuspended in PBS (containing 0.1% BSA and 0.1% sodium azide) at a concentration of about $2x10^6$ cells/ml. Half of

each sample was stained with FITC labelled antibody against Ly9.1. Cells were analysed by flow cytometry for GFP and FITC expression (see section 2.7.2).

2.9.1.4 Detection of GFP expression in CFU-A colonies derived from recipient spleen and bone marrow

CFU-A assays with bone marrow and spleen cells harvested from recipient mice were set up as described in section 2.5.1. Colonies were analysed with a fluorescence microscope to detect any GFP expressing cells.

2.10 Genotyping of *Ubc7*^{Gt246For} mice and embryos

The loci of gene trap integrations, once characterized as potentially unique, can be named and symbolised as members of a series, using the prefix Gt (for gene trap), followed by a vector designation in parentheses, a serial number assigned by the laboratory characterizing the locus, and the laboratory ILAR code (http://www.informatics.jax.org/mgihome/nomen/gene.shtml#gtl). Therefore, the 246th ES clone generated, which had trapped a gene by the pGT0, 1 or 2 vector in the laboratory of Lesley Forrester (For) is symbolized as *Gt(pGT0,1,2)246For*. A gene trap designation becomes an allele of the gene into which it was inserted, once that gene is identified. Therefore, *Gt(pGT0,1,2)246For* is known to disrupt the ubiquitin conjugating enzyme 7 (*Ubc7*) gene; thus the full allele designation for this gene trap mutation is *Ubc7*$^{Gt(pGT0,1,2)246For}$ and its abbreviated form is *Ubc7*^{Gt246For}.

The mutant mouse line *Ubc7*^{Gt246For} was generated by gene trapping using a vector containing a *LacZ* reporter gene (see section 6.2.1.2). Since the trapped gene is ubiquitously expressed β-galactosidase activity detected by X-gal staining of tissues was used to determine the genotype (*Ubc7*$^{+/+}$, *Ubc7*$^{Gt246For/+}$ or *Ubc7*$^{Gt246For/Gt246For}$) of the mice. Ear punches were taken for genotyping adult mice and yolk sac or head was used for embryos.

Ear punches, yolk sacs or embryonic heads were washed in PBS and fixed at room temperature with 0.2% gluteraldehyde, 5mM EGTA (pH7.3), 2mM $MgCl_2$ in 0.1M sodium phosphate for 5 minutes (yolk sac or head) or 15 minutes (ear

punches). The samples were then washed at room temperature in wash buffer (20mM $MgCl_2$, 0.01% deoxycholate, 0.02% Nonidet in 0.1M sodium phosphate (pH 7.3)) three times for 5 minutes (yolk sac or head) or 15 minutes (ear punches). Samples were stained at room temperature (yolk sac or head) or 37°C (ear punches) using X-gal stain (1mg/ml X-gal, 250mM potassium ferrocyanide, 250mM potassium ferricyanide in wash buffer) from 4 hours to overnight. After staining, samples were stored in wash buffer at 4°C. X-gal stain was collected, filtered and frozen for re-use.

After staining with X-gal tissues were examined either by eye or light microscopy. The tissues that stayed clear/yellow were wildtype, heterozygous tissues turned blue and homozygous tissues turned a deep turquoise colour.

2.11 Wholemount *in situ* hybridisation of embryos

2.11.1 Probe synthesis

The murine cDNA (from Hogan E8.5 library) for the 3'UTR and C-terminus of *Wnt8B* was used as a template for generating riboprobes for *in situ* hybridisation. The 720kb cDNA template cloned into pBSII KS- (R129) was a kind gift from Dr John Mason.

1μg DNA plasmid each were linearised by restriction digest with XbaI (for transcription with T3 RNA polymerase to generate the antisense probe) and with XhoI (for transcription with T7 to generate the negative control sense probe).

For synthesis of digoxygenin (DIG)-labelled RNA probe, transcription reactions were set up at room temperature to generate antisense and sense probes using the DIG RNA labelling kit from Roche as per manufacturer's instructions. 13μl dH_20, 2μl 10x transcription buffer (400mM Tris HCl pH8.25, 60mM $Mgcl_2$ and 20mM spermidine), 1μl 0.2M DTT, 2μl nucleotide mix (10mM GTP, 10mM ATP, 10mM CTP, 6.5mM UTP and 3.5mM digoxigenin-UTP), 1μl (1μg/μl) linearised plasmid template cDNA, 0.5μl (100U/μl) placental ribonuclease inhibitor and 1μl (10U/μl) T3 or T7 RNA polymerase were mixed and incubated at 37°C for 2 hrs. A

1µl aliquot was removed and run on a 1% agarose gel containing ethidium bromide. An RNA band ca. 10 fold more intense than the plasmid band was used as an indicator the ca. 10µg probe had been synthesised. 2µl DNase I (RNase free) was added to the remaining transcription reaction and incubated at 37°C for 15 mins.

To precipitate the synthesised probe, 100µl TE (50mM Tris HCl, 1mM EDTA pH8.0), 10µl LiCl and 300µl EtOH were mixed, incubated at -20°C for 30mins, spun in a microfuge at 4°C for 10 mins washes twice in 70% EtOH and air-dried. The probed were then dissolved in TE at ca. 0.1µg/µl and stored at -80°C. 10µl/ml hybridisation mix was used in the hybridisation stage (see section 2.11.3)

2.11.2 In situ *hybridisation*

2.11.2.1 Pre-hybridisation treatment

Dissected E10.5 $mUbc7^{+/+}$ and $mUbc7^{Gt246For/Gt246For}$ embryos (see section 2.1.1) were fixed in 10ml 4% paraformaldehyde in PBS at 4°C o/n. Unless otherwise stated all incubations were carried out at room temperature and solutions were made up in PBT (0.1% Tween 20 in PBS).

Fixed embryos were washed twice in PBT and dehydrated by taking through a MetOH series (25%, 50% and 75% MetOH) incubated for 5 mins each. Embryos were finally washed twice in 100% MetOH (and were stored at -20°C for several days). Rehydration was in the reverse series to before (75%, 50% and 25% MetOH) and with rinsing (twice) in PBT also for 5 mins each.

Embryos were bleached with 6% hydrogen peroxide for 1hr and washed with PBT three times for 5 mins each. Treatment with Proteinase K (10µg/ml) was carried out to allow the RNA probe to penetrate the cells of the embryo. Incubation was for exactly 15 mins after which the embryos were washed immediately with fresh glycine (2mg/ml) to inhibit the action of Proteinase K, followed by two washes with PBT, before refixing with 0.2% glutaraldehyde/4% paraformaldehyde (40µl 25% gluteraldehyde in 5ml 4% paraformaldehyde) in PBS and two washes in PBT for 5 mins each.

2.11.2.2 Hybridisation

Unless otherwise stated, all solutions are made up in dH_20. Embryos were rinsed and incubated at 70°C for 1hr in prehybridisation solution (50% formamide, 5x SSC pH4.5 and 1% SDS). During this incubation, the probes were denatured at 80°C for 10 mins and cooled on ice and added to hybridisation solution (50% formamide, 5x SSC pH4.5, 1% SDS, 50μg/ml yeast RNA and 50μg/ml heparin) at ca. 1μg/ml. Embryos were rinsed in hybridisation solution and then incubated in hybridisation with probe at 70°C o/n on a rotating platform in a hybe oven.

2.11.2.3 Post hybridisation treatment and staining with antibody against digoxygenin

After hybridisation with probe, embryos were washed twice at 70°C for 30 mins each in pre-warmed post-hybridisation solution I (50% formamide, 5x SSC pH4.5 and 1% SDS as prehybridisation), followed by a 10 min wash at 70°C with a 1:1 mixture of solution I and II (0.5M NaCl, 0.1M Tris pH7.5 and 0.1% Tween 20) and 3 washes with solution II at room temperature for 5 mins. To remove unbound probe, embryos were incubated twice with 100μg/ml RNase A in solution II at 37°C for 15 mins each and rinsed in solution II for 5 mins. A final two posthybridisation washes were performed with solution III (50% formamide and 5% SSC pH4.5) at 65°C for 30 mins each.

Embryos were then washed three times in TBST (0.4M NaCl, 2.7mM KCl, 25mM Tris HCl pH 7.5 and 0.1% Tween 20) and preblocked with 10% sheep serum (endogenous alkaline phosphatases were inactivated by heating serum at 65°C for 30 mins) in TBST for 2.5 hrs at room temperature. Incubation with anti-DIG antibody conjugated to alkaline phosphatase (AP) (1μl anti-DIG-AP in 2ml 1% sheep serum in TBST) was carried o/n at 4°C on a rocker.

After staining with antibody, samples were washed thoroughly in 5ml TBST at room temperature, first for three washes of 5 mins each followed by a further five washes for 1 hr each.

2.11.2.4 Staining for alkaline phosphatase activity

All incubations, unless otherwise stated, were carried out at room temperature. Embryos were washed three times in freshly made up NTMT (0.1M NaCl, 0.1M Tris pH9.5, 0.05M $MgCl_2$ and 0.1% Tween 20) before staining with NBT (6.75µl in 2ml NTMT) and BCIP (5.25µl in 2ml NTMT) substrate mix. Samples were stained at 4°C in the dark o/n.

When the reaction was complete, the embryos were washed twice in NTMT for 10 minutes and in PBT for a further 10 mins. The embryos were post fixed in 4% paraformaldehyde and 0.1% gluteraldehyde in PBS for 1 hr, washed twice with PBT and stored at 4°C.

For photography, embryos were washed in 50% and then 80% glycerol in PBT.

2.12 Microscopy and Photography

Cells were examined with a Leitz Labovert light or Zeiss Axiovert 25 fluorescence microscope. eGFP was visualised with uv light through Zeiss filter #44. Embryo dissections were performed using a Zeiss Stemi 2000-C dissecting microscope.

Cells and embryos were photographed with an AxioCam digital camera using AxisVision 3.1 software. For images of the self-renewal assay, 6-well plates were digitally scanned on an Epson Perfection 2480 Photo flatbed scanner.

2.13 Statistical analysis

Statistical analysis was performed using the Mann-Whitney U and Student's *t* Tests to assess significance of the non-parametric data sets.

Chapter Three:

Establishing the conditions for AGM region and ES cell co-culture

3.1 Aim

To develop a method for the co-culture of differentiating ES cells with the AGM region.

3.2 Introduction

Methods for the *in vitro* differentiation of ES cells down haematopoietic lineages are well established as reviewed in Chapter 1. A method for culturing the embryonic AGM region, while maintaining its haematopoietic activity, has also been described (Medvinsky and Dzierzak, 1996). However, these culture conditions are very different. Differentiation of ES cells usually involves the formation of embryoid bodies cultured in suspension, with or without the addition of growth factors or on particular stromal feeder cells. In contrast, AGM regions are cultured on membranes at the interface between the air and the medium to maintain their haematopoietic activity. Both have defined culture media requirements. Thus the first aim was to find a method that allowed the *co*-culture of the differentiating ES cells and the embryonic AGM region, which was optimal for either partner.

3.3 Experimental approach

- Determine the haematopoietic activity of the AGM region in culture using the CFU-A assay for comparison with published data using other assays.
- Identify a genetically marked ES cell line, which would allow the ES cells to be distinguished from the AGM region cells after co-culture and investigate its haematopoietic differentiation capacity in standard conditions.
- Assess whether the organ rudiment explant culture conditions were conducive to ES cell differentiation as there are no reports published on the effect of culturing EBs at the interface between the air and the medium.

3.4 Results

3.4.1 CFU-A activity in haematopoietic tissues

Although the haematopoietic activity has been analysed in the AGM region using other assays (e.g. CFU-S_8 and $_{11}$) (Medvinsky and Dzierzak, 1996; Medvinsky et al., 1993), there are no reports of the use of the CFU-A assay in this regard. The baseline level of CFU-A activity in haematopoietic tissues was therefore determined. The number of CFU-A colonies per $3x10^4$ cells in uncultured AGM region increased from 3 ± 1.4 to 25 ± 23.6 between day 10.5 and 11.5 *post coitus* (*p.c.*). This is similar to the emergence of CFU-S_{11} previously reported, which increase from 0.94 ± 0.42 to 5.98 ± 0.87 per tissue (approximately $2x10^4$ cells) between day 10 and 11 of gestation (Medvinsky et al., 1993). CFU-A activity in the liver at E13.5 was 30 ± 10. 5 colonies per $3x10^4$ cells, adult spleen had 44 ± 10.0 and bone marrow had 88 ± 8.2 colonies (**Figure 3.1**).

Medvinsky and colleagues (Medvinsky and Dzierzak, 1996) have shown that the numbers of CFU-S_{11} expand in organ culture. Hence the capacity of haematopoietic activity, as measured by the CFU-A assay, to expand in culture was tested (**Figure 3.1**). The explant culture system used by Medvinsky and others, in which the embryonic organ rudiment is cultured at the interface between the air and the medium, was used. However, because the system was being optimised for co-culture with ES cells, standard ES cell differentiation medium (see Chapter 2: Materials and Methods section, 2.2.1.3) was used instead of the specialised medium for haematopoietic progenitors (MyeloCult M5300 from Stem Cell technologies). The AGM region from the E10.5 embryo shows the greatest expansion of progenitors in culture (to 91.7 ± 55.3 per $3x10^4$ cells).

The variability in CFU-A activity in E10.5 AGM region after culture is very high. To examine this in more detail, AGM regions from E10.5 C57Bl/6 littermates were cultured individually and their CFU-A activity compared. Even within inbred littermates, the variation in the number of CFU-A colonies after culture was very high, ranging from 17.3 to 160 colonies per $3x10^4$ cells (approximately 0.3-1 embryo equivalents; e.e.) (**Figure 3.2**). This could be due to a variation in the developmental stages found within litters.

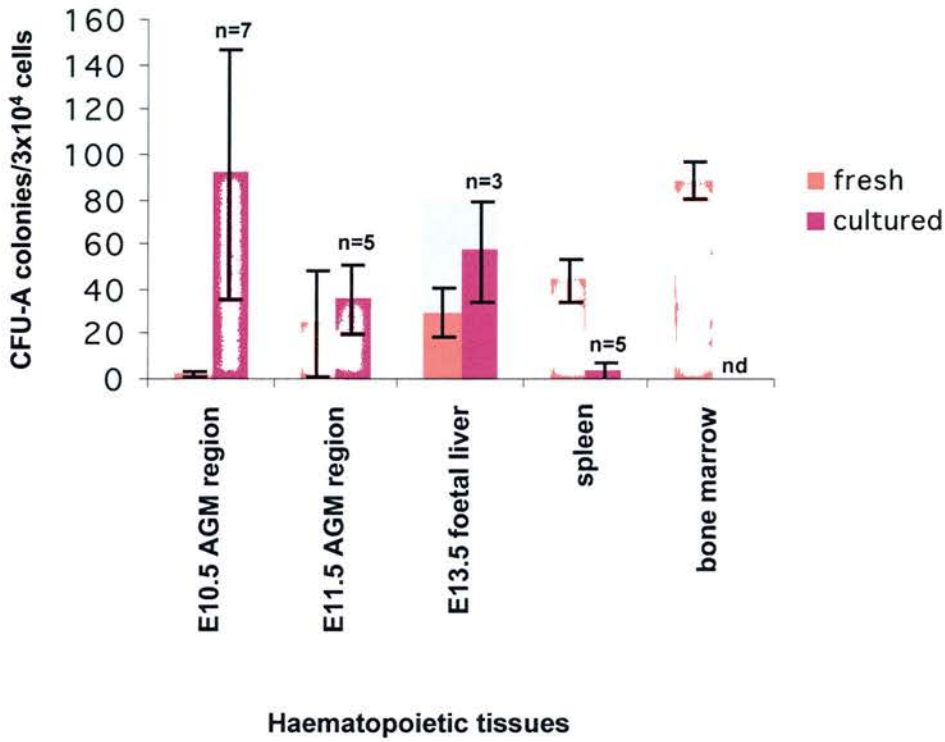


Figure 3.1 CFU-A activity in haematopoietic tissues. The number of CFU-A colonies in the haematopoietic tissues from foetal and adult mice, immediately after dissection (■) and when cultured for 6 days (■). Coloured bars show the number of colonies/3x10^4 cells. Results for fresh tissues are taken from two independent experiments set up in triplicate. Numbers of experiments for cultured tissues are shown. Error bars represent standard deviations of the means. nd = not determined

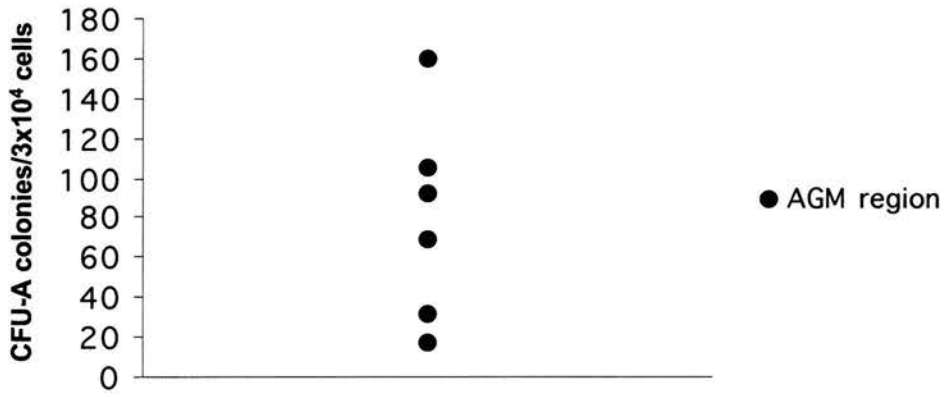


Figure 3.2 Variability of CFU-A activity in E10.5 AGM regions among inbred littermates after organ culture.. Each data point (●) represents the number of CFU-A colonies/3x10^4 cells (typically 0.3-1 e.e.) from the AGM region of individual C57Bl/6 littermates at E10.5 after 6 days of culture. The numbers of colonies are means of triplicate plates.

Given that an increase was observed after six days in culture a more detailed study was carried out to assess the timing of expansion of CFU-A progenitors. A time-course was set up to further compare the AGM region from E10.5 and E11.5 embryos (**Figure 3.3**). In this experiment, the number of CFU-A progenitors in E10.5 AGM increased dramatically between days two and four (26 ± 2.0 to 165 ± 31.7) after which, no further expansion is seen, while only a doubling was observed over the same period in E11.5 AGM region (27 ± 1.7 to 43 ± 7.2). This is in contrast to the published data for cultured AGM region using the CFU-S_{11} assay, where greater expansion is seen in E11.5 embryos. However, as seen here using the CFU-A assay, it has been demonstrated that HSC activity of the AGM region increases dramatically in the first 2-3 days in organ culture after which it is maintained for at least one week, and in addition the HSC activity was reportedly more extensive in E10.5 AGM explant cultures compared to E11.5 (de Bruijn et al., 2001).

Thus in summary, the baseline CFU-A activity found in the AGM region was comparable to the published CFU-S activity and there was expansion of the CFU-A progenitors in culture, demonstrating that the organ culture system was reproducible in the lab and could be useful for co-culture experiments.

3.4.2 CFU-A activity in differentiating ES cells

A marked ES cell line was needed for the co-culture experiments because haematopoietic progenitors in the AGM region can be maintained and expanded in organ culture as shown in the previous section and by others (Medvinsky and Dzierzak, 1996). Therefore, ES cell and AGM region derived cells needed to be distinguishable for two reasons. First, to determine by flow cytometry the contribution of AGM region and ES cells to the co-cultures at the time of harvest for setting up the CFU-A assay. Second, to distinguish by fluorescence microscopy the colonies derived from the ES cells from the endogenous AGM region-derived colonies when scoring the assay. The enhanced Green Fluorescent Protein (eGFP) reporter was chosen because it is detectable in viable cells in contrast to, for example β-galactosidase encoding *LacZ* reporter gene.

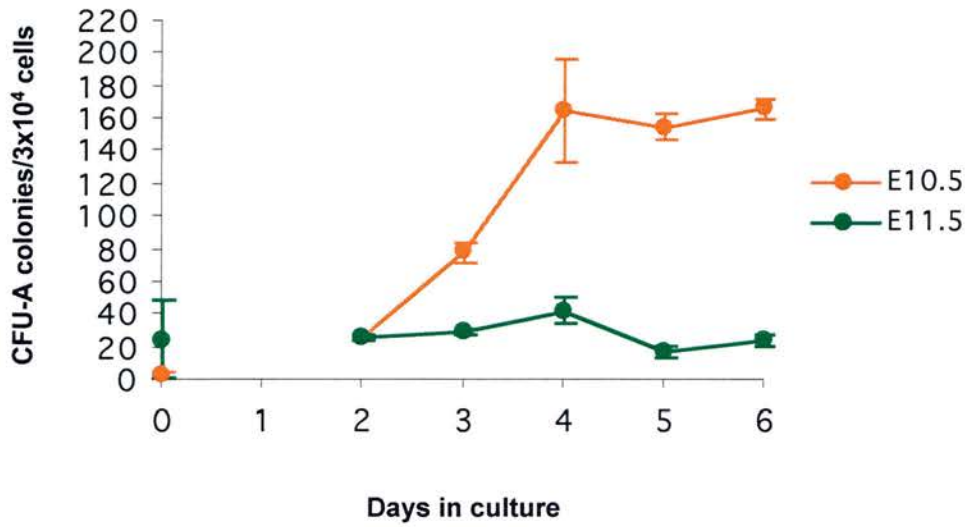


Figure 3.3 CFU-A activity in AGM regions after organ culture. Numbers of CFU-A colonies/3x10^4 cells in pooled AGM regions from E10.5 (—●—) and E11.5 (—●—) mouse embryos over 6 days in culture. Error bars represent standard deviations of the mean for triplicate plates.

Two GFP-marked ES cell lines (GFP#7a and GFP#97a) (Gilchrist et al., 2003) were chosen, which were previously generated from the parental line E14tg2a by Dr Derek Gilchrist (in the lab of Dr Alexander Medvinsky). The cell lines were made in order to generate a reporter mouse, which would express eGFP in the majority of cells in all tissues, particularly those of the haematopoietic lineages, which are prone to silencing transgenes. The eGFP reporter Cre-inducible ES cells were electroporated with a "silent" eGFP construct in which the PGK promoter and eGFP sequences are separated by puromycin resistance gene and stop cassette flanked by loxP sites. Upon Cre-mediated excision of the puromycin resistance gene and stop cassette (by electroporation of a Cre-expressing plasmid when testing the ES cells *in vitro*) the eGFP was "activated" (**Figure A.1** in Appendix). Several lines, including #7a, showed high eGFP expression in all haematopoietic cells after *in vitro* differentiation in methylcellulose. Furthermore, after germline transmission and activation *in ovo* (by crossing with mice where Cre is expressed exclusively in the growing oocyte prior to the completion of the first meiotic division), eGFP was expressed ubiquitously including in 90% of lymphoid and myeloid cells for at least 10 months. The #97a ES cells contained the eGFP-F reporter, which contains a farnesylation signal fused to the c-terminus of eGFP. This signal directs eGFP-F to the inner face of the plasma membrane where it remains bound in both living and fixed cells (Derek Gilchrist, personal communication and Clontech technical information).

Here GFP#7a and #97a ES cells were examined to i) confirm the level of their GFP expression by both fluorescence microscopy and flow cytometry and ii) test their ability to form CFU-A colonies upon differentiation. The GFP expressed by GFP#7a cells was readily detectable by eye with a fluorescence microscope and by flow cytometric analysis the shift in the fluorescence peak is substantial compared to wild type cells. GFP#97a ES cells were not as easily visualised by microscopy probably because the eGFP was confined to the plasma membrane. When analysed by flow cytometry, a substantial GFP^- population was detected, which was not observed in GFP#7a ES cells (**Figure 3.4**). Haematopoietic differentiation was compared to the wild-type EFC-1 (Nichols et al., 1990) ES cells routinely used in the

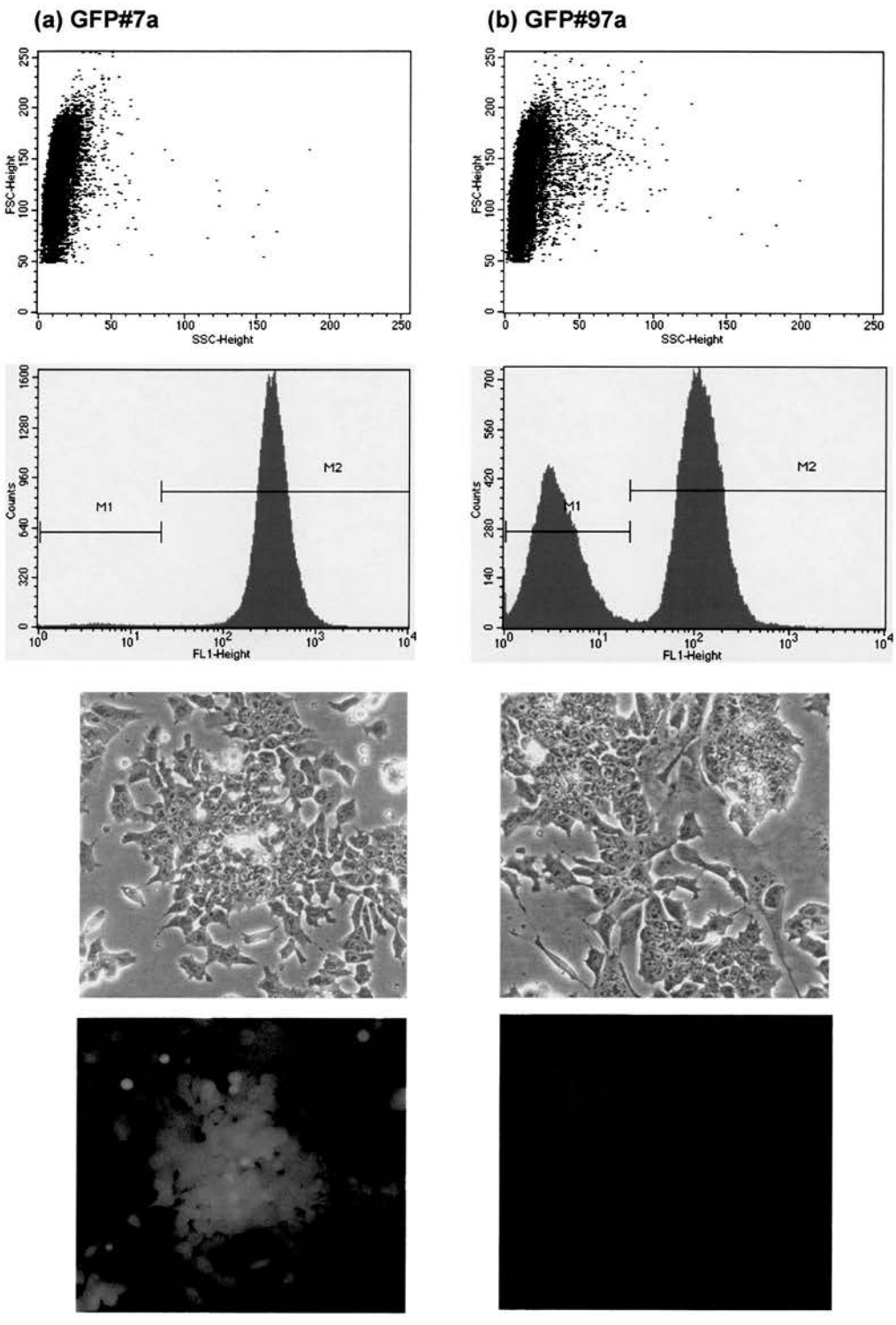


Figure 3.4 GFP expression in #7a and #97a ES cell lines. (a) GFP#7a ES cells with eGFP and (b) GFP#97a ES cells with the membrane bound form of eGFP-F. Top panels show flow cytometric analysis of FSC and SSC and GFP expression in fluorescence channel 1. #7a ES cells are brighter and 100% GFP^+ as shown by the peak along the FL1-H axis and the M2 gate, respectively. Bottom panels show microscopic analysis of the GFP#7a and #97a ES cells with light and fluorescence microscopy. Flow cytometric analysis was performed by Kay Samuel.

lab. ES cell lines can vary in their ability to form CFU-A colonies, so it was essential that this comparison be made. The percentage of EBs, which gave rise to CFU-A colonies after 6 days in suspension culture was similar in all three lines (EFC-1, 42±3.6; GFP#97a, 22±12.3; GFP#7a, 28±7.4; **Figure 3.5a**). However, due to the high level of GFP expression in the GFP#7a cell line, it was chosen for analysis of CFU-A emergence over a time-course of differentiation. The GFP#7a line was equivalent to the EFC-1 line in terms of (i) the percentage of EBs which gave rise to CFU-A colonies, (ii) the timing with which these cells emerge (**Figure 3.5b**) and (iii) the frequency with which CFU-A progenitors are found in dissociated EBs (**Figure 3.5c**).

3.4.3 Culture of EBs: suspension versus air-medium interface

Organ rudiments are often cultured at the interface between the air and the medium (Cudennec et al., 1981), presumably due to oxygen tension. This has also been found to be the best way to culture the embryonic AGM region to allow induction, maintenance and proliferation of HSCs (Medvinsky and Dzierzak, 1996). It was surmised that to fully exploit the capture of this microenvironment *in vitro* for co-culture experiments, it was useful to establish whether EBs could also be cultured as organ rudiments at the air-medium interface and retain the ability to differentiate. The haematopoietic differentiation of GFP#7a EBs was compared when cultured in suspension or at the air-medium interface for up to six days (**Figure 3.6a**). Differentiation was not affected by the method of culture; 34 ± 4.6 and 38 ± 2.5 CFU-A colonies per $3x10^4$ cells plated were found after six days of suspension or interface culture respectively (**Figure 3.6b**). Although there is a 10-fold difference in the CFU-A activity at four days, this did not warrant further consideration because both values are very low and within the expected range for ES cell lines in the lab. The purpose of this experiment was to determine whether haematopoietic differentiation of EBs was at all possible when cultured at the air-medium interface; the data show that it is. Cultures of EBs alone would be set up alongside all co-culture experiments to control for the inter-experimental variability often observed during ES cell differentiation. In comparison, the low level of inter-experimental

variation in the CFU-A activity of adult bone marrow (**Figure 3.1**) indicates that the CFU-A assay itself is not the source of the variability in these experiments but rather the differentiation of the ES cells.

It should be noted that it is not possible to retrieve individual intact EBs from the membranes of the interface cultures so all EBs were dissociated enzymatically to achieve a single cell suspension for plating in the CFU-A assay (see Chapter 2: Materials and Methods, section 2.3.1).

3.5 Discussion

There were two issues to be dealt with when establishing a method for co-culturing ES cells and AGM region. First, the two co-culture components normally require very different culture conditions, so a method that would be conducive to ES cell differentiation while not detrimental to the haematopoietic supporting microenvironment of the AGM region and *vice versa* had to be found. An embryonic organ rudiment culture system, which importantly preserves the structure and function of the AGM region, was chosen as the basis for the co-culture experiments. Since the co-culture system was being optimised for ES cells, standard ES cell differentiation medium was used. The AGM region appeared to respond well to this medium as shown by the expansion of CFU-A progenitors in culture. The question of whether the explant culture system is conducive to ES cell differentiation remained, because there are no reports in the literature on the effect of culturing EBs at the interface between the air and the medium. The data show that interface cultures do not inhibit the haematopoietic differentiation of ES cells, at least as measured by the CFU-A assay.

The second issue was the ability to distinguish between the ES cells and the AGM region, since both contain CFU-A progenitors. Indeed, the simplest strategy to eliminate the colony forming potential of the AGM regions would be by irradiation before co-culture. However, the ability to capture the AGM region microenvironment, including its ability to produce HSCs, was considered an important part of the co-culture system and the effects of irradiation are not entirely known.

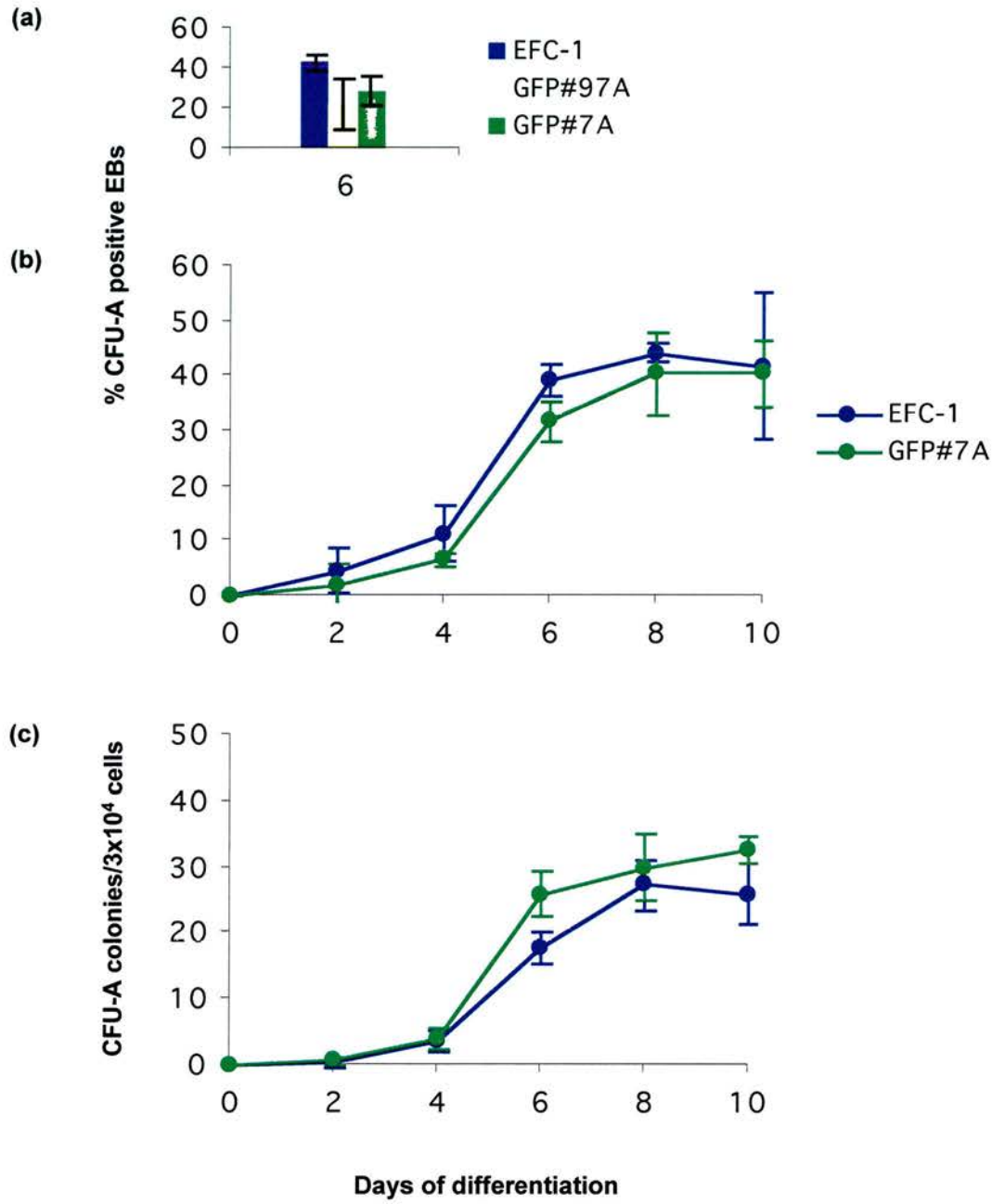


Figure 3.5 CFU-A activity in differentiating unmarked and GFP-marked ES cells. (a) Percentage of EBs giving rise to CFU-A colonies from EFC-1 (■), GFP#97a ( ) and GFP#7a (■) ES cells after 6 days of differentiation. (b-c) CFU-A activity in EBs from EFC-1 (●) and GFP#7a (●) ES cells over a time course from 0 to 10 days of differentiation showing the percentage of EBs giving rise to CFU-A colonies (b) and the number of CFU-A colonies/$3x10^4$ cells after dissociation (c). Error bars represent standard deviations of the means of triplicate plates.

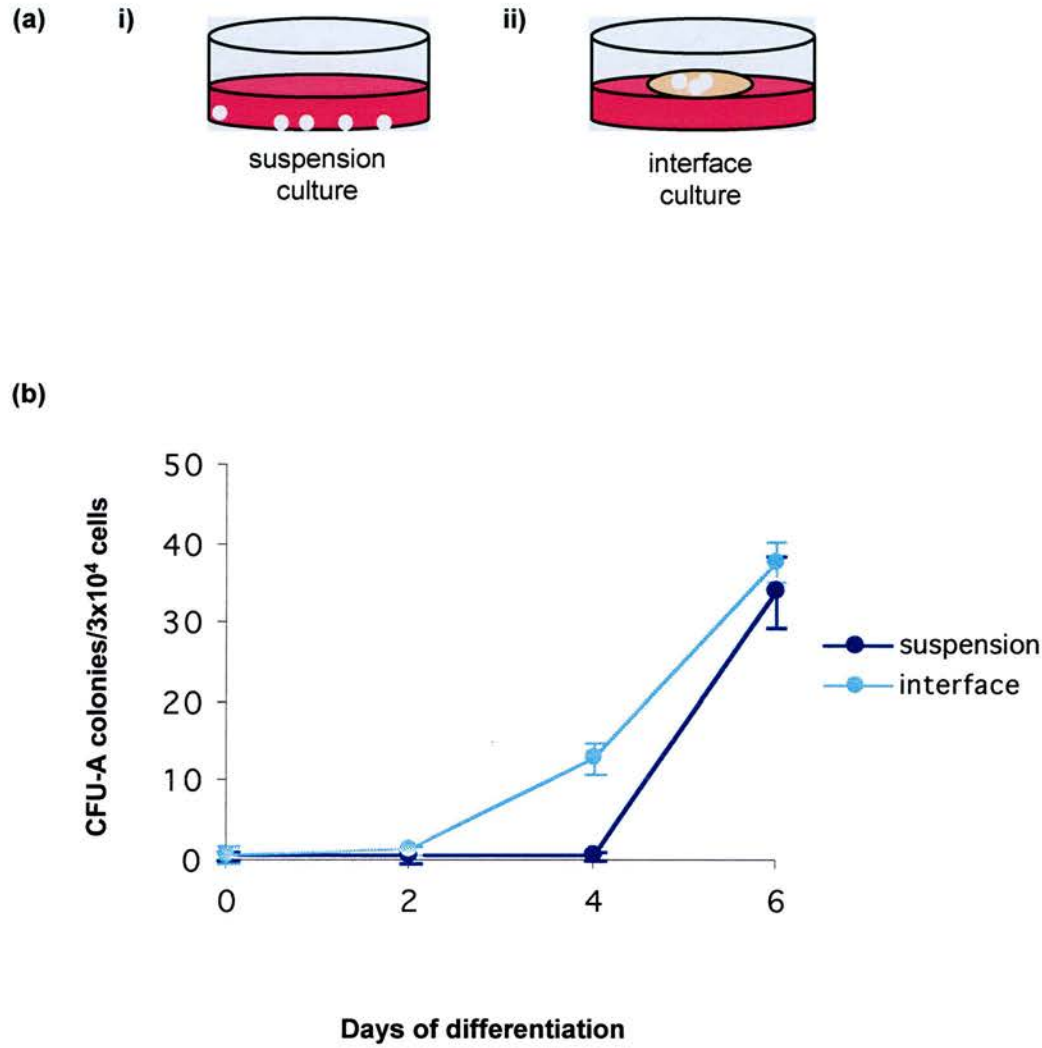


Figure 3.6 Comparing culture of embryoid bodies in suspension and at the air-medium interface. (a) Diagram showing culture of EBs i) in suspension and ii) at the interface between the air and the medium. (b) The number of CFU-A colonies/$3x10^4$ cells over a time-course for EBs in suspension (●) or at the air-medium interface (●). Error bars represent standard deviations of the means of triplicate plates.

Therefore, a highly expressing GFP-marked ES cell line was identified, which produced colonies in the CFU-A assay that were visible by fluorescence microscopy. Furthermore, the GFP-marked cells were easily distinguished from wild type cells by flow cytometry, which was useful for determining the level of contamination by the AGM region cells in the co-cultures. That information made it possible to compare the numbers of CFU-A colonies derived from equivalent numbers of EB cells in co-cultures and control cultures.

The co-culture method established as a result of these experiments is described in Chapter 4.

Chapter Four:

Haematopoietic differentiation of ES cells by AGM region co-culture

4.1 Aim

To examine the effect of the AGM region on the haematopoietic differentiation of ES cells.

4.2 Introduction

4.2.1 The method for AGM region and ES cell co-culture

The co-culture method established as a result of the experiments described in Chapter 3 is illustrated in **Figure 4.1** (see Chapter 2: Materials and Methods for a detailed description of all the procedures outlined).

In brief, EBs were made by the *hanging drop* method from GFP#7a ES cells and cultured at the interface between the air and the medium with the AGM regions dissected from E10.5 or 11.5 embryos. The controls were EBs cultured alone. No cytokines were added to the cultures to assess only the effect of the AGM region on the differentiating EBs. Cultures were harvested, dissociated and plated into the CFU-A (or other haematopoietic colony) assay. Samples of the cells were analysed by flow cytometry to determine the percentage of GFP^+ ES-derived cells. The percentage of GFP^+ EB cells typically obtained from co-cultures comprising of three intact AGM regions and up to 1000 EBs was between 75 and 90% (e.g. FACS histogram in **Figure 4.1** and **Figure 2.6**). CFU-A assays were scored by light and fluorescence microscopy to count the total number of colonies and the green GFP^+ colonies respectively. Using the percentage of GFP^+ ES-derived cells determined by flow cytometry, the number of CFU-A colonies per $3x10^4$ EB cells was calculated to allow the comparison to control cultures containing only EB cells.

A comparison to foetal liver co-culture was also made.

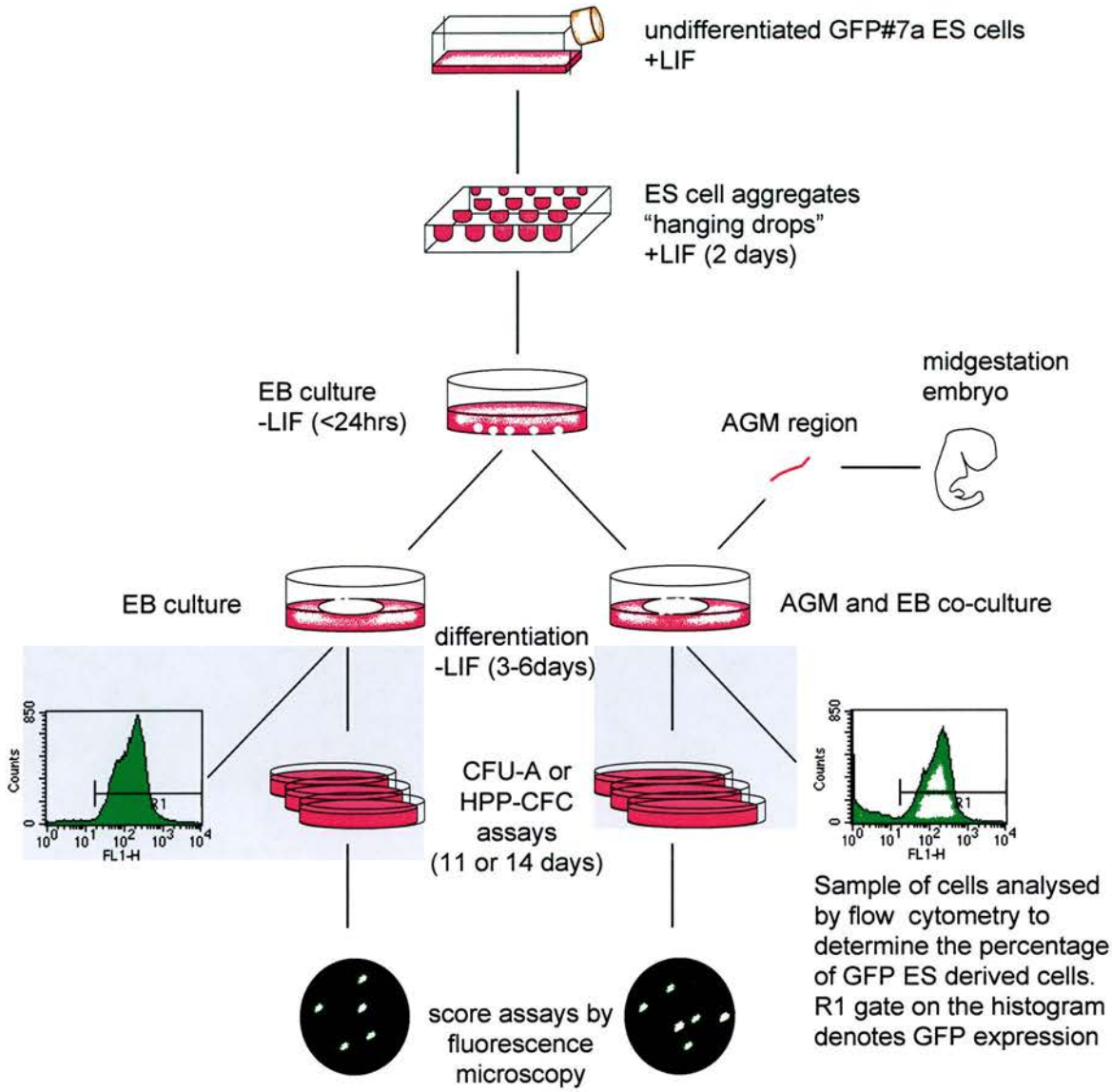


Figure 4.1 Flow diagram illustrating the co-culture set up and subsequent haematopoietic analysis. A detailed description of all the procedures depicted can be found in Chapter 2: Materials and Methods.

4.3 Experimental approach

- Set up a preliminary study to rapidly assess the AGM region co-culture strategy as a method for enhancing haematopoietic differentiation of ES cells and to investigate the inter-experimental variability of the system.
- Compare the effect of the E10.5 and 11.5 AGM region and another haematopoietic tissue, the E13.5 foetal liver, on the haematopoietic differentiation of ES cells.
- Investigate the inductive capacity of the E10.5 AGM region over a more extensive time-course of ES cell differentiation.
- Compare the CFU-A assay with the more widely used HPP-CFC assay, which also detects primitive haematopoietic progenitors over a time-course of differentiation.
- Assess the ability of progenitors from co-cultured EBs to engraft *in vivo* after transplantation into lethally irradiated recipients.

4.4 Results

4.4.1 Preliminary co-cultures to assess the feasibility and variability of the experimental system

The initial co-culture experiments were performed to assess first, whether the AGM region had any effect on the haematopoietic differentiation of ES cells and second, the reproducibility of the system. In three independent experiments EBs were co-cultured with AGM regions from late E10.5 and early E11.5 embryos. All experiments resulted in a greater number of haematopoietic colonies from the co-cultured EBs compared to those cultured alone. The range of increase in CFU-A activity after co-culture was from 1.5- to 4-fold (39 to 151, 69 to 166 and 79 to 120 colonies per $3x10^4$ cells, **Figure 4.2**). $P < 0.01$ as determined by the *t* Test. These preliminary data provide strong evidence to support the hypothesis that exposure of embryonic AGM region to differentiating ES cells enhances haematopoietic differentiation. However, the results obtained are highly variable. ES cell

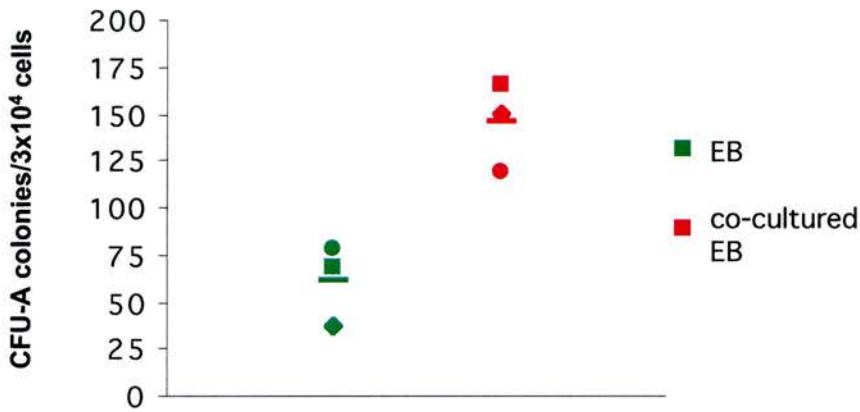


Figure 4.2 Variability of CFU-A activity in differentiating embryoid bodies co-cultured with AGM region. Numbers of CFU-A colonies/3x10^4 cells in EBs cultured alone ( ) and co-cultured with E10.5 -11.5 AGM region ( ) for 6 days. Each data point represents the mean number of colonies in triplicate plates from independent experiments. The shapes correspond to individual matched experiments. Circles ( ) represent E11.5 AGM regions. Diamonds and squares (,) represent E10.5 AGM regions. Bars ( ) show overall means. $P < 0.01$, as determined by the *t* Test.

differentiation is prone to inter-experimental variation as was also found in the level of haematopoietic activity in the spontaneously differentiating EBs cultured alone. Interestingly, there was some correlation between the level of induction and the developmental stage of the embryos from which the AGM regions were dissected. The smallest increase in CFU-A activity came from E11.5 AGM region (indicated by circles in **Figure 4.2**).

Importantly, the co-culture differentiation system is a multi-step process, which inherently introduces variables, increasing the potential for error. Possible sources of error include: (i) the precise gestational age of the embryos from which AGM regions are dissected, (ii) the quality of the dissection and how long it takes, (iii) the number of EBs in contact with the inductive factors from the AGM regions and (iv) the membrane filters being exactly at the air-medium interface; neither too far above the medium nor too submerged.

4.4.2 The effect of the embryonic AGM region and foetal liver on the haematopoietic differentiation of ES cells

The CFU-A activity of EBs after co-culture with AGM regions from E10.5 and E11.5 embryos or foetal liver from E13.5 was compared. The time-points (four and six days of differentiation) were chosen based on the emergence of CFU-A activity in differentiating EBs (Chapter 1: Introduction, section 1.6.4.1; **Figure 3.6** and Hole et al., 1996). The E10.5 AGM region had the most potent effect on haematopoietic differentiation. After six days in co-culture, the number of CFU-A colonies per $3x10^4$ cells plated was 9-fold greater than in EBs cultured alone (15 ± 5.9 to 142 ± 12.7, **Figure 4.3a**). Co-culture with E11.5 AGM resulted in only a 2-fold increase (62 ± 11.4 to 137 ± 34.1 colonies per $3x10^4$ cells, **Figure 4.3b**). In contrast, foetal liver had no effect (62 ± 11.4 to 55 ± 28.8 colonies per $3x10^4$ cells, **Figure 4.3c**).

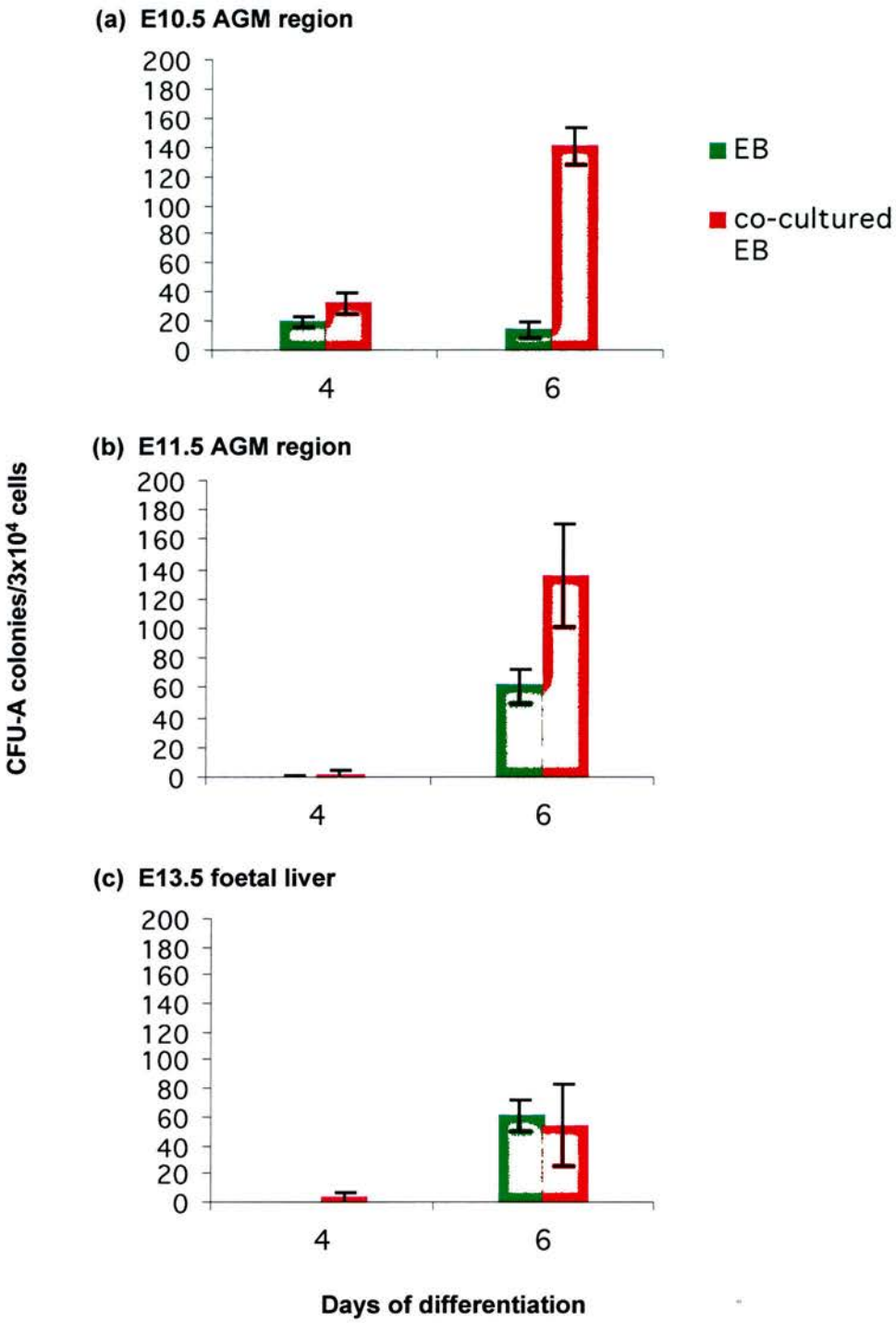


Figure 4.3 CFU-A activity in embryoid bodies after co-culture with AGM region and foetal liver. Numbers of CFU-A colonies/$3x10^4$ cells in EBs cultured alone (■) and co-cultured (■) for either 4 or 6 days with (a) E10.5 AGM region, (b) E11.5 AGM region or (c) E13.5 foetal liver. Error bars represent the standard deviations of the mean of triplicate plates.

4.4.3 Time-course of haematopoietic differentiation of ES cells by AGM region co-culture

In order to analyse the effect of AGM region on differentiating ES cells in more detail, a more extensive time-course was performed and the CFU-A assay was compared to the HPP-CFC.

4.4.3.1 CFU-A assay

Haematopoietic progenitors emerge in a time-course dependent manner with a peak at six days of differentiation. CFU-A activity was increased approximately 20-fold by co-culture (186 ± 27.5 colonies per $3x10^4$ cells) compared to the control EBs cultured alone (10 ± 5.5 per $3x10^4$ cells, **Figure 4.4**).

Interestingly, there was a decrease in haematopoietic activity in EBs at seven days. A likely explanation for this observation is that the progenitors had differentiated into more mature cell types, which are not detected by this assay. An alternative hypothesis was that extended culture of EBs at the air-medium interface may result in cell death. However, there was no evidence for this when cells were stained with propidium iodide (PI) and annexin V and analysed by flow cytometry (data not shown).

4.4.3.2 HPP-CFC assay

The HPP-CFC (High Proliferative Potential-Colony Forming Cell) assay was set up in parallel to the CFU-A assay, as a second, more widely used measure of primitive haematopoietic progenitors.

The emergence of HPP-CFC progenitors in the time-course followed the CFU-A with a peak at six days, although HPP-CFC colony numbers were lower in both control and co-cultured EBs (**Figure 4.5**). The number of colonies in the control EBs cultured alone was as found previously by others using this assay (Palis et al., 2001). There was a 50-fold increase in HPP-CFC activity after co-culture with the AGM region, with the number of colonies per $3x10^4$ cells plated rising from 0.3 ± 0.6 (control) to 57 ± 11 (co-cultured).

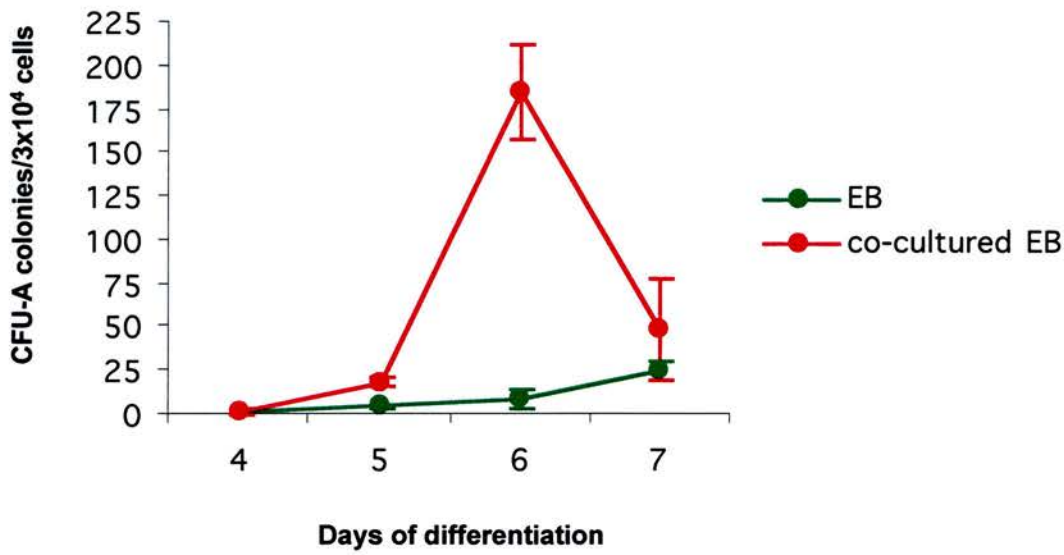


Figure 4.4 Time course of CFU-A activity in embryoid bodies after co-culture with AGM region. Numbers of CFU-A colonies/3x10^4 cells in EBs cultured alone (●) and co-cultured (●) with E10.5 AGM region for up to 7 days. Error bars represent the standard deviations of the mean of triplicate plates.

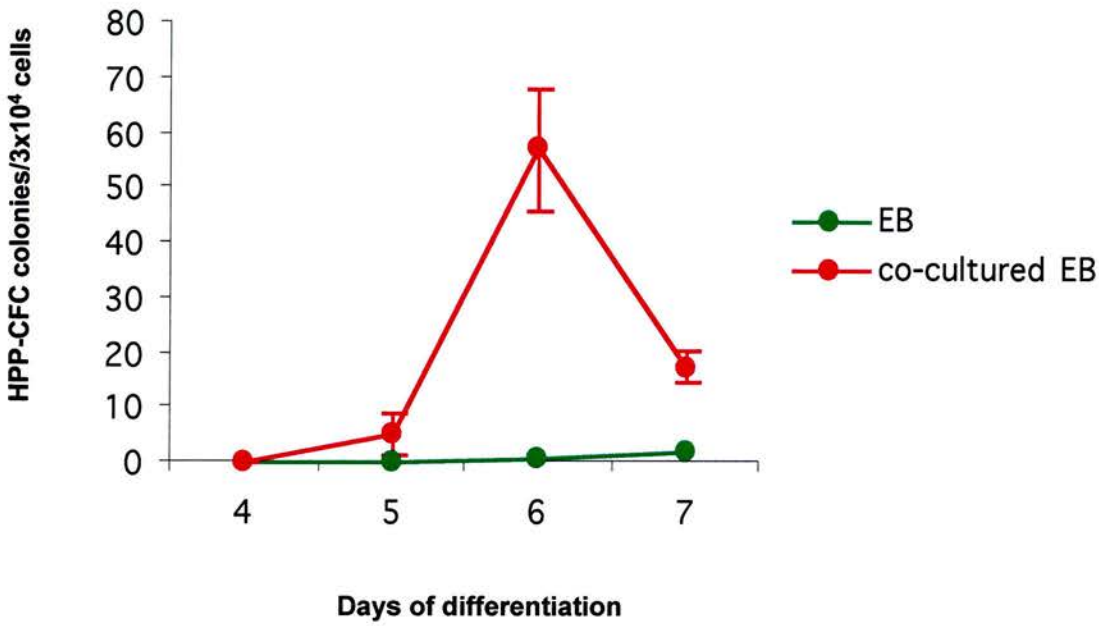


Figure 4.5 Time course of HPP-CFC activity in embryoid bodies after co-culture with AGM region. Numbers of HPP-CFC colonies/3x10^4 cells in EBs cultured alone (●) and co-cultured (●) with E10.5 AGM region for up to 7 days. Error bars represent the standard deviations of the mean of triplicate plates.

4.4.4 Transplantation of co-cultured EB and AGM region cells into adult recipients

To determine whether co-cultured EBs contained LTR-HSCs, the cells were transplanted into adult mice and the contribution to haematopoiesis measured (see Chapter 2: Materials and Methods section 2.8 and **Figure 4.6**). For transplantation experiments the co-cultures were harvested after only five days of differentiation assuming that cells detected by the CFU-A and HPP-CFC assays are derived from LTR-HSCs. Lethally irradiated adult mice were each injected with $1x10^6$ co-cultured cells. It must be noted that the cells harvested from the co-cultures were not sorted prior to injection and therefore approximately 10% were AGM region derived (as measured by flow cytometry, data not shown). One group received $2x10^5$ carrier spleen cells in addition to the experimental cells from the co-cultures. Control animals received either irradiation only or were irradiated and injected with $2x10^5$ carrier spleen cells. Historical controls for EBs cultured alone were used (Hole et al., 1996) in combination with a concurrent transplantation experiment carried out by Helen Taylor, Julie Buchanan and Kay Samuel, to repeat the previous findings by this lab (Hole et al., 1996). The contribution of donor cells to haematopoiesis in the surviving recipients was assessed two and seven months after transplantation.

A marker system was devised to analyse the contribution of donor cells to the haematopoietic compartment. Cells from up to four different sources (host, EB cell, AGM region and carrier spleen) had to be distinguishable, thus a combination of markers was used to recognise them (**Table 4.1**). ES-derived cells were identified by GFP expression. Host animals were female; although the GFP#7a ES cell line is male, the Y-chromosome specific *YMT* gene was not unique to the EB-derived cells because half of the AGM regions in the co-cultures would also be male. However, the AGM regions were dissected from C57Bl/6 embryos, which harbour a different allele of the *GPI-1* gene (1^b) from the mouse strain129/Sv (1^a), the strain of the recipients, carrier spleen cells and ES cells. In addition C57Bl/6 cells do not express the Ly9.1 antigen, which is found on the surface of most 129/Sv cells. Finally, the spleen cells used as carriers were harvested from a female 129/Sv mouse carrying the gene trap integration, GT411 with the *LacZ* reporter (Wallis, Axton and Forrester, unpublished).

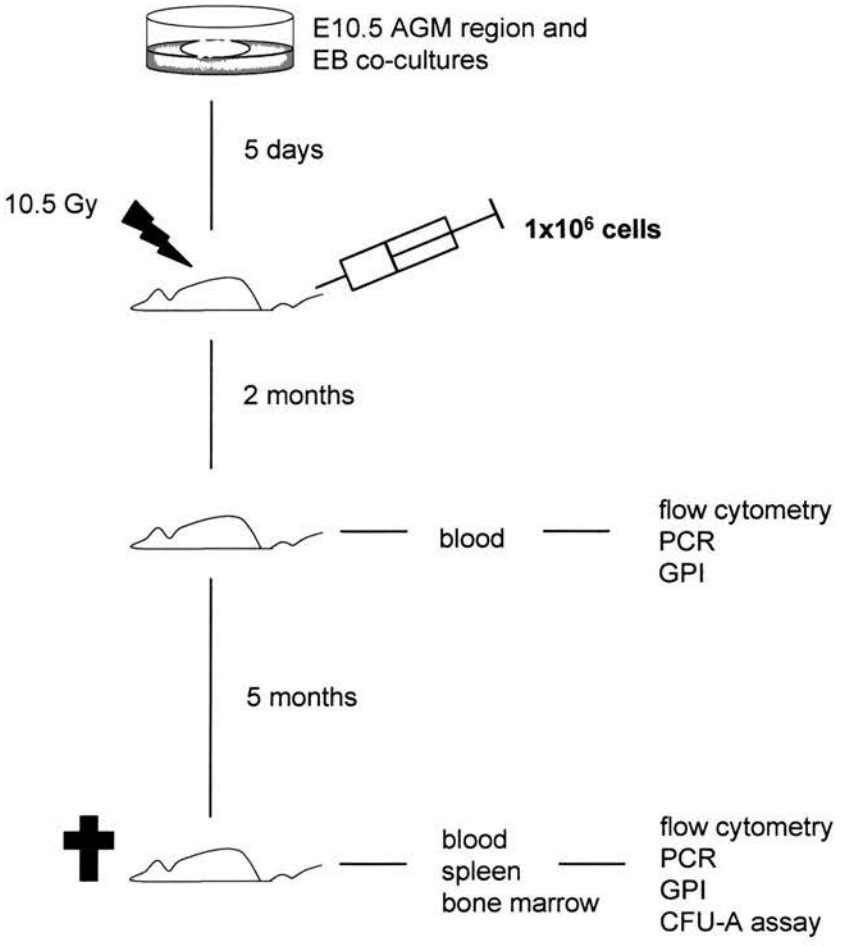


Figure 4.6 Flow diagram illustrating procedure for transplantation experiment. Co-cultured EBs and AGM region were dissociated and $1x10^6$ cells were injected into lethally irradiated female 129 recipient mice (n=5). (See Table 4.1 and 4.2 for cell markers and information on control mice respectively). 2 months after injection blood was taken from surviving mice and analysed for the presence of EB or AGM-derived cells. After a further 5 months the mice were killed, the blood, spleens and bone marrow were harvested for further analysis. See also Chapter 2: Materials and Methods, section 2.9

	GPI-1	GFP	Ly9.1	*YMT*	*LacZ*
Recipient mice 129/Sv female	A	-	+	-	-
ES cells (GFP#7A) 129/Sv male	A	+	+	+	-
AGM region cells C57Bl/6 female/male	B	-	-	+	-
Spleen carrier cells GT411/129/Sv female	A	-	+	-	+

Table 4.1 Markers used to distinguish donor and host cells after transplantation. GPI-1 alloenzymes were distinguished by electrophoretic separation. GFP and Ly9.1 expression was detected by flow cytometry. The presence *YMT* and *LacZ* sequences in genomic DNA was detected by PCR.

group (n=5)	carrier spleen ($2x10^5$ cells)	co-cultured AGM and EB ($1x10^6$ cells)	survival
1	-	-	10-12 days (5/5)
2	+	-	~1 month (5/5)
3	-	+	10-12 days (2/5) ~7 months* (3/5)
4	+	+	10-12 days (5/5)

Table 4.2 Survival data. All recipient mice were irradiated wth 10.5 Gy γ-rays before being injected with cells as indicated. *these mice were healthy by were killed for analysis of donor EB-cell contribution to haematopoietic tissues

Survival of the control mice was as expected; the non-injected irradiation controls died after 10-12 days and the mice injected only with carrier spleen cells survived approximately one month. Three out of the five mice given co-cultured cells survived for the duration of the experiment and were killed seven months after transplantation for analysis. All the mice injected with co-cultured cells accompanied by spleen carriers died unexpectedly after only 10-12 days. It is most likely that these animals developed graft-versus-host (GVH) disease, or more accurately a "graft-versus-graft" response. Mature T cells from the carrier spleen cells would have recognised the AGM region cells as foreign. It is perhaps surprising that this did not merely result in a failure of the AGM region cells to engraft, but apparent systemic immune disease killing the animals. *In vitro* immunologic assays (such as testing for T-cell cytotoxicity by a mixed lymphocyte reaction) could be set up to test this hypothesis. In future experiments this could also be avoided by depleting the T cells from the carrier spleen by irradiation, which would also rule out the possibility of long term engraftment by stem cells in the spleen. In the concurrent but independent transplantation experiment none of the animals injected with $1x10^6$ day 5 EB cells survived. Two out of the five mice, who received carrier spleen cells in addition to EB cells, survived over 7 months as previously reported (Hole et al., 1996) and (data not shown). The survival data for the present study are shown in **Table 4.2**.

Eight weeks after transplantation, peripheral blood was taken from the three surviving mice. The blood was analysed (i) by flow cytometry for any GFP expressing cells and for the presence of Ly9.1, (ii) for the GPI isoenzyme assay and (iii) genomic DNA was extracted for PCR analysis and (see Chapter 2: Materials and Methods, section 2.8.1). No GFP^+ cells were detected in any of the animals by flow cytometry (**Figure 4.7**). From PCR amplification of the genomic DNA with primers specific for the *YMT* gene, it was apparent that all three mice had been repopulated with male cells (**Figure 4.8a**). Due to the absence of any GFP, differentiated ES cells could not be responsible. However, the small number of cells expressing Ly9.1 (**Figure 4.7**) suggested that most of the peripheral blood was not host derived. GPI analysis demonstrated that the peripheral blood consisted of GPI-b cells, confirming that the AGM region cells had engrafted in the host animals and were contributing to

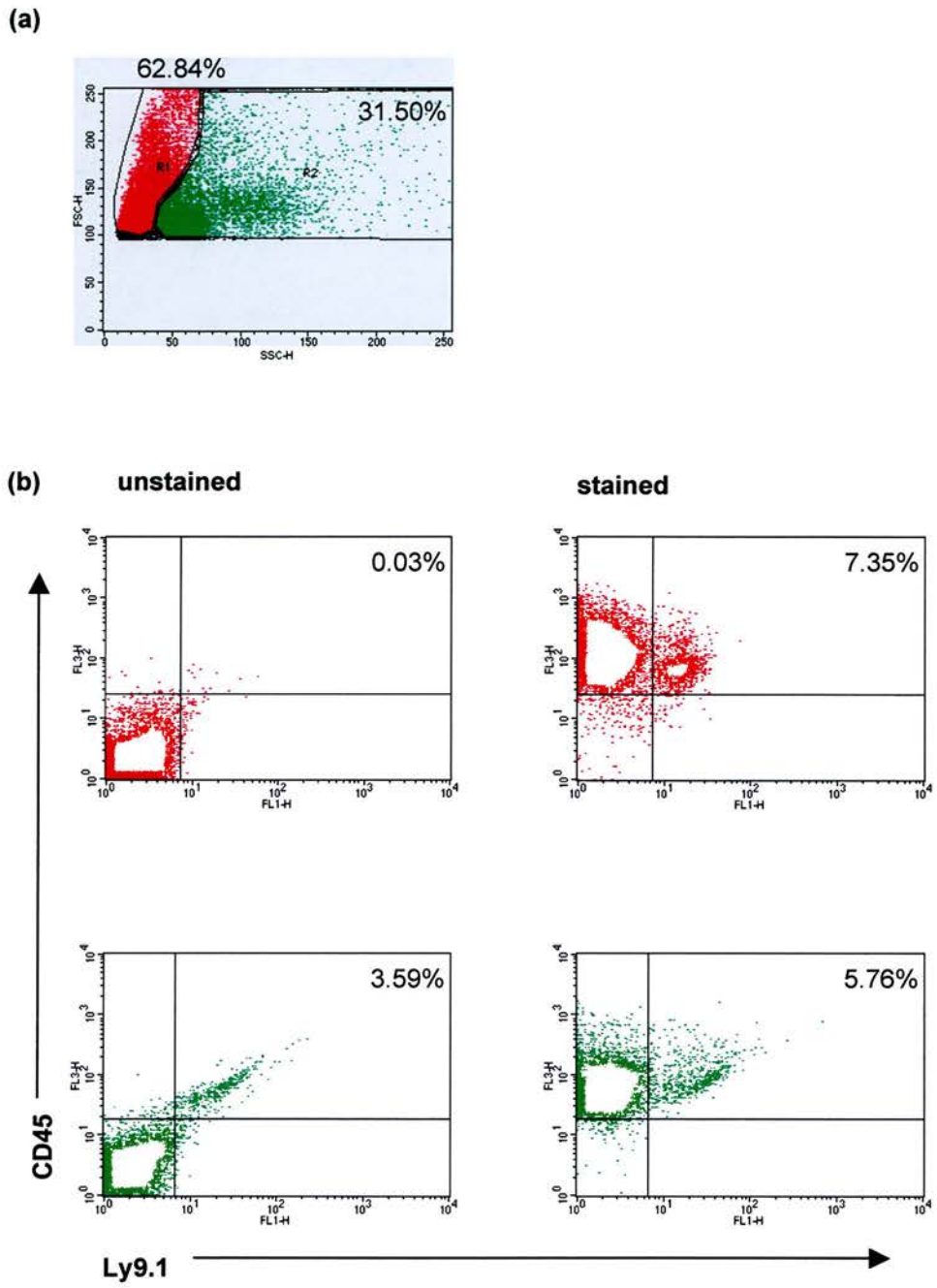


Figure 4.7 Peripheral blood shows no haematopoietic repopulation from ES-derived cells. Flow cytometric analysis of peripheral blood taken from recipient mice 2 months after being injected with $1x10^6$ co-cultured AGM and ES cells. Histograms show data from one representative animal (AK3). (a) FSC and SSC profiles of PB. The R1 gate (●,62.84% total) denotes lymphocytic cells. The R2 gate (●,31.50% total) denotes granulocytic cells. The cells were gated for analysis because the granulocytes have higher levels of autofluorescence, which interferes with the detection of GFP and surface antigen expression. (b) PB unstained and stained with antibodies against CD45 and Ly9.1 is shown as gated in (a). Unstained samples show no GFP^+ cells in the FL1 channel. A small population of $CD45^+$ haematopoietic cells, both lymphocytes (7.32%) and granulocytes (2.16%), express Ly9.1 indicating that they are host derived. Flow cytometric analysis was done by Kay Samuel.

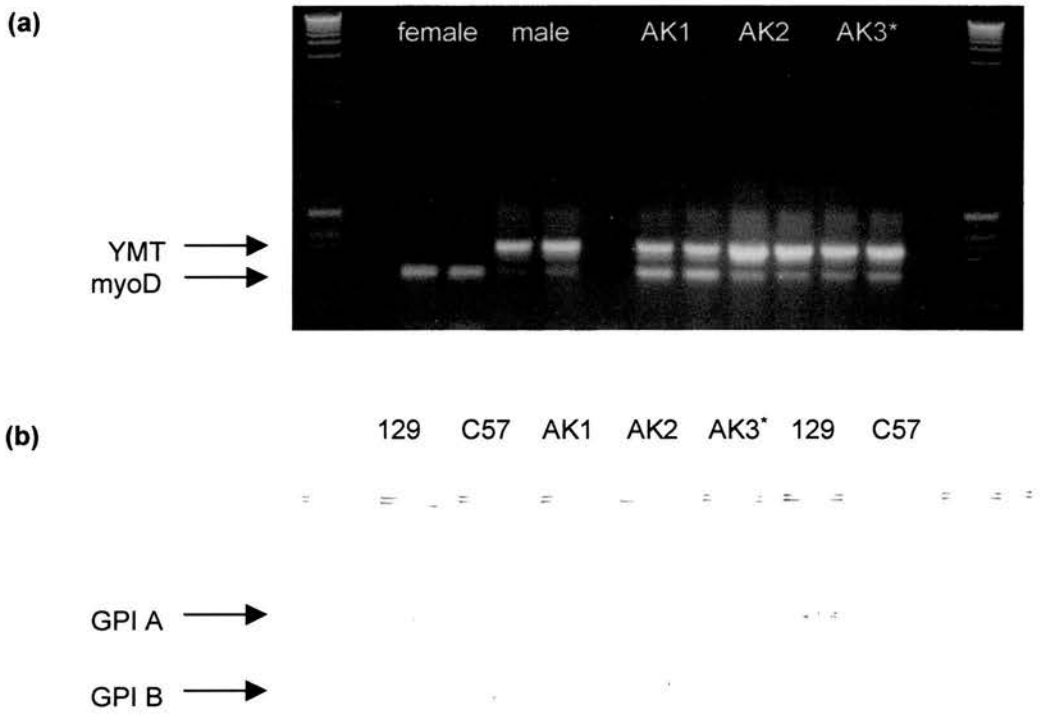


Figure 4.8 Surviving recipient mice are repopulated by AGM region-derived cells. PCR and GPI analysis of peripheral blood taken from female irradiated 129 mice 2 months after being injected with $1x10^6$ co-cultured AGM and ES cells. (a) PCR for Y-chromosome specific *YMT* gene shows all three recipient mice are repopulated with male cells. The amplification of the *MyoD* gene was used as an internal control for the reaction (b) GPI analysis shows all three recipient mice are repopulated with GPI B cells, in this case from the C57Bl/6 mouse strain, donor of the AGM regions. * indicates the recipient animal shown in Figure 4.7

the haematopoiesis (**Figure 4.8b**). In the concurrent control experiment, no GFP^+ cells or *YMT* expression was detected in the two surviving animals that received EB cells with carrier spleen. Interestingly, expression of the *LacZ* reporter gene was detected by PCR suggesting engraftment of the carrier spleen cells (data not shown).

To investigate the possibility of long-term engraftment of EB-derived cells from the co-cultures, the three surviving animals were killed 7 months after transplantation and their haematopoietic tissues (peripheral blood, spleen and bone marrow) were analysed. Again, no GFP^+ cells were detected by flow cytometry (data not shown, as in Figure 4.7). It was concluded from these data that the survival was due to engraftment of AGM region-derived cells and confirmed by the small percentage of cells expressing Ly9.1. The remaining spleen and bone marrow cells were used to set up CFU-A assays in an effort to expand any ES-derived cells that might have been present but were not sufficiently abundant for detection by flow cytometry. The CFU-A colonies were scrutinised by fluorescence microscopy, but none of the progeny were visibly GFP^+ (data not shown).

Again, there was persistent haematopoietic contribution by carrier spleen cells in the animals in the concurrent control experiment (data not shown).

A summary of the results described in this section is shown in **Table 4.3**.

4.5 Discussion

The data demonstrate that the differentiation of ES cells into early haematopoietic progenitors *in vitro* is enhanced by co-culture with the embryonic AGM region. The gestational age of the embryo from which the tissue was dissected is important. As shown in **Figures 4.2** and **4.3**, E10.5 AGM region has a more potent effect on haematopoietic differentiation than one day later in development. *In vivo* HSCs emerge from the AGM region after E10 and by E11.5 their numbers have peaked. One could hypothesise that the E10.5 AGM region is an *inductive* microenvironment for HSCs whereas the E11.5 AGM may be more *expansive*, and this activity is reflected on ES cell differentiation. However, there is no direct evidence for this and it is purely speculative to draw such conclusions at this stage from only these data. To test such a hypothesis one would have to be able to

	2 months			7 months								
	PB			PB			Spl			BM		
	1	2	3	1	2	3	1	2	3	1	2	3
GFP[1]	-	-	-	-	-	-	-	-	-	-	-	-
Ly9.1	-	-	-	-	-	-	-	-	-	-	-	-
YMT	+	+	+	+	+	+	+	+	+	+	+	+
GPI	B	B	B	nd	nd	nd	nd	nd	nd	nd	nd	nd
GFP[2]	nd	nd	nd	nd	nd	nd	-	-	-	-	-	-

Table 4.3 Summary of the analysis of the transplantation experiment. The numbers (1, 2 and 3) represent the three surviving mice AK1, AK2 and AK3. GFP[1] stands for analysis by flow cytometry. GFP[2] denotes visual examination of CFU-A colonies. nd = not determined.

compare the proportion of EBs in culture which show CFU-A activity *and* the number of CFU-A progenitors within each EB after co-culture with AGM region from embryos of various stages and control EBs alone. This is not possible using the co-culture system because individual EBs cannot be harvested intact from the cultures.

The possibility that AGM region cells were promoting the formation of colonies within the CFU-A assay itself was ruled out by plating mixtures of cells from AGM regions and EBs that were cultured separately. No increase in EB-derived CFU-A activity was found (data not shown).

Co-culture of EBs with foetal liver did not result in an increase in haematopoietic activity. There are several possible explanations for this result. First, the foetal liver may not be an inductive environment. Second, the organ rudiment explant culture conditions may not be suitable for culturing pieces of foetal liver. Third, the foetal liver was taken from E13.5 embryos because it is at this stage during development when the haematopoietic activity in the organ has peaked in terms of number of LTR-HSCs. It could be reasoned however, that since HSC activity is present in the foetal liver from day 11 of gestation, earlier stages could have been effective.

Another experiment, which could be suggested, is to co-culture EBs with yolk sac, which has repopulating activity from E11. More significantly, definitive, adult-type HSCs have recently been found to expand in organ culture at E12.5, and may perhaps even be generated in this tissue (Kumaravelu et al., 2002).

Nevertheless, the AGM region is the only tissue tested here that increases haematopoietic differentiation from ES cells; control experiments using limb buds, somitic tissue and foetal head also had to effect (data not shown).

The CFU-A assay is not widely used despite being established in the literature (see Chapter 1: Introduction). Therefore, the emergence of CFU-A over a time-course of EB differentiation was compared to a more widely used assay, the HPP-CFC. These data are shown in **Figure 4.5**. The CFU-A and HPP-CFC assays detect equivalent progenitors in the haematopoietic hierarchy, though the CFU-A are reported to be more mature than the most primitive HPP-CFC (see Chapter 1: Introduction). This may explain why overall there were fewer HPP-CFC colonies

than CFU-A colonies. HPP-CFC activity in differentiating EBs cultured alone was as previously demonstrated by others (Palis et al., 2001), as was the CFU-A activity (H.T., unpublished). A direct comparison of ES cell differentiation using both haematopoietic colony assays has not been reported previously. Co-culture with the E10.5 AGM region resulted in a substantial rise in the number of progenitors detected by both assays peaking at six days of differentiation; where there was a 20-fold increase in CFU-A activity, the increase in HPP-CFC activity was more than 50-fold. No published differentiation protocol has resulted in such a substantial increase in haematopoietic progenitors from ES cells.

Since haematopoietic progenitors arise within spontaneously differentiating EBs it is not possible to determine from these data whether the increase in CFU-A and HPP-CFC after AGM region co-culture is due to *de novo* induction from precursors or proliferation of progenitors. When EBs were allowed to differentiate for three days prior to co-culture with the AGM region, there was no significant increase in haematopoietic activity (**Figure A.2** in Appendix). This is unexpected if the expansion of existing precursors was solely responsible for the increase observed and would support the hypothesis of an inductive role for the AGM region. However, to test such a hypothesis, as stated above, one would need to determine the proportion of EBs in co-culture and alone which exhibit CFU-A activity *and* the number of CFU-A progenitors within each EB. Furthermore, since CFU-A have self-renewal potential as determined by their ability to form secondary (2°) CFU-A colonies on replating, it would be interesting to examine the effect of AGM region co-culture on this proliferative potential both in terms of the proportion of original CFU-A colonies that can generate 2° CFU-A colonies and their frequency within the original CFU-A.

The CFU-A and HPP-CFC assays are not a measure of engraftable HSCs capable of persistent reconstitution of the adult haematopoietic system. That was assessed by transplantation into haemato-compromised animals and the subsequent long-term analysis of haematopoiesis for contribution from the donor cells. Unfortunately no evidence of engraftment by co-cultured ES-derived cells was found and the survival of the experimental animals was due to repopulation by HSCs from the AGM region, which were present because the cells from the co-cultures were not

sorted prior to injection. A previous study based on a similar time-course of ES differentiation (Hole et al., 1996) reported the survival of lethally irradiated mice injected with EB cells but only when accompanied by carrier spleen cells. However, the level of engraftment by the EBs was so low they could only be detected by PCR amplification of the Y chromosome specific sequence after hybridisation. In that study, the carrier spleen cells were not distinguishable from the host animals so their potential long term engraftability could not be determined. Control mice receiving only spleen cells were rescued over the short term, which is the function of carrier spleen cells. These experiments were repeated concurrently with the present transplantation study, but using spleens from genetically marked animals as carriers to enable their detection. The apparent long term reconstitution of the haematopoietic system by spleen cells when co-injected with differentiated ES cells was unexpected. This observation leads to speculation that the EB cells produce a factor or factors, which facilitate engraftment by spleen derived cells. In support of this hypothesis, conditioned media from ES cells and differentiating EBs have been shown to increase the number of initiating cells (IC) in long term cultures (LTC) of primitive human progenitors (Croizat and Bouhassira, 1999) and CFU-S colonies were generated (at a higher frequency than the irradiation controls) after injection of EB derived cells, but no evidence of donor cell contribution was found (Muller and Dzierzak, 1993). Also, in EB and AGM region co-cultures, the increase in haematopoietic activity was not confined to the EB cells but the CFU-A activity of the AGM regions was also increased (data not shown). However more experiments are needed to substantiate this because of the high variation in haematopoietic activity in cultured AGM regions (**Figures 3.1** and **3.3**). Indeed, in support of a facilitative role for differentiating ES cells, the level of stable engraftment by the AGM region cells after co-culture with EBs in the three surviving mice was very high. Unfortunately no control animals were injected only with AGM region cells (due to lack of material) to verify such a possibility.

The possibility of very low level engraftment cannot be ruled out for the transplantation experiment described above, but it is unlikely because bone marrow and spleen cells harvested from transplanted mice were plated into the CFU-A assay and no GFP^+ colonies were found on examination using fluorescence microscopy.

Any EB derived HSCs would probably have been out-competed by HSCs from the AGM region. In future experiments, co-cultured EB cells should be sorted from AGM region cells prior to injection.

A CFU-A assay was set up at day 6 of differentiation (one day after transplantation) with cells co-cultured in parallel to the transplanted cells. In this particular experiment very low numbers of CFU-A colonies were generated by both co-cultured and control EBs (data not shown). This was not a good indicator of cell differentiation and/or quality.

There could be several additional reasons for the failed engraftment of EB cells. Cells expressing low levels of MHC Class I are recognised and cleared by the animal's NK cells. MHC I is not highly expressed by early gestation embryos before the development of the immune system (Ozato et al., 1985) and was similarly not found on the surface of ES cells (K.S., unpublished). However, when a chromium release assay was set up to measure the killing of ES cells by NK cells *in vitro*, no such activity was found (**Figure A.3** in Appendix). A criticism of this experiment however, is that the assay should also have been performed with cells from differentiated EBs to recapitulate the transplanted population. The expression of MHC I on differentiated mouse ES cells is unknown, but undifferentiated human ES cells have been found to express very low levels of MHC I, which is slightly elevated upon differentiation. Furthermore, only limited cytotoxic NK cell activity against both undifferentiated and differentiated cells was observed in this study (Drukker et al., 2002).

Although reconstitution is possible with a single HSC, perhaps $1x10^6$ EB cells does not contain a sufficient number of HSCs. If AGM region co-culture can give rise to 200 CFU-A colonies per $3x10^4$ cells, this still only represents 0.7% of the total EB cell population (0.2% in the case of HPP-CFC) and the number of HSCs from which they descended is presumably even smaller. A possibility would be to sort the EB cells on the basis of surface markers associated with HSCs (e.g. KTLS; see section 1.2.2). Preliminary flow cytometric analysis showed an increase in the number of cells from EBs expressing c-kit and CD34 after AGM region co-culture (**Figure A.4** in Appendix). However, Sca-1 is not detected at significant levels in differentiating ES cells. The *Ly-6* gene locus encoding Sca-1 shows strain-dependent

allelic variation. The 129 mouse, from which ES cells are most frequently derived, is a *Ly-6* strain which only expressed Sca-1 in on about 25% of HSCs in the bone marrow (Spangrude and Brooks, 1993) and might partly attribute to the low levels of Sca-1 detected on differentiated ES cells and may therefore not be a useful marker for ES-HSC.

The significant increase in repopulation of ES-derived HSCs after intra bone marrow injection (Kurt and see Chapter 1: Introduction, section 1.5.3) indicates that homing to the bone marrow is defective. Therefore it would be interesting to determine whether AGM region co-culture enhances the surface expression of antigens, associated with homing to and entry into the bone marrow, such as the chemokine receptor CXCR4 (Peled et al., 1999) or adhesion molecules, for example VLA-4/VCAM-1 (Chan and Watt, 2001). In addition, since the ectopic expression of *HoxB4* has recently been found to confer ES derived cell with LTR-HSC activity and postulated to be a molecular "switch" for definitive haematopoiesis (Kyba et al., 2002); see section 1.5.3), it would be interesting to see if AGM region co-culture induces expression of this gene.

Chapter Five:

Haematopoietic differentiation of ES cells by stromal cell co-culture

5.1 Aim

To examine the effect of stromal cell lines derived from the haematopoietic tissues (AGM region and foetal liver) of the midgestation mouse embryo on the differentiation of ES cells.

5.2 Introduction

The experiments in Chapter 4 demonstrate that co-culture with the AGM region, using an explant culture system, significantly increases the haematopoietic differentiation of ES cells *in vitro*. However, the biological mechanisms involved in this process are unknown. In an attempt to gain a mechanistic insight, co-culture experiments using stromal cell lines derived from the embryonic AGM region and liver were set up. These experiments were designed to address three important questions, which may be useful in the identification of the haematopoietic promoting factor(s). (i) What is the phenotype of the cell or cells within the AGM region which support the haematopoietic differentiation of ES cells, (ii) are the haematopoietic promoting factors dependent on cell-cell contact or are they secreted and (iii) is the increased haematopoietic activity a result of *de novo* induction or proliferation of committed progenitors.

5.2.1 Stromal cell lines

The most extensive and well characterised panel of stromal cell lines derived from tissues of the midgestation embryo was generated by members of Elaine Dzierzak's laboratory (Oostendorp et al., 2002a; Oostendorp et al., 2002b).

Mice transgenic for the temperature-sensitive form (*tsA58*) of the SV40 *Tag* immortalising gene under the control of the β-actin or PGK promoters were generated for the purpose of establishing the cell lines. Control BL1b mice, which express the *LacZ* reporter gene under the control of *Ly6E*, since Sca-1 is reportedly expressed on most stromal lines (Montecino-Rodriguez et al., 1994), were also used. Stromal cell lines were derived from all three transgenic lines, but with much greater efficiency from those harbouring the immortalising *tag* gene. More than 100 clonal

cell lines were established from the liver, gastrointestinal region and subdissected AGM region of E10 and E11 transgenic embryos. Most of these lines were tested for their ability to maintain haematopoietic progenitors and HSCs (Oostendorp et al., 2002a; Oostendorp et al., 2002b).

In the present study, three of the cells lines found by Oostendorp and others to be most supportive of HSCs, UG26.1B6, EL08.1D2 and AM20.1B4 (a kind gift from Prof. Dzierzak) were tested for their effect on differentiating ES cells. The AM20.1B4 line was derived from the dorsal aorta and surrounding mesenchyme of the AGM region dissected from an E10 *tag* expressing embryo. UG26.1B6 was derived from the urogenital ridge of the AGM region from a *tag* expressing embryo at E11. EL08.1D2 was derived from the liver of an E11 control BLb1 embryo (**Figure 5.1**).

5.3 Experimental approach

- Measure the CFU-A activity in EBs after co-culture on primary cultures of adherent cells derived from disassociated AGM regions as an alternative co-culture strategy to the explant method, which could generate preliminary data on the effect of contact compared to non-contact culture.
- Compare the expression of a selected panel of surface markers on the three candidate stromal cell lines by flow cytometric analyses.
- Determine the effect of co-culture with the three candidate stromal cell lines on the haematopoietic differentiation of ES cells as measured by the CFU-A assay.
- Examine the effect of conditioned medium from the stromal cell lines on the haematopoietic differentiation of ES cells to determine if cell-cell contact is required.
- Measure the CFU-A activity in individual EBs cultured on the candidate stromal cell lines to investigate whether any increase in haematopoietic differentiation was a result of *de novo* induction or proliferation of committed progenitors. A hypothesis is that *de novo* induction of haematopoietic cells would result in an increase in the proportion of EBs that exhibit CFU-A activity, whereas an

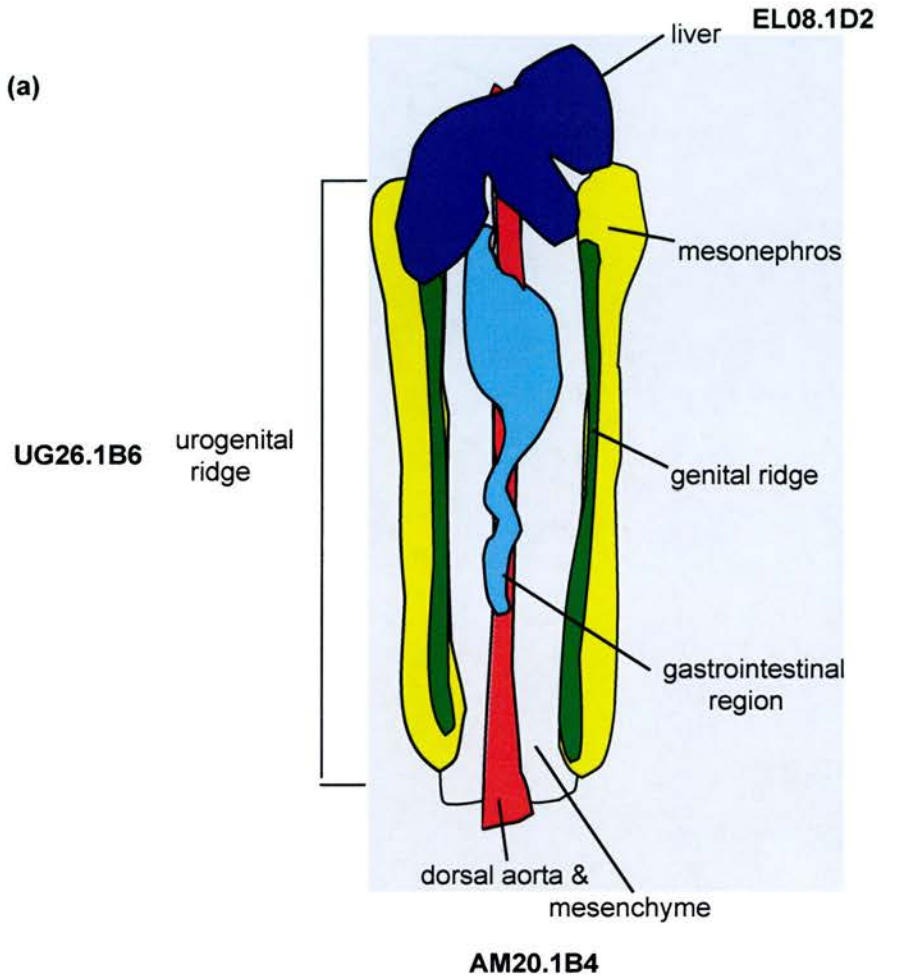


Figure 5.1 Anatomical origin of the stromal cell lines. (a) Diagram illustrating the midgestation AGM region, liver and gut from the ventral side, to highlight the subregions from which the stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2 were derived. Adapted from Oostendorp *et al*, 2002. (b) Photographs of the corresponding region dissected from an E11 embryo, taken from a dorsal and a lateral view respectively.

increase in the number of colonies generated from each EB would indicate the proliferation of progenitors.

- Investigate the capacity of the stromal cell lines for supporting ES cell self-renewal.
- Assess the effect of stromal cell line co-culture on the differentiation of ES cells into cardiomyocytes.

5.4 Results

5.4.1 CFU-A activity in EBs after co-culture on primary adherent AGM region cells.

AGM regions from E10.5 embryos were dissociated to obtain a single cell suspension, which was then seeded onto gelatin coated 24-well plates. The adherent cells formed confluent monolayers within a week in culture onto which, day 1 EBs produced in hanging drops were plated at a frequency of one EB per well. In addition, half of the EBs were cultured in transwell inserts, which inhibit direct contact between the AGM region cells and the differentiating ES cells but allow secreted molecules to pass freely (**Figure 5.2a** and Chapter 2: Materials and Methods, section 2.2.4.2). This co-culture experiment was set up to determine (i) if the haematopoietic activity of differentiating ES cells is also increased by culture on primary cells from dissociated AGM regions (with a view to using the stromal cells lines) and (ii) whether direct cell-cell contact is required.

50% of the EBs plated onto gelatin contained CFU-A progenitors after six days of differentiation (5 days in co-culture) (**Figure 5.2b**), which was as expected (Hole et al., 1996; Lako et al., 2001), see section 3.4.2 and **Figure 3.5a** and **b**). The number of CFU-A colonies derived from these individual EBs cultured on gelatin ranged from 0 to 3, with a mean of 0.92 colonies per EB including those with no CFU-A activity (**Figure 5.2c**). Similar observations have been made previously (Hole et al., 1996). 70% of the EBs cultured directly on AGM region derived cell monolayers gave rise to colonies in the CFU-A assay and each contained an average of 10.5 CFU-A colonies per EB, ranging from 0 to 47. When EBs were cultured in

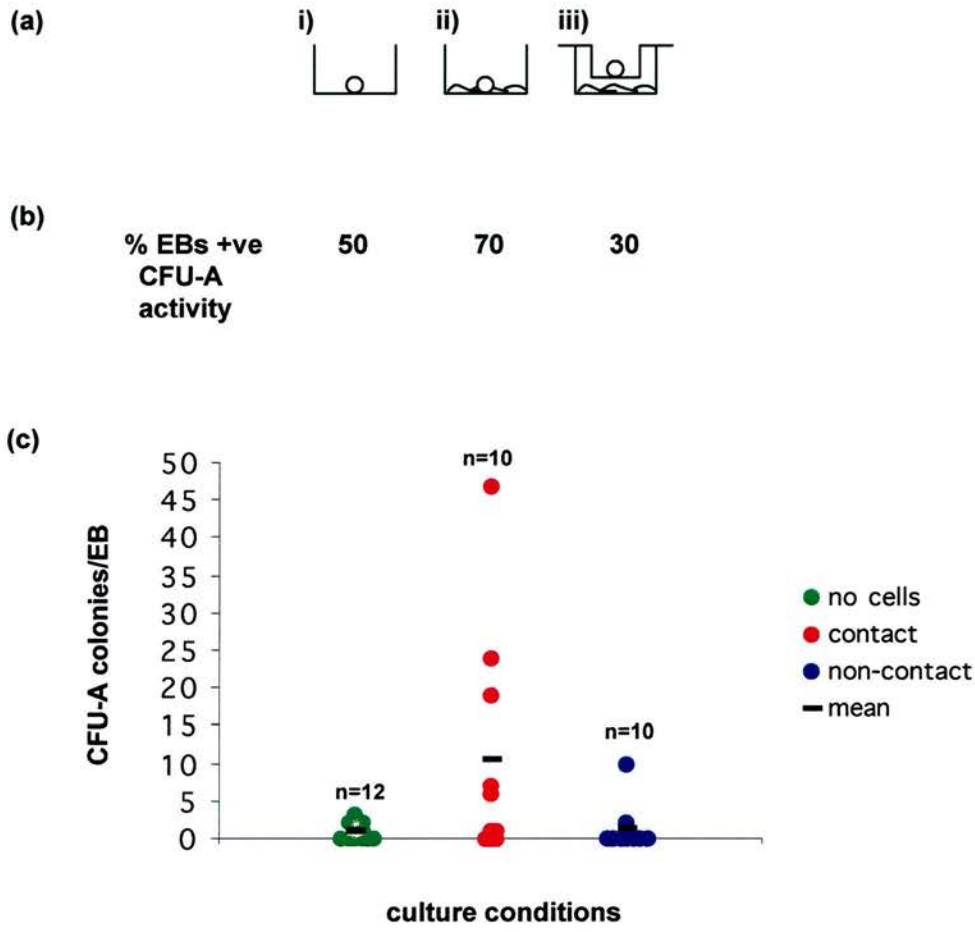


Figure 5.2 CFU-A activity in individual embryoid bodies after co-culture on AGM region derived cells. (a) Diagram illustrating the co-culture of EBs on AGM region derived cells either i) on gelatin with no AGM cells, ii) in direct contact and iii) in non-contact cultures using transwell inserts. Single EBs were cultured per well in a 24-well plate for 6 days. (b) The proportion of EBs (%) that produced CFU-A colonies. (c) The number of CFU-A colonies per EB after culture on gelatin (●), in direct contact with AGM region cells (●) and in non-contact cultures (●). Each data point represents the number of colonies from an individual EB. Bars (▬) indicate the mean number of colonies. Numbers of individual EBs analysed are shown.

transwell inserts over the AGM region derived cells only 30% showed CFU-A activity. The number of colonies ranged from 0 to 10, but the mean was only 1.3 colonies per EB, because although one EB generated 10 colonies, the majority (70%) displayed no activity in this assay. The primary cultures of adherent AGM region cells alone did not give rise to any colonies in the CFU-A assay (data not shown).

These data support the use of primary cultures of dissociated AGM region derived cells to provide an effective environment for enhancing the haematopoietic differentiation of ES cells with approximately 10 fold increase in CFU-A activity. (However, the data are not significant with >95% confidence in a non-parametric test, $p < 0.138$ for contact cultures compared to control EBs cultured on gelatin as determined by the Mann-Whitney U Test.) These data also suggest that direct cell-cell contact is important.

It was next investigated whether the haematopoietic-enhancing capacity could be maintained in clonal cells lines derived from subdissected AGM regions **(Figure 5.1)**.

5.4.2 Expression of surface antigens on the stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2

The stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2 were examined by flow cytometry for the expression of a small panel of cell surface antigens. These included markers associated with haematopoietic cells (e.g. CD45, c-kit and Sca-1), endothelial cells (e.g. CD31 and Flk-1) or stromal cells (e.g. CD29, CD44, CD49e, CD106 and Sca-1). Sca-1 is a known marker of LTR-HSCs, but also associated with stromal cells and is found on several other tissues (see Chapter 1: Introduction, section 1.2.2). A complete list is provided in **Table 5.1**.

Markers for haematopoietic (except Sca-1) and endothelial lineages were not detected on any of the three cell lines. In contrast they were found to express surface antigens associated with stromal cells (**Table 5.1** and **Figure 5.3**). In this analysis CD49e (α_5 integrin) expression was higher on the AM20.1B4 stromal cell line and Sca-1 expression was lower on UG26.1B6 cells compared to the other cell lines.

		UG26.1B6	AM20.1B4	EL08.1D2
CD29	Integrin β_1 chain	+	+	+
CD31	Pecam-1	-	-	-
CD44	Polymorphic glycoprotein 1	+	+	+
CD45	Common leukocyte antigen	-	-	-
CD49e	Integrin α_5 chain	+	++	+
CD51	Integrin α_v chain	+/-	+/-	+/-
CD61	Integrin β_3 chain	-	-	-
CD106	VCAM-1	+	+	+
CD117	c-kit	-	-	-
Sca-1	Ly6A/E	-	+/-	+/-
Flk-1	VEGF-R2	-	-	-

Table 5.1 Relative expression levels of surface antigens on stromal cell lines derived from the embryonic AGM region and liver.

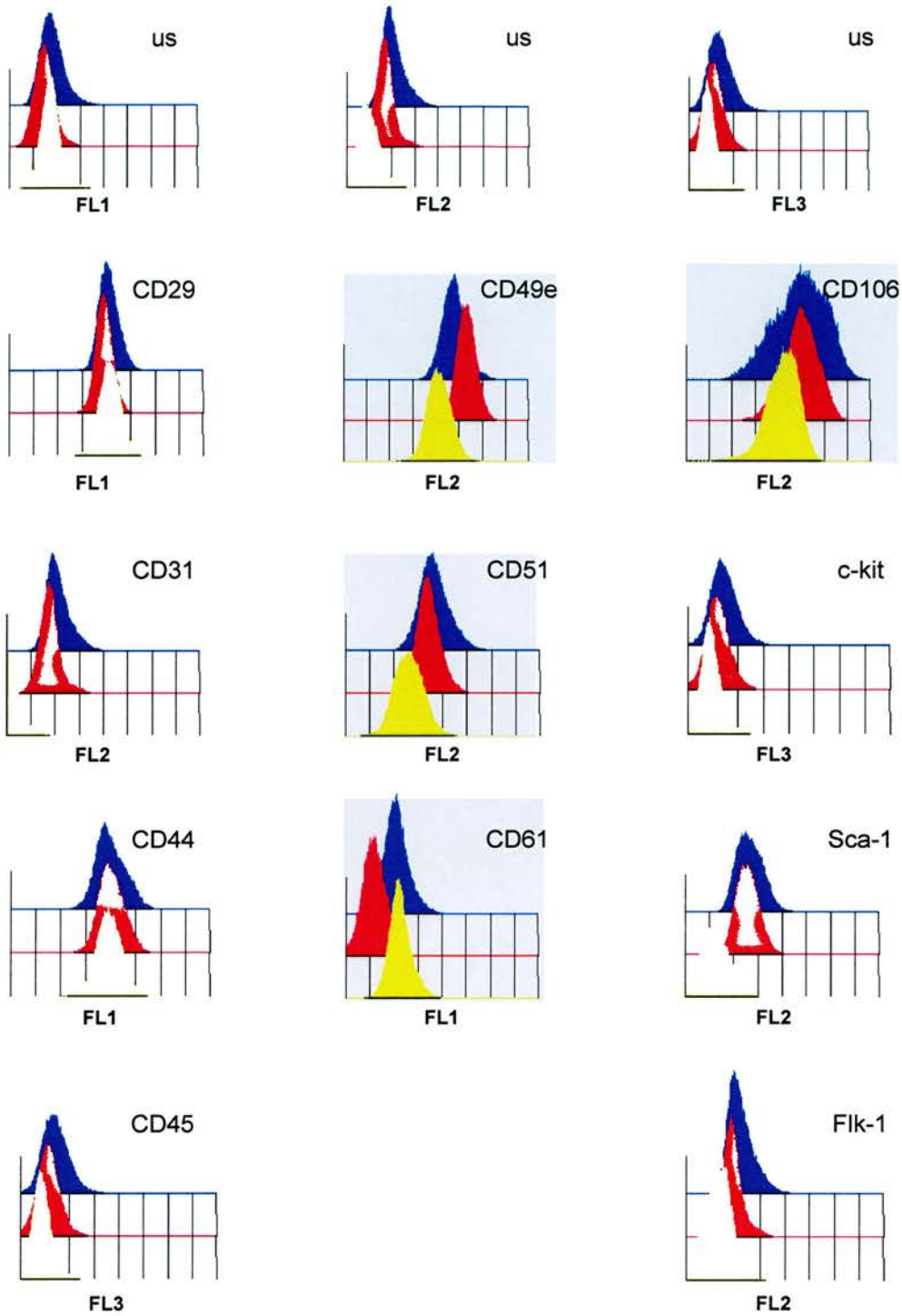


Figure 5.3 Surface antigen expression on stromal cell lines derived from the embryonic AGM region and liver by flow cytometry. Top panels show unstained cells in fluorescence channels 1, 2 and 3. Detection of antibodies against CD29, CD31, CD44, CD45, CD49e, CD51, CD61, CD106, c-kit, Sca-1 and Flk-1 are shown on AM20.1B4 (■, UG26.1B6 ( ) and EL08.1D2 ■).

Apart from these subtle differences, there was no marked difference between the three cell lines in terms of surface marker expression.

5.4.3 Haematopoietic differentiation of ES cells after co-culture on stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2

The CFU-A activity of EBs after co-culture on stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2 was compared after six days of differentiation (see Chapter 2: Materials and Methods, section 2.2.4.3). Only co-culture with the stromal cell line derived from the dorsal aorta and surrounding mesenchyme of the AGM region (AM20.1B4, see **Figure 5.1**) resulted in an increase in CFU-A activity (145.2 ± 15.3 colonies/$3x10^4$ cells) compared to EBs cultured on gelatin (39.7 ± 4.95 colonies/$3x10^4$ cells); a 3.5 fold rise. In contrast, a reduction in the number of CFU-A colonies was observed in EBs co-cultured with the other cell lines, UG26.1B6 (10.3 ± 0.8 colonies/$3x10^4$ cells) and EL08.1D2 (8.7 ± 1.1 colonies/$3x10^4$ cells) by approximately 4 fold in both cases (**Figure 5.4**).

5.4.4 The effect of conditioned medium from the stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2 on the haematopoietic differentiation of ES cells

The CFU-A activity of EBs cultured for six days in ES differentiation medium containing 50% conditioned medium from the stromal cell lines AM20.1B4, UG26.1B6 or EL08.1D2 was compared. Control EBs were cultured in ES differentiation medium containing 50% fresh stromal medium to rule out any effect from the medium (see Chapter 2: Materials and Methods, section 2.2.4.4). CFU-A activity was assessed both in terms of the number of colonies per $3x10^4$ cells derived from a population of dissociated EBs and the proportion of EBs which gave rise to the colonies determined by plating intact EBs into the assay.

Conditioned medium had no consistent effect on the haematopoietic differentiation of ES cells (**Figure 5.5**). The data shown are compiled from two independent experiments. In one experiment, culture of EBs in conditioned medium

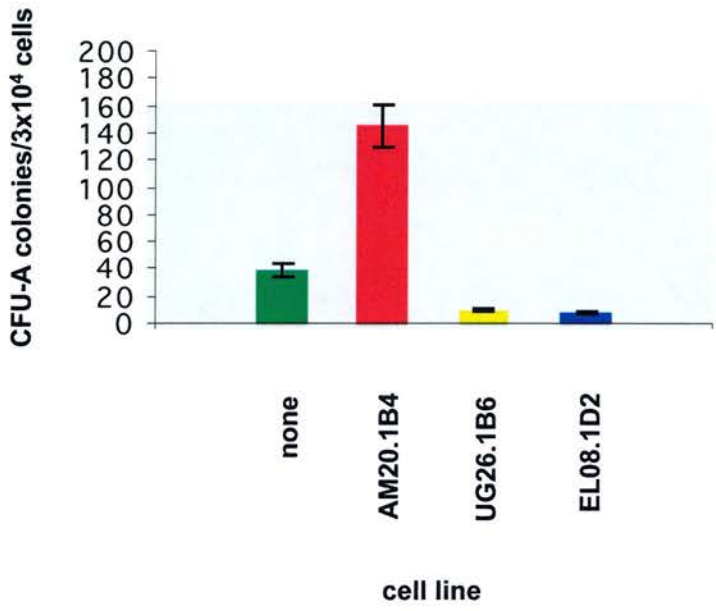


Figure 5.4 CFU-A activity in embryoid bodies after co-culture on stromal cell lines derived from the embryonic AGM region and liver. Numbers of CFU-A colonies/3x10^4 cells in EBs cultured for 6 days on gelatin (, AM20.1B4 ( ), UG26.1B6 ( ) and EL08.1D2 ( ) cells. Error bars represent the standard deviation of the mean of triplicate plates. The data shown is representative of three independent experiments.

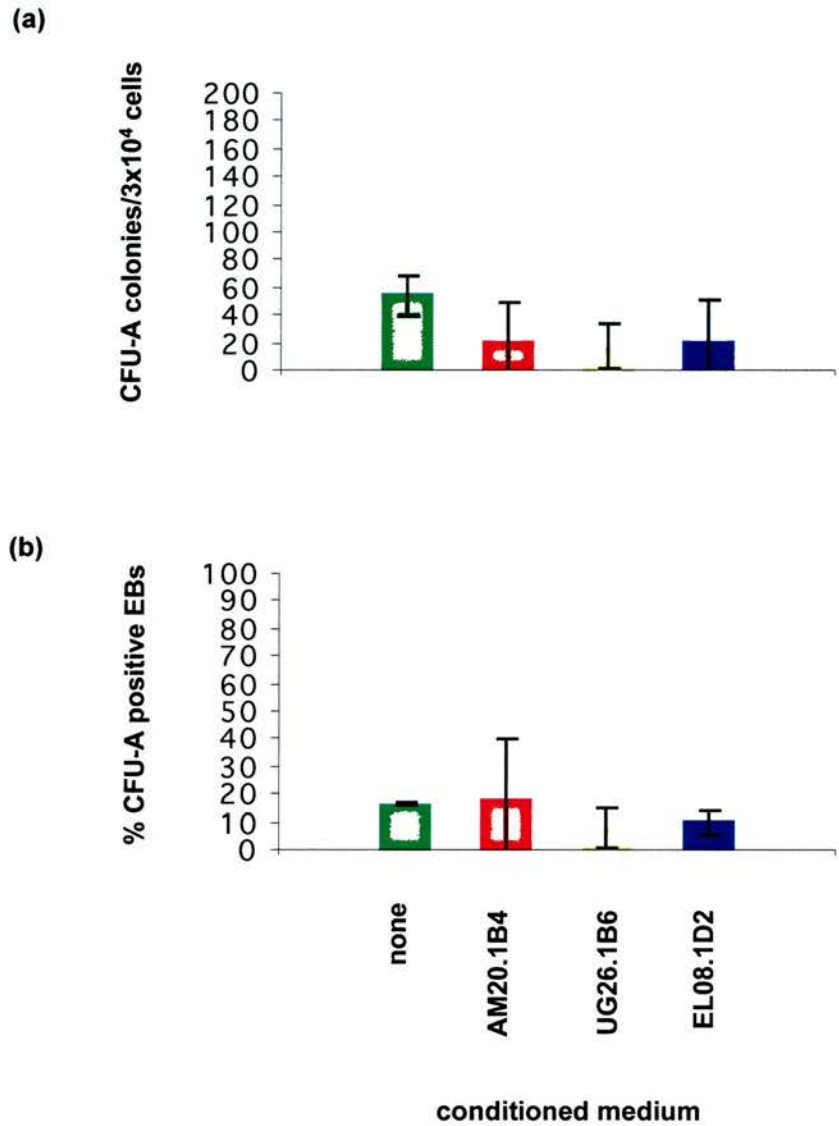


Figure 5.5 CFU-A activity in embryoid bodies after culture in conditioned medium from stromal cell lines derived from the embryonic AGM region and liver. (a) Numbers of CFU-A colonies/3x10^4 cells and (b) percentage of EBs which gave rise to CFU-A colonies after culture for 6 days in "standard" medium (50% ES differentiation medium/50% stromal medium, ■) or ES differentiation medium containing 50% conditioned medium from AM20.1B4 (■), UG26.1B6 ( ) and EL08.1D2 (■) cells. Error bars represent the standard deviation of the mean of two independent experiments.

from all three stromal cells lines resulted in a significant reduction in CFU-A activity per $3x10^4$ cells derived from a population of dissociated EBs compared with control EBs (data not shown). However this was not observed in the second experiment, which explains the high variability. Furthermore, there was an increase in the proportion of EBs harbouring CFU-A activity after culture in conditioned medium from AM20.1B4 cells in one experiment (data not shown); again this observation was not repeated. Thus, the possibility that conditioned medium has some effect on the haematopoietic differentiation of ES cells cannot be ruled out, but this effect is not consistent. Therefore the data support a cell-cell contact dependent mechanism, but do not rule out a high concentration of a short-range secreted factor.

5.4.5 CFU-A activity in single EBs after co-culture on stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2

The experiments so far have demonstrated that co-culture of EBs with the stromal cell line AM20.1B4 enhances their haematopoietic activity after six days of differentiation and that direct cell-cell contact is likely to be an important factor. However, the co-culture experiments do not discriminate between an increase in haematopoietic activity from *de novo* induction of precursors to a haematopoietic fate or proliferation of committed progenitors. To address this issue, single EBs were co-cultured on the stromal cell lines and the CFU-A activity was measured for each EB individually (see Chapter 2: Materials and Methods, section 2.3.2). Furthermore, CFU-A activity was assessed at six and ten days of differentiation to determine the effect of prolonged co-culture.

16.7% of control EBs allowed to differentiate for six days in the absence of any stromal cells generated colonies when dissociated and plated in the CFU-A assay (**Table 5.2**). Similarly, 16.7% of EBs cultured on AM20.1B4 cells showed CFU-A activity. However, the mean number of colonies per EB (including EBs with no activity) was 0.6 (0 - 12) in controls and 2 colonies (0 - 24) after co-culture on AM20.1B4 stromal cells, a 3.3 fold increase confirming previous experiments see section 5.4.3 and **Figure 5.4**). Only 4.2% of EBs produced CFU-A colonies after co-

culture on UG26.1B6 or EL08.1D2 cells, and the overall mean number of colonies was 0.04 (0 - 1) and 0.13 (0 - 3) respectively (**Figure 5.6** and **Table 5.2**).

After ten days of differentiation, 87.5% of control EBs contained CFU-A activity as compared to 91.7% after co-culture on AM20.1B4 cells. The mean numbers of colonies per EB were 9.9 (0 - 62) and 47.5 (0 - 158) respectively. Therefore co-culture on AM20.1B4 cells resulted in a 4.8 fold rise after 10 days. Interestingly, after ten days of differentiation, there was a dramatic rise in the CFU-A activity in EBs co-cultured on UG26.1B6 stromal cells. 95.8% contained CFU-A progenitors and the mean number of colonies per EB was 50.7 (0 - 105). CFU-A activity in EBs co-cultured on the EL08.1D2 cells remained very low (4.2% of EBs with the mean from of 0.9 colonies per EB). This stromal cell line does not support ES cell derived haematopoiesis and the data even suggest a negative effect on haematopoietic differentiation (**Figure 5.6** and **Table 5.2**). These data demonstrate that AM20.1B4 and UG26.1B6 stromal cells support the proliferation of early haematopoietic progenitors in differentiating EBs. These data also show that their respective timings of action during EB differentiation are distinct, indicating that there is a difference in the mechanism by which co-culture with AM20.1B4 and UG26.1B6 cells results in an increase in CFU-A activity.

To further examine the action of the AM20.1B4 and UG26.1B6 stromal cells on the haematopoietic differentiation of ES cells, these two cell lines were mixed prior to co-culture. It was hypothesised that perhaps this would have an additive effect. In this particular experiment, the overall level of CFU-A activity was lower than previously (**Figure 5.7** and **Table 5.2**). After six days control EBs had differentiated well with 37.5% containing CFU-A activity and a mean of 1.2 (0 - 7) colonies per EB. However, after ten days only 41.7% of control EBs generated CFU-A colonies and each EB had fewer CFU-A progenitors (an overall mean of 0.9 colonies, ranging from 0 to 6, were produced per EB). Nevertheless the stromal cell line AM20.1B4 supported the haematopoietic differentiation of ES cells with 50% of EBs with CFU-A activity at day six and 95.8% at day ten, with 2.5 (0 - 11) and 21.3 (0 - 50) colonies per EB respectively. These data suggest that AM20.1B4 cells may have an *inductive* role on the haematopoietic differentiation of ES cells because the *proportion* of EBs that generated colonies in the CFU-A assay increased, but

Expt	none		AM20.1B4		UG26.1B6		EL08.1D2		AM20.1B4 UG26.1B6	
	d 6	d 10	d 6	d 10	d 6	d 10	d 6	d 10	d 6	d 10
1	16.7	87.5	16.7	91.7	4.2	95.8	4.2	4.2	nd	nd
2	37.5	41.7	50	95.8	12.5	41.7	nd	nd	20.8	100

Table 5.2 Percentage of EBs co-cultured on stromal cell lines derived from the embryonic AGM region and foetal liver that gave rise to CFU-A colonies. d = day of EB differentiation, nd = not determined

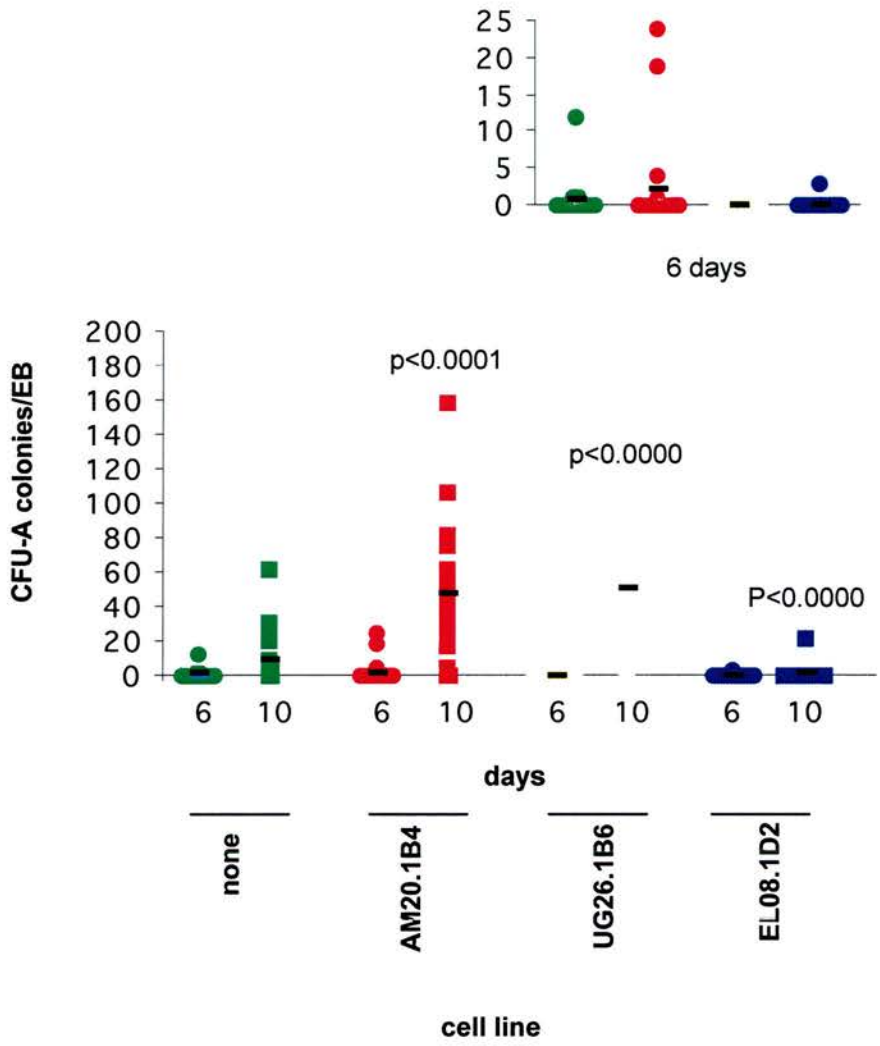


Figure 5.6 CFU-A activity in individual embryoid bodies after co-culture on stromal cell lines derived from the embryonic AGM region and liver. Numbers of CFU-A colonies per EB cultured in individual wells of a 24-well plate for 6 and 10 days on gelatin (■), AM20.1B4 (■), UG26.1B6 ( ) and EL08.1D2 (■) cells. Bars (▬) indicate the mean number of colonies per EB from one experiment. Inset shows the data at 6 days of differentiation. P values are shown as compared to control EBs by the Mann-Whitney U Test. Data at day 6 of differentiation were not significant with >95% confidence by a non-parametric test (Mann-Whitney U).

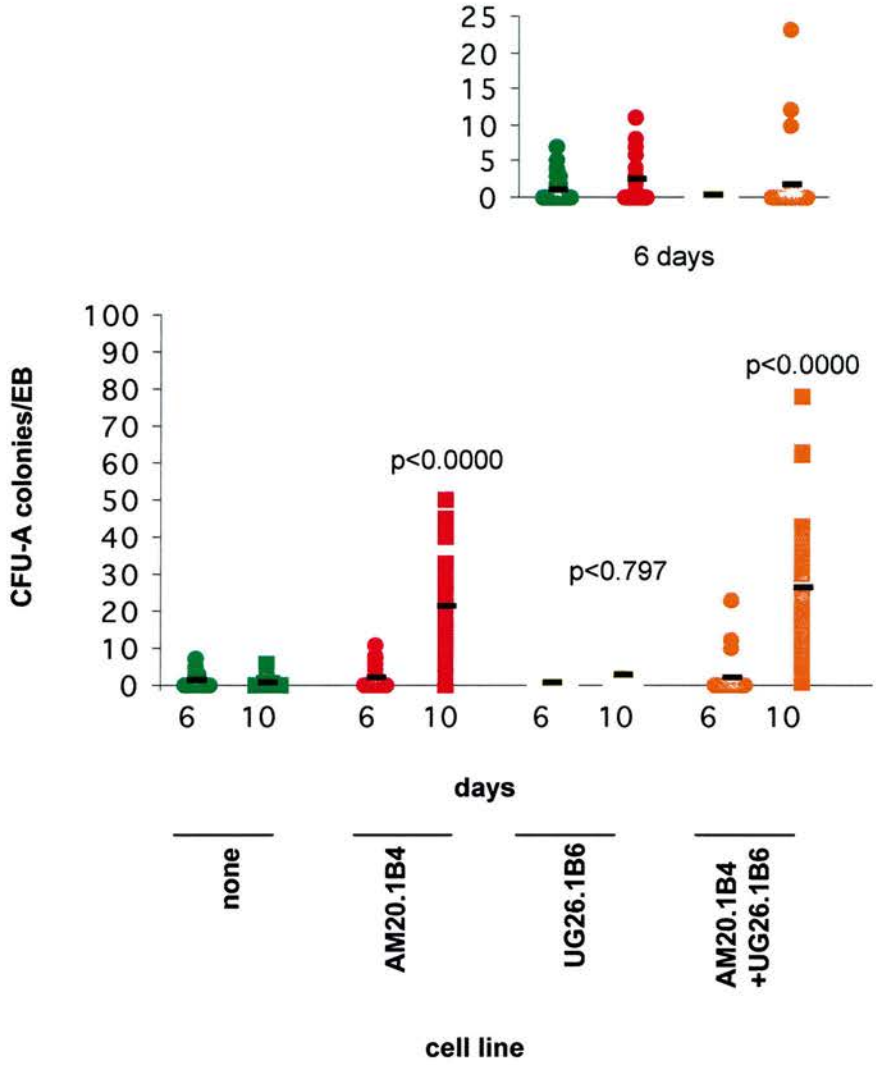


Figure 5.7 CFU-A activity in individual embryoid bodies after co-culture on stromal cell lines derived from the embryonic AGM region. Numbers of CFU-A colonies per EB cultured in individual wells of a 24-well plate for 6 and 10 days on gelatin (■), AM20.1B4 (■), UG26.1B6 ( ) and a mixture of AM20.1B4 and UG26.1B6 (■) cells. Bars (▬) indicate the mean number of colonies per EB from one experiment. Inset shows data at six days of differentiation. P values are shown as compared to control EBs. Data at day 6 of differentiation were not significant with >95% confidence as determined by a non-parametric test (Mann-Whitney U).

combined with the previous experiment (**Table 5.2** and **Figure 5.6**) the data are not statistically significant with a 95% confidence limit as determined by the two-sample t-test. Co-culture on the UG26.1B6 stromal cells again resulted in decreased haematopoietic activity after six days of differentiation compared to control EBs, 12.5% EBs with CFU-A activity and an overall mean of 0.5 colonies (0 - 7) per EB. Again at day ten, co-culture of EBs with UG26.1B6 cells resulted in an increase in haematopoietic activity. The percentage of co-cultured EBs which gave rise to CFU-A colonies was equal to control EBs (41.7%), but the overall mean number of colonies per EB was higher at 2.6, ranging from 0 to 21 (compared to 0.9 colonies), supporting a proliferative role.

When EBs were cultured on mixtures of the stromal cell lines AM20.1B4 and UG26.1B6, 20.8% of EBs gave rise to CFU-A colonies at day six and there was an average of 2.0 colonies (0 - 23) per EB. This level of CFU-A activity is between the levels after co-culture on the stromal cell lines separately and does not support a combined effect. However, after ten days of differentiation, 100% of EBs displayed CFU-A activity with a mean of 26.1 colonies (1 - 78) in each EB, which indicates a combined effect but only at later time points.

5.4.6 The self-renewal capacity of ES cells after passage on stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2

The decrease in CFU-A activity observed in EBs co-cultured with UG26.1B6 (for the first six days of differentiation) and EL08.1D2 lead to the possibility that these stromal cell lines might be inhibiting or delaying the differentiation of ES cells. To test this, the self-renewal capacity of ES cells after passage on the stromal cell lines was assessed. ES cells were serially passaged on all three irradiated stromal cell lines in the absence of LIF for up to six passages. Control cultures of ES cells on gelatin both in the presence and absence of LIF were set up as standards for routine maintenance in LIF and for the rate of decline in self-renewal in the absence of LIF. ES cells were harvested at each passage and samples were plated at low density in varying concentrations of LIF (0, 1, 10 100U/ml), allowed to grow for six days and then stained for alkaline phosphatase expression as a marker of

undifferentiated ES cells. After six days the relative numbers of colonies containing AP expressing cells were compared for a gross assessment of the extent of their differentiation and thus the self-renewal capacity of the clonogenic cell (see Chapter 2: Materials and Methods, section 2.6 and **Figure 2.5**). The experiment described in this section was set up as a crude measure of the support of undifferentiated ES cells, however the following observations from two independent experiments clearly show how a detailed quantitative analysis would be beneficial.

As expected, ES cells cultured on gelatin in the presence of LIF maintained a relatively stable self-renewal potential. ES cells cultured on gelatin in the absence of LIF gradually lost their capacity to self-renew, as indicated by a decline in the number of colonies expressing AP. It must be noted that within four passages, ES cells cultured without LIF appeared to regain self-renewal capacity. This result was unexpected but could possibly have occurred because the experimental conditions enriched for cells with increased self-renewal/decreased differentiation potential. Serial passage on AM20.1B4 cells resulted in an initial fall in the ability to give rise to colonies expressing AP when replated in LIF, but appeared to become stable after 3-4 passages. Passage on EL08.1D2 cells resulted in the gradual decline in AP expressing colonies. ES cells cultured on UG26.1B6 lost their self-renewal capacity very rapidly, after two passages only one or two colonies stained for AP on replating (**Figure 5.8**). Interestingly, ES cells cultured on either of the three stromal cell lines did not regain their ability to generate AP expressing colonies in contrast to control ES cells passaged on gelatin without LIF. Although $1.5x10^6$ cells were seeded at each passage, the overall cell numbers followed the pattern of AP expression/self-renewal potential (data not shown). The other intriguing observation was the persistence and size of differentiated AP^- colonies after culture on either stromal cell line, but particularly on UG26.1B6 cells compared to ES cells passaged on gelatin in the absence of LIF. Colonies were counterstained with trypan blue in an attempt to make this more visible (**Figure 5.8**, for enlargement of bottom panel see **Figure A.5** in Appendix).

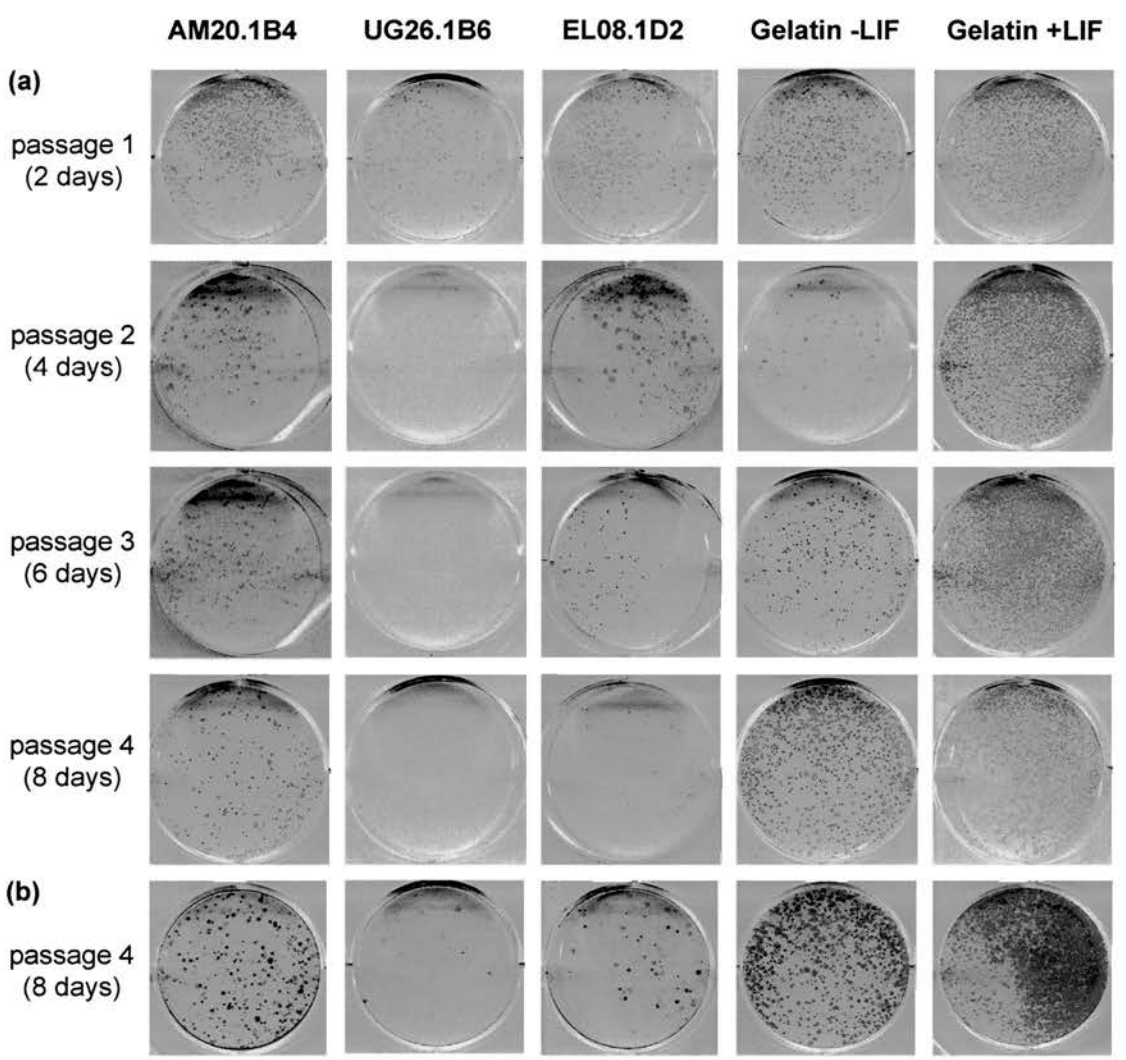


Figure 5.8 Self-renewal of ES cells passaged on stromal cell lines derived from embryonic AGM region and foetal liver. (a) The frequency of colonies containing ES cells expressing alkaline phosphatase (AP) that arise after plating 1000 cells in LIF, after serial passage for up to 8 days on AM20.1B4, UG26.1B6 or EL08.1D2 stromal cells compared to control ES cells passaged on gelatin either in the presence or absence of LIF. (b) Counterstaining of colonies from passage 4 with trypan blue. (see Appendix: Supplementary data Figure A.5 for enlargement of (b). Pictures are representative of duplicate wells from two independent experiments.

5.4.7 Cardiac differentiation of ES cells after co-culture on stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2

It was also interesting to determine if the differentiation of ES cells enhanced by the stromal cell lines was specific to the haematopoietic lineage or occurs at the level of commitment to a mesodermal fate. Therefore, the effect of the stromal cell lines AM20.1B4, UG26.1B6, EL08.1D2 and a mixture of AM20.1B4 and UG26.1B6 on the differentiation of ES cells into cardiomyocytes was assessed by scoring the percentage of "beating" EBs at day ten of differentiation in the co-culture experiments described above. The level of cardiac differentiation in control EBs was low with 4.2% recorded as "beating". The UG26.1B6 stromal line was highly supportive of differentiation to a cardiac fate with 77.1% ± 14.7 EBs "beating" compared to 4.2% ± 0 in control EBs; an 18 fold rise. The number of "beating" EBs co-cultured on AM20.1B4 cells was highly variable between experiments with a mean of 16.7% ± 26.6; a four fold increase relative to control EBs. Co-culture on a mixture of these two stromal cell lines resulted in 70.8% of EBs containing cardiomyocytes. EL08.1D2 cells did not effect the cardiac differentiation of ES cells, the percentage of "beating" EBs was equal to the control cultures at 4.2% (**Figure 5.9**). Since the number of "beating" EBs was scored prior to their dissociation for plating in the CFU-A assay, it was possible to determine whether commitment to cardiomyocytes was mutually inclusive or exclusive of haematopoietic commitment. There was no correlation between CFU-A activity and cardiomyocyte differentiation of individual EBs in these experiments (data not shown).

5.5 Discussion

Overall, explant cultures of intact AGM regions provide the best microenvironment for the haematopoietic differentiation of ES cells; after six days of differentiation CFU-A activity is increased 20 fold compared to control EBs cultured alone (**Figure 4.4**). Primary cultures of dissociated AGM region cells provided good support, though those data are preliminary, increasing CFU-A activity 10 fold

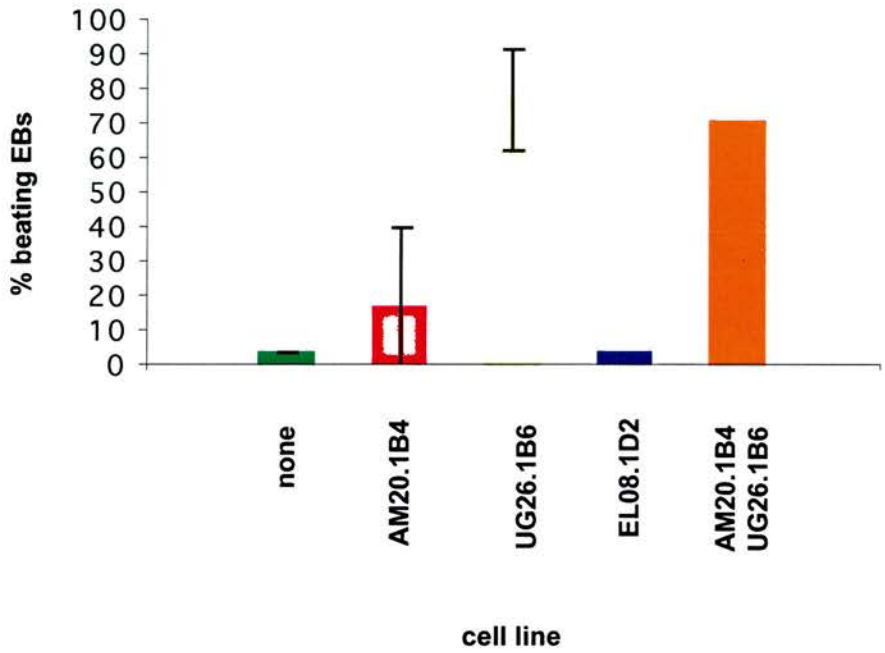


Figure 5.9 Cardiac differentiation of embryoid bodies co-cultured on stromal cell lines derived from embryonic AGM region and liver. The percentage of EBs which were "beating" after 10 days on gelatin (, AM20.1B4 ( ), UG26.1B6 ( ), EL08.1D2 ( ) and a mixture of AM20.1B4 and UG26.1B6 ( ) cells. The numbers are from 24 EBs cultured individually per well of a 24-well plate. Error bars represent the standard deviation of the means from two independent experiments. Where there are no error bars the data shown is from one experiment.

compared to control EBs cultured alone (**Figure 5.2**). At six days of differentiation the CFU-A activity was only 3.5 times that of control EBs when co-cultured on the most supportive stromal cell line AM20.1B4 (**Figure 5.4**). There are several possible explanations for these observations. There is evidence that in explant cultures the AGM region develops as *in vivo*, and thus the appropriate factors for the development of haematopoietic progenitors are likely to be present at the appropriate times. Primary cultures of dissociated AGM regions might be more supportive than stromal cell lines for two reasons. First, primary cultures contain more than one cell type and second, the effect of irradiation on the stromal cells (the primary cultures were unirradiated) is unknown. However, when differentiation is extended to ten days, a 20-fold rise in CFU-A activity was achieved by co-culture on AM20.1B4 cells (**Figure 5.6** and **5.7**). Here the cell lines may have an advantage over the explant cultures, where a decrease in haematopoietic activity was seen after seven days of differentiation (**Figure 4.4** and **4.5**), because the cell lines provide a constant source of the inductive signals. Perhaps then explant co-cultures could be improved further by adding fresh AGM regions to the co-cultures every few days.

In the Introduction to this chapter, it was proposed that the experiments described above were set up to gain an insight into first, the phenotypes of cells from the AGM region that supports the haematopoietic differentiation of ES cells; second, whether cell-cell contact is required and third, if the increased haematopoietic activity is due to the induction of precursors *de novo* or proliferation of committed progenitors.

5.5.1 How do the effects of the stromal cell lines on the haematopoietic differentiation of ES cells compare with previous findings by others using these cell lines?

In this study, by six days of differentiation, only the stromal cell line AM20.1B4 enhances the haematopoietic differentiation of ES cells and the stromal lines UG26.1B6 and EL08.1D2 appear to have a negative effect (**Figure 5.4, 5.6** and **5.7**). This is in contrast with previous findings in other studies where, of these three cell lines AM20.1B4 was by far the least supportive of bone marrow derived

haematopoietic progenitors in long term cultures as measured by the LTC-CFC assay, while UG26.1B6 were most supportive (Oostendorp et al., 2002a). Here, UG26.1B6 stromal cells do support and enhance haematopoietic activity of EBs when co-culture was extended up to day ten of differentiation and AM20.1B4 cells continue to enhance the haematopoietic activity of EBs at this time point (**Figure 5.6** and **5.7**). However, EL08.1D2 cells continue to have a markedly inhibitory effect (**Figure 5.6**) in contrast to their ability to support bone marrow derived progenitors (Oostendorp et al., 2002a). AM20.1B4 stromal cells are more effective at maintaining LTR-HSCs from E11 dorsal aorta compared with the UG26.1B6 line (Prof. Elaine Dzierzak, personal communication). Taken together, these data highlight a biological difference in maintaining embryonic-derived (from the AGM region or EBs) compared to adult-derived HSCs (from bone marrow).

5.5.2 Is the increase in haematopoietic activity in the EBs after co-culture a result of the de novo *induction of precursors or the proliferation of committed progenitors?*

In the embryonic AGM region both *de novo* induction and proliferation of HSCs occurs. It is assumed therefore that the AGM region is a source of signals for both. Although the data are preliminary, co-culture of EBs on primary AGM region cells supports this, since both the proportion of EBs with CFU-A activity *and* the number of colonies generated from each EB is increased by co-culture. As for the stromal lines, the data show that UG26.1B6 cells provide a proliferative effect because co-culture on this cell line does not result in a large increase in the percentage of EBs that contain CFU-A progenitors (**Table 5.2**). The increased haematopoietic activity is also dependent on the "baseline" CFU-A activity in control EBs and occurs only after an extended period of differentiation (**Figure 5.6** and **5.7**). Oostendorp and others (Oostendorp et al., 2002a) have reported that adult bone marrow derived HSCs maintain their ability to repopulate irradiated recipients even after four weeks in co-culture with UG26.1B6 stromal cells and that this cell line was the most supportive clone tested. However, co-culture with this stromal line did not induce LTR-HSC activity in a candidate pre-HSC population of $CD34^{+}c\text{-}kit^{+}$ cells

from early E10 yolk sac and AGM region tissues, at a time before HSC activity is detected.

The most convincing evidence that the stromal cell line AM20.1B4 has an *inductive* effect on the haematopoietic differentiation of ES cells is that co-culture can result in an increase in the proportion of EBs which give rise to colonies in the CFU-A assay (**Table 5.2**). The effects of co-culture on pre-HSCs from yolk sac and AGM region, as described above, would be interesting and could confirm an inductive role for AM20.1B4 cells, but this experiment has not yet been performed using this clone (Prof. Elaine Dzierzak, personal communication).

Combining the AM20.1B4 and UG26.1B6 cells could, in theory, have exploited the inductive effect of the AM20.1B4 line and the proliferative effect of the UG26.1B6 cells resulting in greater CFU-A activity in the EBs than from either cell line used alone. Although 100% of the EBs contained haematopoietic progenitors, the mean number of CFU-A colonies per EB was not as high as had been hypothesised. A possible explanation is that the negative effect UG26.1B6 cells have on the haematopoietic differentiation during the first days of co-culture might have quenched the inductive effect of the AM20.1B4 cells. This may have been exacerbated by a larger proportion of UG26.1B6 cells within the cultures because that cell line grows more quickly than the AM20.1B4 line and equal numbers of cells from both lines were seeded and then grown to confluence over two days prior to irradiation and co-culture. It would be useful to fully establish the time course of activity by the stromal cells during EB differentiation. Then a two-step co-culture strategy could be employed that might exploit the respective inductive and proliferative properties of the cells lines more fully and might also reflect more accurately the development of HSCs *in vivo*.

The apparently inhibitory effect on the haematopoietic activity of EBs co-cultured on UG26.1B6 cells during the first days of differentiation and on EL08.1D2 cells throughout is interesting. Both cell lines are unable to induce repopulating activity in pre-HSCs from the yolk sac and AGM region (Oostendorp et al., 2002a). Such inductive activity has been previously reported using the AGM region derived stromal cell line AGM-S3 (Matsuoka et al., 2001). The foetal liver at E11-12 is not yet a highly supportive microenvironment for HSCs, as shown by organ culture

experiments (Medvinsky and Dzierzak, 1996) and by the small number of supportive stromal cell lines generated from this tissue in the panel by Oostendorp (Oostendorp et al., 2002b). The primary haematopoietic function of the E11-12 liver may be to generate mature cells from HSCs migrating from the AGM region and yolk sac (Kumaravelu et al., 2002). Perhaps EL08.1D2 cells might have a *differentiating* effect on ES derived haematopoietic progenitors, which could be measured using an assay that detects more mature cells such as the methylcellulose assay. Oostendorp and his colleagues (Oostendorp et al., 2002b) tested the stromal cell line supernatants for CFC-promoting activity on adult bone marrow cells in methylcellulose cultures by using conditioned medium from the stromal cell lines in place of the pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM). They found that all cell lines produced supernatants that were able to support the differentiated growth of the CFCs. Supernatant from the primary line EL08 from which the clone EL08.1D2 was derived, was one of the most efficient, supporting a *differentiative* role of the EL08.1D2 cells. Interestingly, EL08.1D2 cells were found to be by far the most supportive (several hundred fold), as compared to other stromal cell lines including the other two in this study, of human cord blood $CD34^+$ cells in long term culture as measured by the CAFC assay. In fact their long term repopulating ability was also maintained by co-culture on this clone and UG26.1B6 cells (Kusadasi et al., 2002). The UG26.1B6 and EL08.1D2 cell lines might also be involved in regulating the *de novo* induction of haematopoietic progenitors.

The assessment of self-renewal capacity of ES cells after serial passage of ES cells on the stromal cell lines provided further insight into the inductive and proliferative properties of these stromal cell lines **(Figure 5.8)**. The experiment was originally set up to examine whether any of the stromal cell lines could support undifferentiated ES cells, because of the decreased CFU-A activity in EBs after co-culture on UB26.1B6 and EL08.1D2 cells.

The AM20.1B4 line was the only line to provide any support for undifferentiated ES cells. This was unexpected since CFU-A activity in EBs was enhanced. After two passages (four days) the number of colonies expressing AP appeared to have stabilised, indicative of the intriguing possibility that a balance between self-renewal and differentiation had been established. An indicator of self-

renewal and differentiation potential of the clonogenic cells is the presence of colonies with both AP^+ ES cells and AP^- differentiated cells ("mixed" colonies see **Figure 2.5**). The three types of colonies (ES cell, mixed and differentiated) were not quantified in this experiment due to the number of cells plated, but it will be interesting to determine whether an increased number or proportion of "mixed" colonies are a consequence of culture on AM20.1B4 (or other) stromal cell lines. UG26.1B6 cells promoted the differentiation of ES cells very rapidly (within 2-3 passages), with no support of undifferentiated self-renewing ES cells. Co-culture of EBs on EL08.1D2 cells resulted in almost no differentiation into cardiomyocytes or haematopoietic progenitors, raising the possibility that this cell line might be inhibiting or delaying the differentiation of ES cells, but EL08.1D2 cells did not maintain undifferentiated ES cells. Taken together, these data suggest that EL08.1D2 stromal cells promote differentiation into lineages not measured by the assays used in this study. In addition, when an ES cell line expressing GFP targeted to the mesodermal specific *brachyury* gene (Fehling et al., 2003); a kind gift from Prof. Gordon Keller) were cultured on EL08.1D2 cells, no GFP^+ cells were detected by fluorescence microscopy at the time point (up to 5 days) when control *brachyury-GFP* ES cells cultured on control OP9 cells were mostly GFP^+ (>90%) (A.K., unpublished). GFP^+ colonies were also generated after co-culture with AM20.1B4 cells and UG26.1B6, but these were not quantified (A.K., unpublished). These data are indicative of a signal from EL08.1D2 stromal cells that blocks or delays the commitment to mesodermal fates.

Two additional and potentially significant observations were made. First, although the control ES cells cultured on gelatin without LIF regained their self-renewal capacity after three passages (six days), ES cells cultured on either of the stromal cell lines did not. In the case of UG26.1B6 and EL08.1D2 cells this suggests a strong drive towards differentiation, but for AM20.1B4 cells that have a limited capacity to support undifferentiated ES cells it is surprising. A possible explanation could be that a balance self-renewal and differentiation has been established, but again, quantification of the colonies is necessary to explore this possibility.

Second, ES cells cultured on the stromal cell lines had an increased tendency to generate large differentiated AP^- colonies compared to control ES cells cultured on

gelatin in the absence of LIF. To highlight this, duplicate wells were counterstained with trypan blue (**Figure A.5** in Appendix). Large, differentiated colonies are not usually generated by ES cells that have been cultured without LIF, probably because of a decrease in seeding efficiency and proliferation in differentiated ES cells, or because of cell death on differentiation, which has previously been detected by DNA laddering (Mehlen et al., 1997) and the TUNNEL assay (A.K., unpublished). These data are in support of a production of factors by the stromal cell lines, especially UG26.1B6, that promote cloning efficiency, cell proliferation or cell survival.

5.5.3 Are the factors that increase the haematopoietic activity of differentiating EBs dependent on direct cell-cell contact or secreted molecules?

Data from two experiments support a contact dependent mechanism. First, when using primary cultures of adherent AGM region derived cells as "feeders" the CFU-A activity in co-cultured EBs was only increased (compared to the control EBs cultured alone) when there was direct contact; no increase was observed when EBs were cultured in transwell inserts suspended over the AGM region cells (**Figure 5.1**). Second, there was no significant effect on the CFU-A activity, either in the proportion of EBs containing progenitors or the total number of colonies produced from dissociated EBs, when cultured in conditioned medium from the stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2 (**Figure 5.6**). However, the haematopoietic activity of the EBs in these experiments was assessed after only six days of differentiation; it may be interesting to extend the culture especially because the increase in CFU-A activity caused by co-culture on UG26.1B6 cells was not detected at day six. However, Harvey and Dzierzak (Harvey and Dzierzak, 2004) demonstrated that the support of haematopoietic progenitors, isolated from E11 aorta, was more efficient in contact co-cultures using UG26.1B6 cells than non-contact co-cultures. This was also observed in co-culture experiments with two other stromal cell lines derived from the urogenital ridge. Furthermore, contact with the UG stromal clones (including UG26.1B6) was essential for the maintenance and expansion of midgestation aortic HSCs. Another group (Xu et al., 1998)

demonstrated that the expansion of clonogenic progenitors (CFU-GM, BFU-E and CFU-mix) from human cord blood-derived $CD34^+$ cells by the E10.5 AGM region stromal cell line AGM-S3 was dependent on direct cell-cell contact. Ohneda *et al* (Ohneda et al., 1998) reported that $lin^-CD34^+Sca\text{-}1^+c\text{-}kit^+$ foetal liver HSCs were expanded 2,600 fold after direct co-culture on the AGM region derived endothelial cell line DAS 104-4, but only 70 fold after non-contact co-culture, and only the cells harvested from contact co-cultures were capable of repopulating the haematopoietic system of lethally irradiated adult mice. It would also be interesting to investigate the role of cell-cell contact in the increase in cardiomyocyte differentiation resulting from EB and UG26.1B6 clone co-culture. Again the possibility of a high concentration of short-range secreted molecules cannot be ruled out.

5.5.4 What are the phenotypes of the cells responsible for the haematopoietic-promoting activity of differentiating ES cells?

Confirming the findings of both Oostendorp *et al* (Oostendorp et al., 2002a; Oostendorp et al., 2002b) and Charbord *et al* (Charbord et al., 2002), the stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2 do not express endothelial or haematopoietic lineage surface antigens, but they do express CD44, V-CAM, integrin subunits α_5 and $\beta_1$and low levels of Sca-1 (**Figure 5.3** and **Table 5.1**). Similar observations have been made on examining stromal cell line AGM-S3 (Xu et al., 1998). CD44, VLA-4 and VLA-5 (consisting of integrin subunits $\alpha_4\beta_1$ and $\alpha_5\beta_1$, respectively) and V-CAM have been found to play important roles in haematopoietic development by allowing haematopoietic progenitors to interact with the stromal cell and extra-cellular matrix components of their "niches" (Chan and Watt, 2001). Sca-1 has been associated with the haematopoietic supporting activity of stroma (Remy-Martin et al., 1999; Satoh et al., 1997). However, although there is a variable effect of co-culture with the stromal cell lines both on the differentiation of ES cells observed in the present study and on embryonic and adult haematopoietic progenitors in other studies (Charbord et al., 2002; Oostendorp et al., 2002a; Oostendorp et al., 2002b), no overt correlation to the levels of membrane antigen expression was found. It is unlikely the higher expression of integrin α_5 (CD49e) is

solely responsible for the inductive properties of the AM20.1B4 stromal cell line. Interestingly, the AM20.1B4 cell line was the only clone (out of the three used in this study), on which surface expression of SCF was detected by flow cytometry (Oostendorp et al., 2002b), which is also in support of a direct cell-cell contact dependent mechanism.

Although the conditioned medium collected from the stromal cell lines had no effect on ES cell differentiation in this study, the production of haematopoietic cytokines is nonetheless interesting. The stromal cell lines were found to express the transcripts of several haematopoietic cytokines including TPO, SCF, FL, G-CSF, IL-1β, Il-6, Il-11 and Oncostatin M (Oostendorp et al., 2002a; Oostendorp et al., 2002b). Again there was no correlation to the cytokine profile and haematopoietic supporting activity of the supernatants taken from the cell lines. Il-3 was not detected in any of the cell lines examined by RT-PCR but this may not be significant to the haematopoietic inducing function of the cell lines because Il-3 acts in combination with other cytokines and the inactivation of its receptor *in vivo* does not result in a major haematopoietic defect (Nicola et al., 1996; Nishinakamura et al., 1996). Interestingly, the three stromal cell lines used here expressed Il-6 and the Il-6α receptor. Il-6 has recently been reported to be an important factor in the haematopoietic-supporting activity of bone marrow stroma (Rodriguez Mdel et al., 2004). The detection of low levels of LIF transcript in UG26.1B6 cells by RT-PCR by Oostendorp and colleagues (Oostendorp et al., 2002b) was surprising since passage on this cell line appears to promote differentiation of ES cells, as determined by the loss of AP expressing colonies, at a faster rate than when cultured on gelatin in the absence of LIF (**Figure 5.8**). However, the levels of LIF could be too low to maintain ES cells in an undifferentiated state. EL08.1D2 do not appear to express the LIF transcript and this data is unavailable for AM20.1B4 cells (Oostendorp et al., 2002b). Another interesting observation by Oostendorp *et al* (Oostendorp et al., 2002b) was the expression of TGF-β1 by most of the stromal cell lines tested (though again AM20.1B4 cells were excluded from this analysis). TGF-β1 is thought to have a strong negative effect on HSCs (Ohta et al., 1987) and it might play a regulatory role in controlling numbers of early progenitors and perhaps help explain the initial negative effect on haematopoietic differentiation of ES cells by the

UG26.1B6 cells, which express high levels of TGF-β1. Co-culture of EBs on UG26.1B6 cells greatly increased the differentiation to "beating" cardiomyocytes (**Figure 5.9**). TGF-β1 is known to be involved in cardiac development and function (Azhar et al., 2003) and induces cardiomyocyte differentiation of ES cells (Behfar et al., 2002).

Taken together, these data show that a complex network of interactions and signals controls the induction, proliferation and differentiation events during haematopoietic development and that subtle differences in expression levels are more likely to be responsible than single factors. Such an extensive panel of stromal cell lines is a powerful tool for elucidating these interactions and for the isolation of novel factors. For example, Ohneda *et al* (Ohneda et al., 2000) identified a novel chemokine Weche from the AGM region derived endothelial cell line DAS 104-4.

5.5.5 Summary

It is important to note for the present study that the stromal cell lines AM20.1B4, UG26.1B6 and EL08.1D2 were derived from embryos at different developmental stages. The AM20.1B4 clone was derived from E10 AGM region, whereas UG26.1B6 and EL08.1D2 cells were derived from E11 genital ridge and liver respectively. Explant cultures of intact E10.5 AGM regions were more effective at promoting the haematopoietic differentiation of ES cells than the E11.5 equivalents, while liver explant cultures had no effect (**Figure 4.3**). Perhaps EB and stromal cell line co-cultures merely reflect this observation.

In summary, co-culture experiments with the stromal cell lines and primary cultures of dissociated AGM regions have shown that (i) both induction and proliferation of haematopoietic progenitors contribute to the increase in CFU-A activity of differentiating ES cells, (ii) the factor(s) responsible are dependent on direct cell-cell contact and (iii) no obvious correlation to a specific cell phenotype has been established. However, explant cultures of intact AGM regions provide the best microenvironment for the haematopoietic differentiation of ES cells; after six days of differentiation CFU-A activity is increased 20 fold compared to control EBs cultured alone (**Figure 4.4**).

Further investigations are underway to elucidate the time course of haematopoietic progenitor induction/proliferation by these three stromal cell lines during ES cell differentiation by a combination of colony assays and co-culture of ES cells transgenic for reporter genes under the control of loci associated with haematopoietic commitment and differentiation, such as *brachyury*, *Flk1*, *Tal1/Scl*, *c-kit* and *Aml1/Runx1*. The possibility of using the stromal cells as an induction screen for the selection of genes involved in early haematopoietic commitment identified by gene trapping strategies is also being explored.

Chapter Six:

Investigating a potential haematopoietic role of a novel mouse homologue to the ubiquitin conjugating enzyme 7 (Ubc7)

6.1 Aim

To investigate a potential haematopoietic phenotype of a mutant deficient in the mouse homologue to the yeast ubiquitin conjugating enzyme (Ubc7).

6.2 Introduction

During a screen designed to identify novel developmentally regulated genes using a gene trap strategy, one of the integrations, GT246, trapped the *Ubc7* gene and functionally disrupted its encoded protein. The GT246 ES cells were injected into blastocysts and transmitted the gene trap through the germline. Generation of homozygous animals showed that absence of mUbc7 resulted in an embryonic lethal phenotype. The cause of a lethal phenotype can be difficult to dissect out. Strategies can be implemented to do this, such as tissue restricted or inducible deletions or the generation of chimeras with ES cells homozygous null for the gene trap integration to examine whether any lineages are devoid of ES cell progeny, but they can be difficult, time consuming and expensive. A rapid *in vitro* approach would be useful.

It was previously noted that some of the homozygous mutant embryos appeared anaemic, so haematopoietic tissues (yolk sac and AGM region) were examined. Two observations made from the experiments described in this thesis could be applied to rapidly investigate a potential haematopoietic defect in the mutants *in vitro*. First, the increase in CFU-A progenitors observed in AGM region explant cultures (see Chapter 3) and second, the ability of the AGM region to enhance the haematopoietic differentiation of ES cells in co-culture (see Chapter 4). It was postulated that a mutation affecting the generation and/or expansion of early haematopoietic progenitors would affect the formation of colonies in the CFU-A assay. Subsequently, distinguishing between a defect intrinsic to the HSC/progenitors, or a non-autonomous defect in the inductive/supportive elements of the stromal microenvironment, could potentially be done by investigating the capacity of the mutant AGM region to increase the haematopoietic potential of

normal ES cells during *in vitro* co-culture. This chapter describes the experiments, which were carried out to identify the cause of death in the mutant embryos.

The gene trap screen, the identification of the trapped sequence and the initial characterisation of the phenotype *in vivo* were done by Dr Julie Wallis under the direction of Dr Lesley Forrester.

6.2.1 Background

Mutational analysis has been important for our understanding of embryonic development and the genes involved. Random mutational strategies using irradiation, which causes large chromosomal rearrangements (Russell, 1951), or chemical agents such as ENU, which induces point mutations (Russell et al., 1979) have generated a large number of mutants (Balling, 2001; Justice et al., 1999; Thomas et al., 1998). Such screens are phenotype driven looking primarily for mutants with specific phenotypes before investigating the genetic cause. Similarly, random insertional mutagenesis with retroviral vectors has resulted in a number of transgenic mouse strains, but this strategy often results in multiple integrations and a tendency for integration into specific regions. Directed mutagenesis by homologous recombination in ES cells to "knock-out" specific sequences have been very useful, but prior knowledge of the genomic structure is essential to construct the targeting vectors. Several genetic loci have also been identified using entrapment strategies, such as gene trapping (see section 6.2.1.1).

6.2.1.1Gene trapping

Gene trapping is a technique, which is used to identify and characterise the function of novel genes. The random insertion of a non-homologous construct, containing a promoterless reporter gene (e.g. *LacZ*), into the intron of an active gene in ES cells generates a fusion between the reporter gene and the endogenous trapped gene (**Figure 6.1**). There are three important consequences of such insertion events: (i) expression of the reporter gene is controlled by the endogenous gene, (ii) the fusion transcript generated allows the endogenous gene sequence to be cloned and (iii) insertion of the vector may result in the disruption of the trapped gene and thus

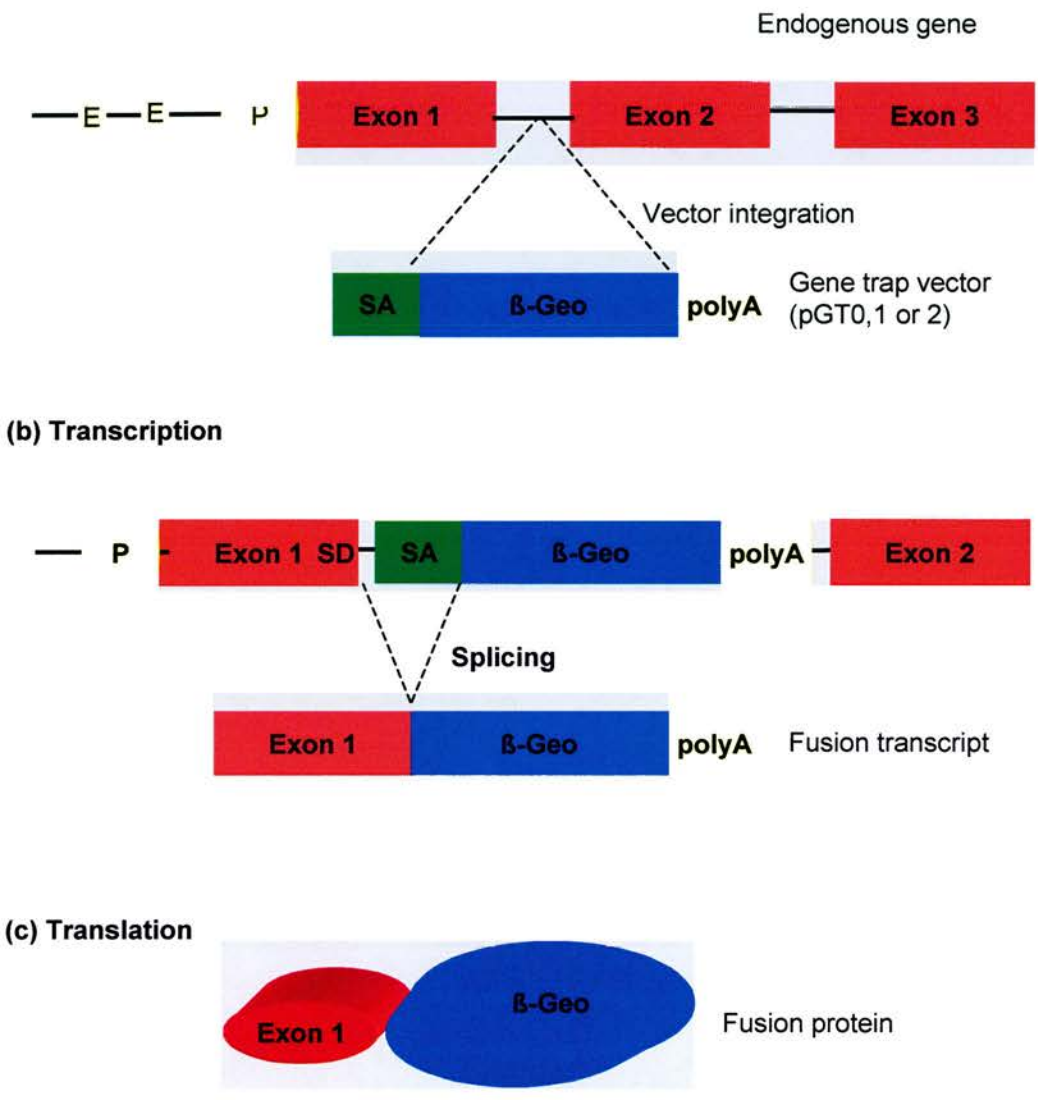


Figure 6.1 Gene trapping. (a) A gene trap vector containing a splice acceptor, β-geo (Lac Z reporter fused to the neomycin resistance gene) and a polyadenylation signal integrates into an intron of a gene at random. (b) The gene trap is now under transcriptional control of the endogenous promoters and enhancers. The presence of the splice acceptor in the construct and the endogenous splice donor results in a fusion transcript between the trapped gene and β-geo, which is (c) translated to from a fusion protein. The trapped protein is often truncated, depending on the site of integration. E = enhancer, P = promoter, SA = splice acceptor, SD = splice donor

the biological consequences may be analysed after germ-line transmission. Several important genes have been identified using gene trapping strategies for example *Hs2st*, encoding heparan sulfate 2-sulfotransferase the transcripts of which were disrupted by the vector integration resulting in renal agenesis (Bullock et al., 1998).

A concerted international effort is being made to achieve saturation of the mouse genome by generating gene trap integrations representing all of the ca. 30,000 genes. A biotechnology company, Lexicon Genetics, has created to date the largest library of ES cell lines (OmniBank; http://www.lexicon-genetics.com). They claim to have achieved close to 60% coverage of the genome in 200,000 tags. Several academic groups initiated the International Gene Trapping Consortium (IGTC; http://www.igtc.ca) and are making their trapped ES cell lines available in the public domain without restriction. So far that library covers 32% of the genome in 27,000 tags and, one fifth of those are not represented by Lexicon (Skarnes et al., 2004).

Trapped genes are identified or characterised by (i) the sequence around the integration site, (ii) the expression pattern in heterozygous mice after germline transmission through chimeras or (iii) the phenotype, if any, of animals homozygous for the integration after breeding heterozygotes (Gossler et al., 1989; Skarnes et al., 1992). The *in vivo* approaches are very laborious and expensive, particularly because only 15-30% of the integrations, which have randomly trapped genes expressed in undifferentiated ES cells, exhibit restricted expression patterns during embryogenesis (Friedrich and Soriano, 1991; Wurst et al., 1995). The unique capacity of ES cells for multulineage differentiation *in vitro* has been exploited to develop screening strategies to select gene trap clones for germline transmission that are likely to be involved in the development of a particular lineage (Hirashima et al., 2004; Stanford et al., 1998).

An *in vitro* pre-selection screen for genes responsive to retinoic acid (RA) was performed to enrich for insertions into genes that are involved in embryogenesis (Forrester et al., 1996). RA, the active metabolite of vitamin A, is known to have a profound effect on development *in vivo*. The application of exogenous RA to embryos (Conlon and Rossant, 1992; Kessel and Gruss, 1991; Morriss-Kay et al., 1991) or differentiating EC cells in culture (Kondo et al., 1992) can alter the expression of developmentally regulated genes and is therefore applied to *in vitro*

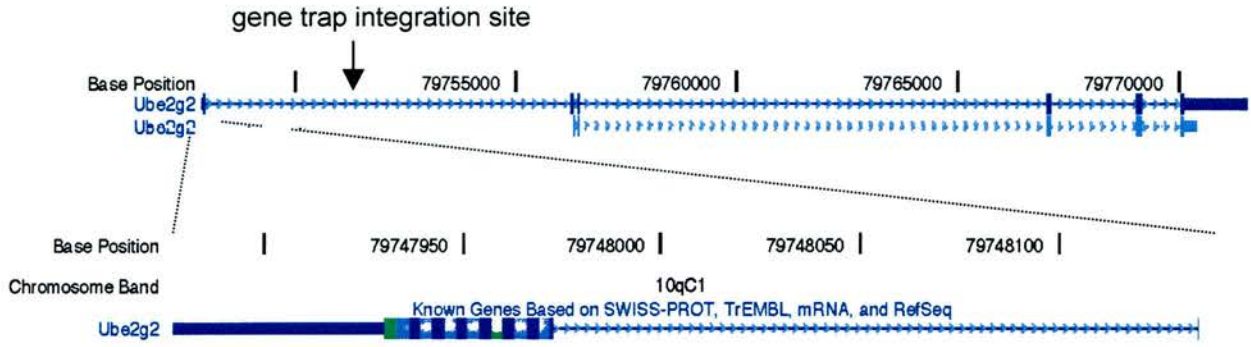
models of tissue specification and differentiation using ES cells. For example RA enhances differentiation down neural lineages and represses commitment to haematopoietic or, at certain concentrations, cardiac fates (Bain et al., 1996; Wobus et al., 1997). The screen that resulted in the trapping of GT246 was designed to enrich for genes involved in cardiogenesis and, as described below, colonies where β-galactosidase activity was repressed by RA treatment were picked for further analysis.

6.2.1.2 The GT246 gene trap event

Gene trap vectors pGT0, 1 and 2, containing an En-2 splice acceptor site (in a different reading frame in each of the three constructs) fused to *β-geo* (*LacZ* reporter fused to the neomycin resistance gene) **(Figure 6.2a)**, were introduced into E14 ES cells by electroporation. Electroporated cells were plated in the presence of G418. Resistant colonies were picked, and replicate cultures were treated with RA. Clones where the expression of LacZ was suppressed by the addition of RA were then differentiated into "beating" cardiomyocytes, which were recorded on video and then again stained with X-gal. Those clones where β-gal was expressed in the "beating" regions were selected for further analysis.

Endogenous gene sequences upstream of the gene trap vector's *β-geo* sequence were isolated by Rapid Amplification of cDNA Ends (RACE) -PCR cloning. A 115bp DNA sequence of one clone, GT246, was used to search the mouse Expressed Sequence Tag (EST) database and a 2030bp contig was constructed from these ESTs. Interestingly, there appeared to be two contigs, one had a 925bp alternatively spliced 3' end. A BLAST search of the predicted 152 amino acid (aa) sequence identified it as the mouse ubiquitin conjugating enzyme homologous to Ubc7 in *S. cerevisiae* (GenBank™ accession number X 69100) with 63% identity. MUbc7 is highly conserved showing 94% identity to the human E2G2 protein (GenBank™ accession number AF 296657), 74% identity to Ubc14 (GenBank™ accession number NP 493381) in *C. elegans* and 58% identity to Ubc13 (GenBank™ accession number T 45703) in *A. thaliana*. The genomic structure consisting of six exons was obtained from the UCSC web browser (http://www.genome.ucsc.edu) **(Figure 6.2)** and is located on chromosome 10. A

(a) Genomic structure of *mUbc7*



(b) Northern analysis of *Ubc7* expression

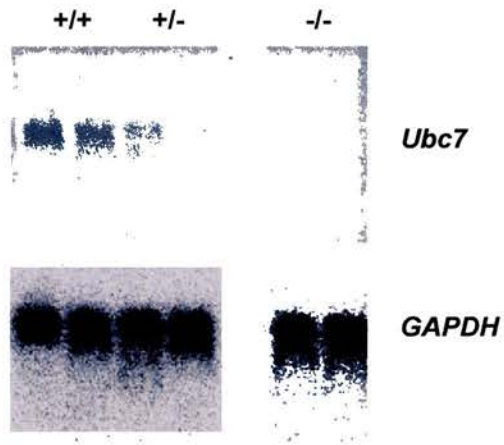


Figure 6.2 Consequences of the gene trap integration GT246 into the mouse ubiquitin conjugating enzyme Ubc7. (a) Schematic representation of the genomic structure of *Ubc7*. The site of integration of the gene trap vector between exons 1 and 2 is shown. (b) Northern blot showing the relative levels of *Ubc7* mRNA in wildtype, heterozygous and homozygous GT246 embryos. The probe was a P32dATP labelled 1.1kb EcoRI/NotI fragment of EST W18096(J131). 10mg E10.5 RNA was loaded in each lane except the second -/- lane, where 10.9mg was loaded. GAPDH was used as a control. The gene trapping, RACE-PCR, cloning, sequence analysis and Northern hybridisation was done by Dr Julie Wallis. aa = amino acid, +/+ = wildtype, +/- = *mUbc7*$^{Gt246For/+}$, -/- = *mUbc7*$^{Gt246For/Gt246For}$

165aa splice variant of mUbc7, which differs at the 3' end (GenBank™ accession number AAH10321), has been reported to play a role in the degradation of unassembled T cell receptor subunits from the endoplasmic reticulum (Tiwari and Weissman, 2001).

The gene trap vector integrated into an intron and was spliced onto the end of exon 1 at aa15, upstream of the predicted catalytic domain (aa24-141) of the 152aa protein producing a functional knockout (**Figure 6.2**). Northern analysis, using a probe against the 3' end of the gene showed complete loss of the *Ubc7* transcript in the homozygous embryos, and lower levels of expression in the heterozygotes compared to wildtype (**Figure 6.2(b)**).

6.2.1.3 The Ubc7/E2G2 ubiquitin conjugating enzyme

The ubiquitin conjugating enzymes (Ubc or E2) are constituents of the ubiquitin-proteosome pathway, a major system for the selective breakdown of proteins in eukaryotes. Target proteins are conjugated to the activated ubiquitin polypeptide as a tag for degradation by the 26S proteosome complex. This process involves the sequential action of three classes of enzymes. First the ubiquitin activating enzyme (Uba or E1) forms a thiolester bond with the C terminus of ubiquitin, which is transferred to a specific cysteine residue on the E2. E2 enzymes donate ubiquitin to the target protein by catalysing the formation of an isopeptide bond, directly or with the involvement of an ubiquitin-protein ligase enzyme (Ubr or E3) (**Figure 6.3**). Targets of this pathway are proteins whose levels need to be tightly regulated, including cyclins, cyclin-dependent kinase inhibitors, transcription factors, the tumour suppresser protein p53 and various oncoproteins (Ciechanover, 2003; Pickart, 2004).

6.2.1.4 The mUbc7$^{-/-}$ phenotype

Animals homozygous for the GT246 gene trap integration (*Ubc7*$^{Gt246For/Gt246For}$) did not survive and the embryos died around day 11.5 of gestation on both inbred (129) and outbred (MF1) backgrounds. Staining of E8.5 and E10.5 embryos with X-gal showed *LacZ* expression was ubiquitous with higher levels in the forebrain (**Figure 6.4**). The mutant embryos are generally smaller than their littermates. At

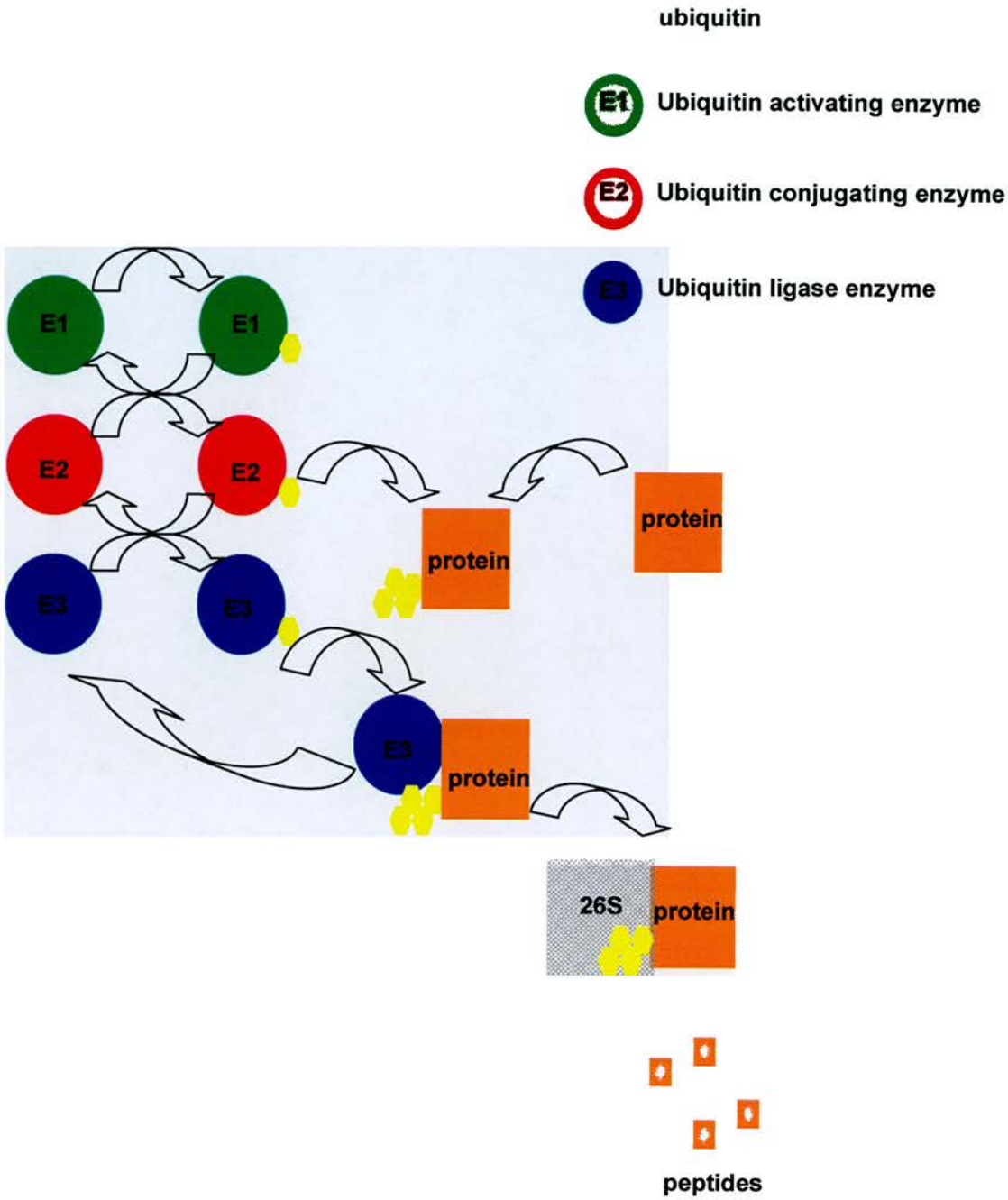


Figure 6.3 Ubiquitin-dependent protein degradation pathway

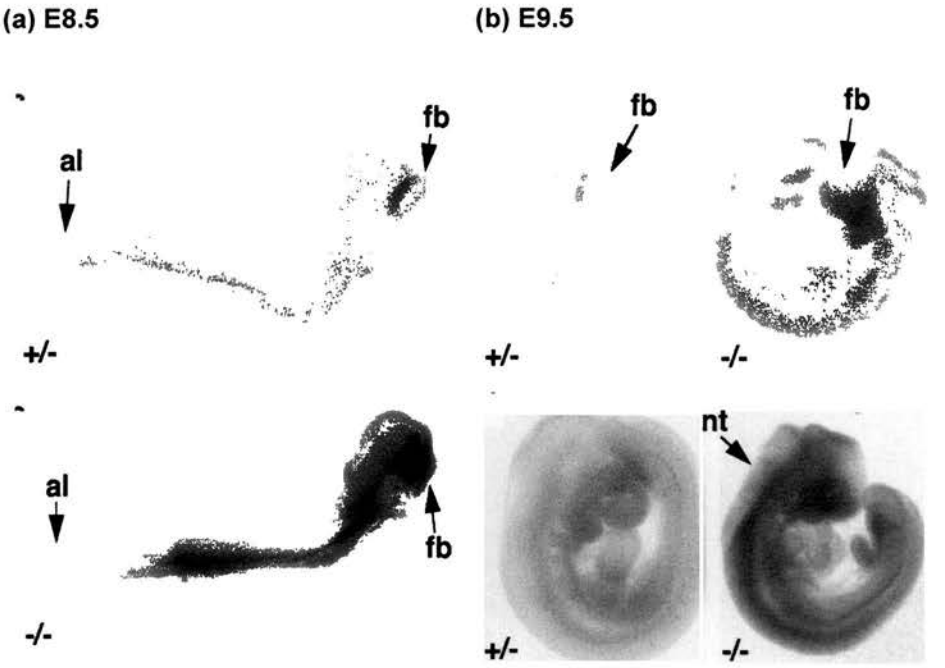


Figure 6.4 Expression and phenotype of the GT246 gene trap integration into *mUbc7*. Heterozygous and homozygous embryos at E8.5 and E10.5 stained with X-gal shows the ubiquitous expression of of the LacZ reporter with higher levels in the forebrain. (a) At E8.5 the allantois of *Ubc7*$^{-/-}$ embryos appears shortened and cylindrical in shape compared to heterozygous embryos which are phenotypically normal. (b) At E9.5 the neural tube has failed to close in *Ubc7*$^{-/-}$ embryos. X-gal staining was done by Julie Wallis. +/+ = wildtype, +/- = *mUbc7*$^{Gt246For/+}$, -/- = *mUbc7*$^{Gt246For/Gt246For}$ al = allantois, fb = forebrain, nt = neural tube

E8.5 the allantois appears shortened and cylindrical in shape (**Figure 6.4(a)**). At E10.5 the homozygotes have an abnormal neural tube, which has failed to close (**Figure 6.4(b)**). Heterozygous embryos look phenotypically normal and survive (**Table 6.1**). Histological sections of the E10.5 head revealed that the neuroepithelium of the mutant embryos is defined and smooth and does not appear to be damaged. Wholemount *in situ* hybridisation with a probe against *Wnt8B* shows that the forebrain in the mutant is specified but the neural folds fail to fuse (**Figure 6.5**).

It seems unlikely that the early embryonic death of the $Ubc7^{Gt246For/Gt246For}$ mice is due to the complete failure of neural tube closure. Of the fifty known single gene mutations that result in neural tube defects (NTD) in mice, most also cause severe embryonic lethal syndromes, but the NTD is a non-specific feature (Copp et al., 2003; Juriloff and Harris, 2000). Furthermore, the absence of a protein, such as Ubc7, with an essential role in a basic cellular pathway could be hypothesised to effect multiple distinct tissues or biological systems in an organism.

6.2.1.5 Possible haematopoietic phenotype

The rare embryos that survived beyond E11.5 appeared to be anaemic, which indicated that Ubc7 may be involved in haematopoiesis. It has been reported that haematopoietic progenitors and bone marrow stroma have binding sites for ubiquitin, which may participate in the homing and binding of haematopoietic cells to the stroma of the bone marrow and spleen (Parakh and Kannan, 1993). Ubiquitin may also be involved in the binding of haematopoietic cells to the stroma in the early embryo, which would by mediated by an ubiquitin conjugating enzyme, such as Ubc7. Therefore, the absence of Ubc7 could result in a failure to conjugate ubiquitin to the haematopoietic or stromal cells so that they were unable to bind to each other, disrupting haematopoiesis.

Gene targeting studies have revealed many genes involved in the generation and maintenance of haematopoiesis (reviewed in section 6.5 and Chapter 1: Introduction). The main haematopoietic defects resulting from the absence of these genes are threefold: (i) disruption of primitive and definitive haematopoiesis, (ii) disruption of definitive haematopoiesis only or (iii) impairment of haematopoietic

age	genotype		
	+/+	+/-	-/-
E9.5	6	14	8
E10.5	24	41	20
E11.5	4	12	0
adult (6-8 wks)*	61	105	0

Table 6.1 Frequencies of midgestation embryos and adult mice according to genotype. +/+ = wildtype, +/- = *mUbc7*$^{Gt246For/+}$, -/- = *mUbc7*$^{Gt246For/Gt246For}$, wks = weeks, * = age when genotyped

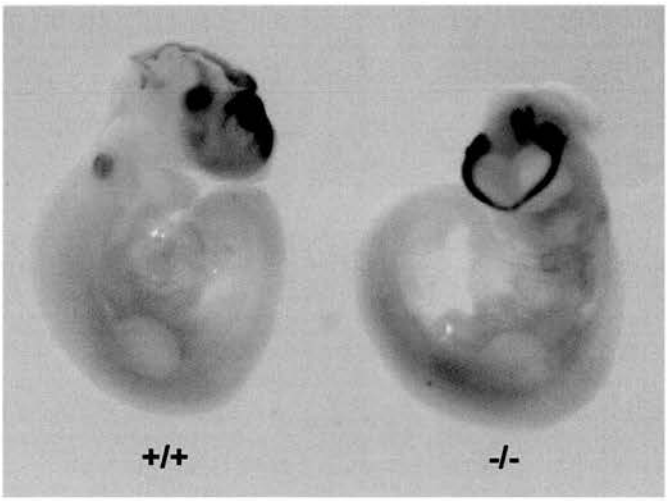


Figure 6.5 Expression of *Wnt8B* at E10.5 highlights failure of neural tube closure in homozygous embryos. *In situ* hybridisation with a probe against *Wnt 8B*, which is expressed in a restricted pattern where the neural folds meet and fuse to form the dorsal midline of the prosencephalon of the developing forebrain. +/+ = wildtype, -/- = *mUbc7*$^{Gt246For/Gt246For}$

precursor migration and colonisation. This chapter describes the experiments performed to examine the mutant embryos for a putative haematopoietic phenotype.

6.3 Experimental approach

- Determine whether there is a defect in primitive haematopoiesis by comparing the numbers of haematopoietic progenitors in the yolk sacs of wildtype, heterozygous or homozygous embryos at E9.5 and E10.5, as measured by the CFU-A assay.
- Determine whether there is a defect in definitive haematopoiesis by comparing the numbers of haematopoietic progenitors in the AGM regions of wildtype, heterozygous or homozygous embryos at E9.5 and E10.5, as measured by the CFU-A assay.

6.4 Results

6.4.1 Primitive haematopoiesis

To measure the level of primitive haematopoietic activity in the mutant $Ubc7^{Gt246For/Gt246For}$ embryos compared to their wildtype and heterozygous littermates, the CFU-A assay was set up with cells isolated from individual dissected yolk sacs from E9.5 and E10.5 embryos. $Ubc7^{Gt246For/Gt246For}$ embryos could be identified immediately on dissection by the open neural tube, but all of the embryos were genotyped by the level of β-galactosidase activity in the head after staining with X-gal (see Chapter 2: Materials and Methods, section 2.9).

Initially, the intact yolk sacs were viewed under a dissecting microscope to determine whether those from homozygous embryos appeared more anaemic in comparison to those from wildtype or heterozygous embryos, potentially indicating defective erythropoiesis. Although the apparent development of the vasculature and the "redness" visible within the vessels varied between the embryos (particularly at E10.5), no obvious correlation to genotype was observed (data not shown) and further quantitation of these observations was not carried out.

There was also no significant difference in the CFU-A activity of yolk sacs from wildtype, heterozygous or homozygous null littermates at 9.5 and 10.5 days of gestation (**Figure 6.6** and **Table 6.2**). At E9.5 the number of CFU-A colonies (per $3x10^4$ cells) ranged from 0.3 to 3.7 from wildtype-, 0 to 0.3 from heterozygous- and 0 to 14.5 in homozygous yolk sacs. The numbers of CFU-A colonies (per $3x10^4$ cells) in the yolk sacs from E10.5 littermates showed a high level of variation, which was irrespective of genotype ranging from 0.3 to 103.3 from wildtype-, 1 to 124.7 from heterozygous- and 0 to 137 from homozygous embryos. The CFU-A activity in yolk sacs at E9.5 was much lower than at E10.5. A similar time course has also been shown using the HPP-CFC assay (Palis and Yoder, 2001). Different strains were used as the sources of the yolk sacs for the two time points, E9.5 yolk sacs were dissected from F1 CBA x 129 embryos, while outbred MF1 mice were used for experiments at E10.5, because although the initial experiments were done on 129/Sv x CBA/Ca mice, the outbred MF1 mice were used subsequently as they produce larger litters. The variation between the two experiments conducted with E10.5 yolk sacs (illustrated respectively by red and blue open circles in **Figure 6.6(b)**) could reflect a slight difference in gestational age of the litters or variable quality of the disassociation of the yolk sacs for plating in the CFU-A assay.

6.4.2 Definitive haematopoiesis

Ubc7$^{Gt246For/Gt246For}$ embryos die prior to foetal liver haematopoiesis, so in order to determine whether there was a defect in definitive haematopoietic activity in the mutant embryos compared to their wildtype and heterozygous littermates, the AGM region was examined. In the AGM region, the production of HSCs peaks by day 11.5 of gestation (see Chapter 1: Introduction) as does the CFU-A activity (**Figure 3.1**). Here the CFU-A assay was again used to measure haematopoietic activity, however no viable homozygous embryos were obtained for analysis at E11.5. Therefore, AGM regions dissected from E10.5 embryos were used, but at this age the number of detectable CFU-A progenitors in the AGM region is very low (**Figure 3.1**). CFU-A activity in the AGM region increases during organ culture (**Figure 3.1**), so this strategy was used to expand the number of haematopoietic progenitors to

Tissue	E	Genetic back-ground	Organ cult.	CFU-A data									[1]Stat. sign.
				+/+			+/-			-/-			
				range	mean	n	range	mean	n	range	mean	n	
YS	9.5	CBA/129	No	0.3-3.7	2	2	0-0.3	0.8	4	0-14.5	4.5	4	No
YS	10.5	MF1	No	0.3-103.3	36.4	9	1-124.7	63.1	8	0-137	58.2	7	No
AGM	9.5	both	Yes	3.3-9.6	30.5	4	1-77.3	31.7	10	0-4.7	2.6	4	Yes
AGM[2]	9.5	MF1	Yes	3.3-10	6.7	2	1-77.3	25.8	8	0-3.3	1.7	2	Yes
AGM[2]	9.5	CBA/129	Yes	12.7-96	54.5	2	40-70.7	55.4	2	2.4-4.7	3.6	2	nd
AGM	10.5	MF1	Yes	11.3-114.7	74.0	10	23.3-129.7	93.7	19	64-106	81.6	5	No
AGM	10.5	CBA/129	Yes	76-191	104.7	5	0-240.5	101.4	14	0-42.3	9.0	8	Yes

Table 6.2 Summary of haematopoietic activity in yolk sacs and AGM regions from *Ubc7*$^{+/+, +/-}$ and $^{-/-}$embryos. The range and mean number of CFU-A colonies per $3x10^4$ cells from yolk sacs or AGM regions are shown. [1]The presence of a significant difference between the numbers of colonies from homozygotes compared to heterozygous and wild type tissues as determined by the Mann-Whitney U test is indicated. [2]The data for CFU-A activity in AGM regions from E9.5 embryos was pooled for representation in Figure 6.8 because the number of F1 embryos was too small for statistical analysis, but is shown separately here. +/+ = wildtype, +/- = *mUbc7*$^{Gt246For/+}$, -/- = *mUbc7*$^{Gt246For/Gt246For}$, n = the number of tissues compared. nd = not determined.

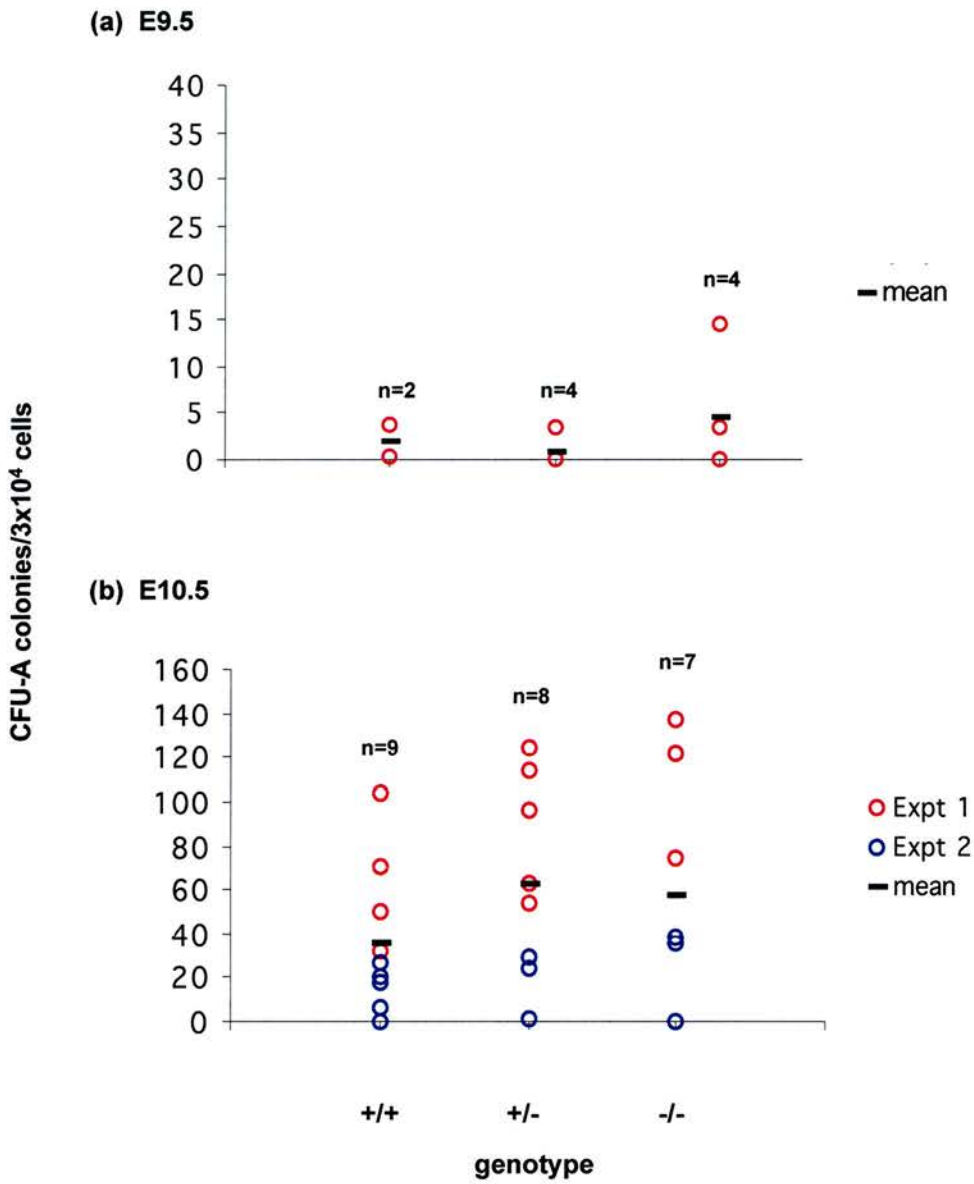


Figure 6.6 Primitive haematopoiesis in *Ubc7* deficient embryos. CFU-A activity in yolk sacs dissected from (a) E9.5 CBA x 129 (b) E10.5 MF1 embryos. Each data point represents the number of colonies per $3x10^4$ cells from individual yolk sacs. Bars (—) show the mean number of colonies. The number of yolk sacs of each genotype analysed is shown. +/+ = wildtype, +/- = *mUbc7*$^{Gt(246Fut/+}$, -/- = *mUbc7*$^{Gt(246Fut/Gt(246Fut}$

measurable levels. The experiments were done using both outbred (MF1) and F1 (129/Sv x CBA/Ca) mouse strains. All the data are summarised in **Table 6.1**.

In the outbred (MF1) strain the number of CFU-A colonies (per $3x10^4$ cells) in E10.5 AGM regions after six days in organ culture ranged from 11.3 to 114.7 from wildtype-, 23.3 to 129.7 from heterozygous- and 64 to 106 from homozygous littermates (**Figure 6.7(a)**). No significant difference was found between the homozygous and wildtype or heterozygous littermates ($p < 0.81$ and $p < 0.27$ respectively) according to the Mann-Whitney U Test.

In the F1 (CBA x 129) mice there were significantly fewer CFU-A colonies (per $3x10^4$ cells) detected in the cultured AGM regions of $Ubc7^{Gt246For/Gt246For}$ embryos (0 to 42.3) compared to wildtype (76 to 191) and heterozygous (0 to 240.5) littermates, $p < 0.003$ and $p < 0.001$ respectively as determined by the Mann-Whitney U Test (**Figure 6.7(b)**).

Cultured AGM regions from E9.5 embryos were then analysed to rule out the possibility that the lower overall CFU-A activity in the F1 at E10.5 was not simply because the embryos were dying, although those embryos had been excluded from the analysis. The data from MF1 and CBA x 129 embryos were combined because the number of embryos obtained was small. At E9.5 there were significantly fewer CFU-A colonies (per $3x10^4$ cells) from homozygous null AGM regions after six days in culture (0 to 4.7) compared to wildtype (3.3 to 96) and heterozygous embryos (1 to 77.3), $p < 0.05$ and $p < 0.016$ respectively as determined by the Mann-Whitney U Test (**Figure 6.8**).

6.4.3 Potential cardiac phenotype

Analysis of embryos at E9.5 reveals a consistent heart defect associated with loss of Ubc function. A comparison of wild type or heterozygous control embryos with $Ubc7^{Gt246For/Gt246For}$ embryos, identified a subtle rightward displacement of the left ventricle and bulbo-ventricular groove (data not shown). Furthermore, one of the four homozygous null embryos examined at E9.5 had additional heart defects (**Figure 6.9**). The bulbus cordis and outflow tract region are displaced such that they

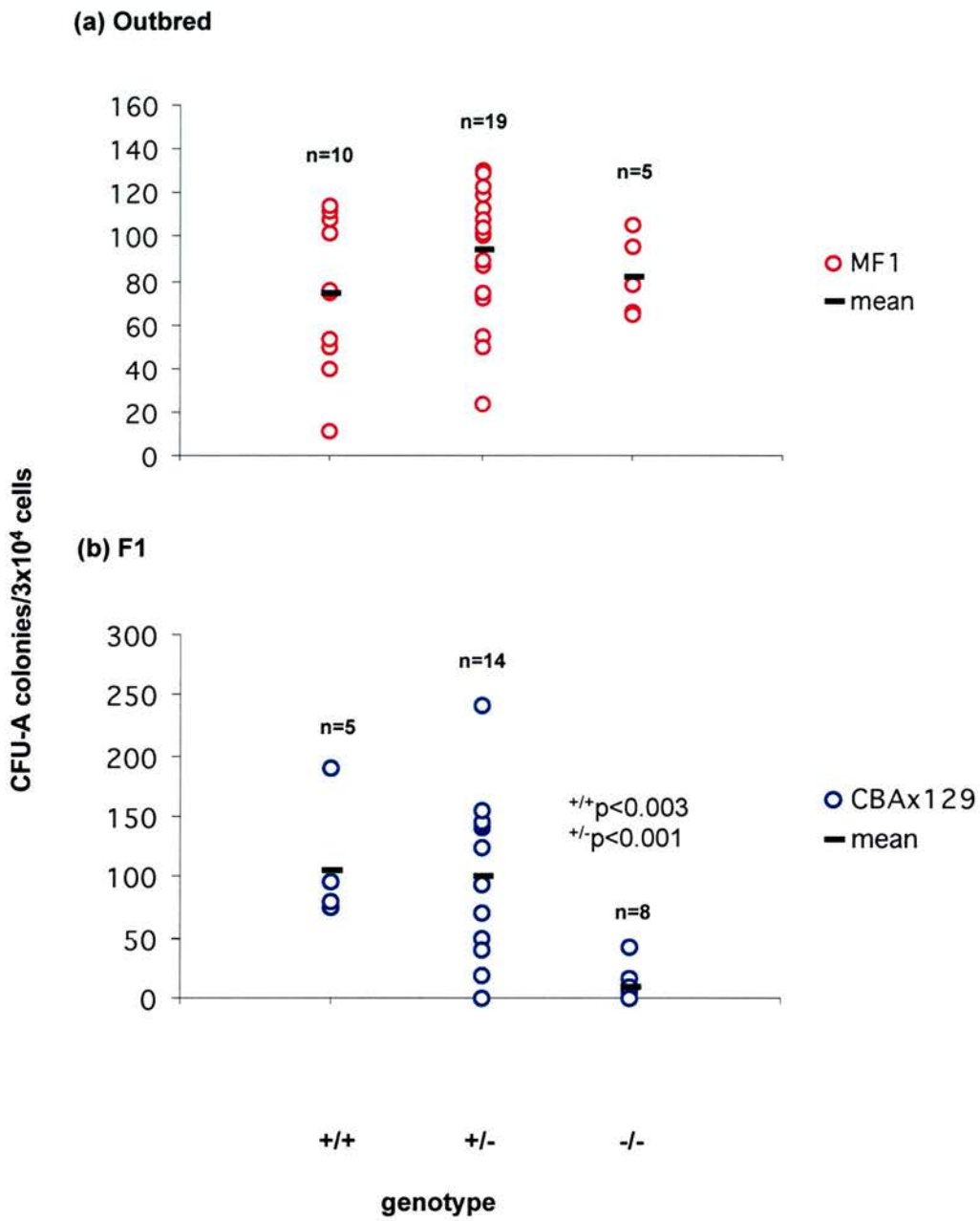


Figure 6.7 Definitive haematopoiesis in *Ubc7* deficient embryos at E10.5. CFU-A activity in cultured E10.5 AGM regions dissected from (a) outbred MF1 and (b) F1 CBA x 129 embryos. Each data point represents the number of colonies per $3x10^4$ cells from individual AGM regions cultured for 6 days. Bars (—) show the mean number of colonies. The number of AGM regions of each genotype analysed is shown. P values are indicated for homozygous embryos against wildtype ($^{+/+}$p) and heterozygous ($^{+/-}$p) embryos respectively. +/+ = wildtype, +/- = $mUbc7^{Gt246For/+}$, -/- = $mUbc7^{Gt246For/Gt246For}$

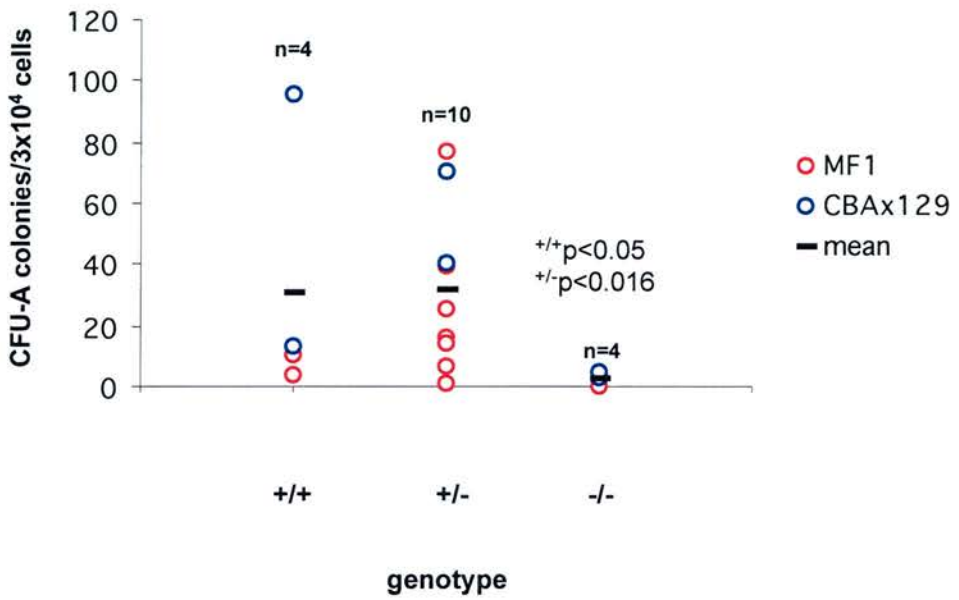


Figure 6.8 Definitive haematopoiesis in *Ubc7* deficient embryos at E9.5. CFU-A activity in cultured E9.5 AGM regions dissected from outbred MF1 (○) and F1 CBA x 129 (○) embryos. Each data point represents the number of colonies per $3x10^4$ cells from individual AGM regions cultured for 6 days. Bars (—) show mean number of colonies. The number of AGM regions of each genotype analysed is shown. P values are indicated for homozygous embryos against wildtype ($^{+/+}$p) and heterozygous ($^{+/-}$p) embryos respectively. +/+ = wildtype, +/- = *mUbc7*$^{Gt246For/+}$, -/- = *mUbc7*$^{Gt246For/Gt246For}$

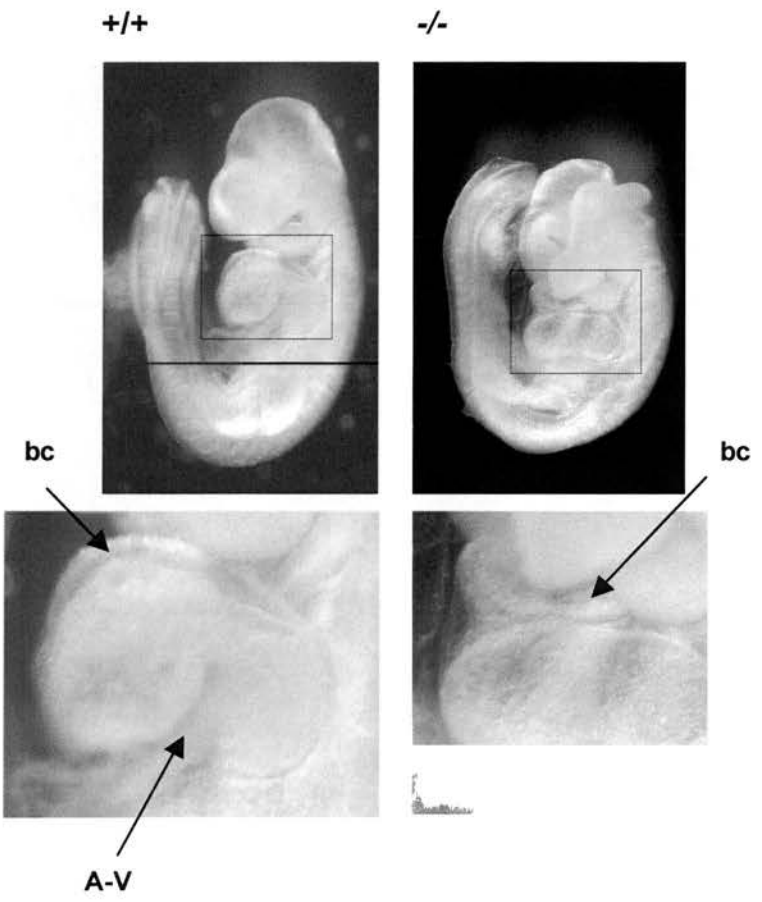


Figure 6.9 Cardiac defect in *Ubc7* deficient embryos. Embryos at E9.5 showing normal wildtype heart and defect in mutant. The bulbus cordis and outflow tract region are displaced such that they sit rostral of the left ventricle and not right lateral as in controls. In addition, the A-V canal fails to form. The lower panels show the heart region as indicated. +/+ = wildtype, -/- = $mUbc7^{Gt246For/Gt246For}$, bc = bulbus cordis

sit rostral of the left ventricle and not right lateral as in controls. The atrio-ventricular (A-V) canal also fails to form (Alistair Watt, personal communication).

The time of death coincides with that of known genetic mutations affecting the looping of the heart tube during development (Lin et al., 1997; Lyons et al., 1995; Niederreither et al., 2001), but histological sections are needed to confirm whether the heart is abnormal in these embryos.

6.5 Discussion

Haematopoiesis in the early embryo is sustained by yolk sac-derived precursors (erythromyeloid cells) until haematopoiesis from intraembryonic sites is initiated. Null mutations affecting primitive haematopoiesis are normally lethal between E8.5 to 10.5 for example, *Flk1* (Shalaby et al., 1995), *Tal1/Scl* (Robb et al., 1995), *Lmo2* (Warren et al., 1994) which is slightly earlier than the mutation caused by the GT246 gene trap integration into the *Ubc7* gene. However GATA-1 deficient embryos die at E10.5 to 11.5 due to an arrest in primitive erythropoiesis (Pevny et al., 1991), so a primitive haematopoietic defect was considered possible. The data demonstrate however that Ubc7 deficiency is not detrimental to primitive, yolk sac-derived haematopoiesis.

Definitive, adult-type HSCs are generated from the AGM region of the midgestation embryo and subsequently colonise the other haematopoietic organ rudiments, such as the foetal liver. Targeted disruption of genes that affect definitive haematopoiesis usually die during mid to late gestation (although some are viable until birth or beyond) depending on the nature of the defect. For example, deficiency of the transcription factor GATA2 affects both primitive and definitive haematopoiesis leading to early embryonic death around E10.5 to 11.5 from defective expansion of the various lineages (Tsai et al., 1994). *CBFβ* null embryos die between E11.5 and 14.5 with haemorrhaging of the CNS and a block in foetal liver haematopoiesis (Wang et al., 1996b). *AML1/Runx1/Cbfα2* null embryos die around E11.5 to 13 from a similar phenotype to $CBF\beta^{-/-}$ embryos (Wang et al., 1996a). It has been shown that AML1/Runx1 is required for the formation of the

clusters of haematopoietic cells found on the ventral wall of the dorsal aorta of the AGM region (North et al., 1999).

A potential lethal phenotype involving definitive haematopoiesis is unlikely, but cannot be ruled out from these experiments. CFU-A activity in $Ubc7^{Gt246For/Gt246For}$ AGM regions at E10.5 after explant culture was reduced, but only in the F1 (129/Sv x CBA/Ca) embryos and not on an outbred (MF1) background. CFU-A activity in the AGM regions from mutant E9.5 embryos was decreased in both strains, but limited numbers were available for analysis. One possibility for the observed reduction in homozygous embryos at E9.5 is that the development of intraembryonic haematopoiesis is delayed and subsequently recovers but only in the outbred animals. Although smaller than their heterozygous and wildtype littermates, homozygous embryos did not appear morphologically retarded, but the numbers of somite pairs should be counted to confirm this.

Since no consistent haematopoietic defect was observed in the mutant AGM regions, it was not considered important to set up ES cell co-culture experiments to test the stromal microenvironment (see Introduction to this chapter). It may nevertheless be interesting find out if the F1 (129/Sv x CBA/Ca) homozygous null AGM regions are impaired in their ability to enhance the haematopoietic differentiation of ES cells compared to on an outbred MF1 background.

Further *in vitro* experiments, including colony assays other than the CFU-A, which measure more mature haematopoietic progenitors, such as the CFC-mix, GM-CFC or CFU-E, could be performed on the AGM regions of these mutant embryos for a more detailed investigation of their haematopoiesis. *In vitro* haematopoietic analyses may be misleading, haematopoietic progenitors isolated from Mll deficient AGM regions after organ culture could differentiate into macrophages *in vitro*, but were unable to reconstitute sublethally irradiated $RAG\gamma C^{-/-}$ recipients (Ernst et al., 2004). The *in vivo* CFU-S or competitive repopulation assays could be used for quantitative analysis on short term or long term HSCs respectively.

These experiments do not assess whether there is a defect in the migration of AGM region derived HSCs to the foetal liver or a defect in the hepatic environment causing impaired foetal liver haematopoiesis, but one would expect the embryos to survive for longer if either were the case. For example HSCs lacking β1 integrin can

differentiate into different lineages but are unable to colonise the foetal liver or adult haematopoietic organs (Hirsch et al., 1996; Potocnik et al., 2000). Embryos homozygous mutant for *jumonji*, encoding a putative transcription factor, (also generated by a gene trapping strategy) die between E10.5 and 15.5 exhibiting defective definitive haematopoiesis in the foetal liver (these animals also have a neural tube defect, which is dependent on genetic background, (Kitajima et al., 1999). *Jumonji* is not expressed in wildtype HSCs isolated from foetal liver, but it is expressed in foetal liver fibroblasts, and mutant *jumonji* deficient HSCs reconstitute irradiated adult recipients, indicating a defective hepatic environment for haematopoiesis. Interestingly, the authors report of unpublished data purporting normal development of haematopoietic cells in AGM region explant cultures. Both of these mutations result in an accumulation of haematopoietic precursors in the circulation; it would be interesting to find if such a phenomenon is evident in $Ubc7^{Gt246For/Gt246For}$ embryos.

Importantly, the gene trap integration into the *mUbc7* gene resulted in a functional disruption of the protein and the embryonic lethal phenotype was penetrant. In contrast, the reduced haematopoietic activity measured by the CFU-A assay in AGM regions after organ culture was strain dependent, observed only in F1 129/Sv x CBA/Ca embryos. Therefore the cause of death in the mutant embryos is likely to be something other than a haematopoietic defect.

There appeared to be a cardiac defect in the homozygous mutant embryos. The heart develops from bilaterally symmetric cardiogenic primordia that coalesce at the midline to form a primitive heart tube. At E8.5 the heart tube initiates rightward looping which positions the chambers. Further maturation of the looped cardiac tube gives rise to the future right and left ventricles and the common atrial chamber. The subtle change in cardiac morphology observed in the $Ubc7^{Gt246For/Gt246For}$ embryos at E9.5 could be a result of looping defects in the developing heart, which in turn may be caused by abnormal rates in cell proliferation. The cell cycle is one of the many processes in which the ubiquitin proteosome-dependent degradation pathway has been shown to be involved (Ciechanover, 2003). Furthermore mutations affecting looping of the heart tube are normally lethal around E10.5 (Kitajima et al., 1999; Lin et al., 1997; Lyons et al., 1995; Niederreither et al., 2001), but less severe defects

could survive longer *in utero*. The apparent cardiac defect is very subtle, which could explain why it was not picked up previously even though the gene trap screen was designed to identify novel cardiac genes, and thus histological sections are needed to confirm this observation.

Finding the defects in these mutant embryos is difficult because of the ubiquitous expression of the gene. In an attempt to address the biological function of Ubc7, it would be useful to identify its target proteins. To this end a proteomics approach is being taken and 2D gel analysis is being performed on proteins extracted from E9.5 and 10.5 embryos, based on the assumption that a deficiency in an ubiquitin conjugating enzyme may lead to a build-up of target proteins that should otherwise have been degraded. For example, mutations in the *PARK2* gene, encoding the E3 ubiquitin ligase parkin, causes autosomal recessive juvenile Parkinsonism (AR-JP), which is one of the common familial form of Parkinson's disease. An accumulation of target proteins is thought to be one component leading to apoptosis of dopaminergic neurons in the pathogenesis of the disease (Coelln et al., in press). Potential target proteins from the 2D gel electrophoresis could then be identified by mass spectrometry.

mUbc7 is the first specific E2 implemented in endoplasmic reticulum-associated degradation (ERAD) in mammalian cells (Tiwari and Weissman, 2001). The ERAD pathway is a quality control mechanism which, guards against the accumulation of aberrant proteins by ensuring their proteolysis (McCracken and Brodsky, 2003). ERAD substrates are selected in the ER and are "retro-translocated" into the cytoplasm for degradation by the proteosome. One mechanism for the extraction of the target proteins from the ER is through polyubiquitinisation by the enzymes of the ubiquitin dependent degradation pathway. Tiwari and Weissman (2001) found mUbc7 to be membrane associated by co-localisation with mUbc6 and overexpression of a catalytically inactive form of mUbc7 delayed the degradation of T-cell receptor subunits (TCRα and CD3-δ) from the ER.

In addition to its function in T-cell receptor subunit degradation in adult mice described above, the gene trap integration into *mUbc7* will eventually lead to the function of the encoded protein during early embryonic development.

Chapter Seven

Summary and Prospective

7.1 Summary and Prospective

The data from this study show that the differentiation of highly proliferative haematopoietic progenitors from ES cells, as measured by the CFU-A and HPP-CFC assays, is dramatically enhanced by embryonic AGM region co-culture. This increase is likely to be due to at least proliferation and perhaps also *de novo* induction of early progenitors within EBs. Analysis of replating efficiencies from CFU-A colonies from co-cultured EBs would show self-renewal capacity and be more indicative of repopulation potential *in vivo*. A comparison of self-renewal potential between EB-derived HPP-CFC and CFU-A would allow their relationship to be examined further.

Cloned stromal cell lines from the AGM region are not as conducive to haematopoietic differentiation of ES cells as intact embryonic organ rudiments and the data suggest that inductive and/or proliferative factors are provided. The co-culture system could be useful for identifying the factor(s) driving these processes. Elaine Dzierzak and her colleagues are already investigating this by DNA microarray on the stromal cell lines. The co-culture system is ideal for analysis by an array or proteomics approach to investigate the inductive/proliferative factors affecting haematopoietic development in EBs by stromal cell line- or intact AGM region explant co-cultures. A stromal cell line based induction screen for candidate genes identified by gene trapping is being designed.

Significant advances have recently been made by others in achieving engraftment of ES-derived HSCs but experiments are underway to further investigate the LTR-HSC activity of co-cultured EBs. Reliably and efficiently obtaining LTR-HSC from ES cells is important not only for studying the factors involved in their development but for exploring their potential future clinical use in cell therapies. Linda Lako and her colleagues are currently employing this AGM region co-culture system for the haematopoietic differentiation of human ES cells. The debate is ongoing as to whether hES cells or multipotential adult somatic stem cells will play a larger or more useful role in stem cell-based therapies.

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Appendix:

Supplementary data

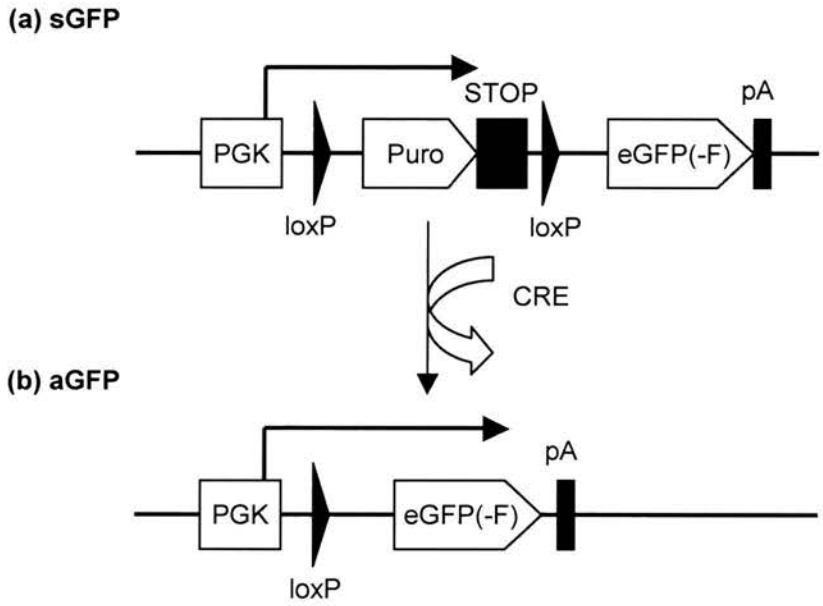


Figure A.1 Strategy for generation of eGFP expressing ES cells #7a and #97a. Cre-mediated excision of the stop cassette transforms silent eGFP into active eGFP. (a) ES cells transfected with seGFP construct are puromycin resistant and eGFP negative. After Cre-mediated recombination the cells become puromycin sensitive and eGFP expressing. This strategy was designed for the generation of silent eGFP mice by Dr Derek Gilchrist and the activated ES cell lines #7a and #97a were a kind gift. Figure adapted from Gilchrist *et al*, 2003.

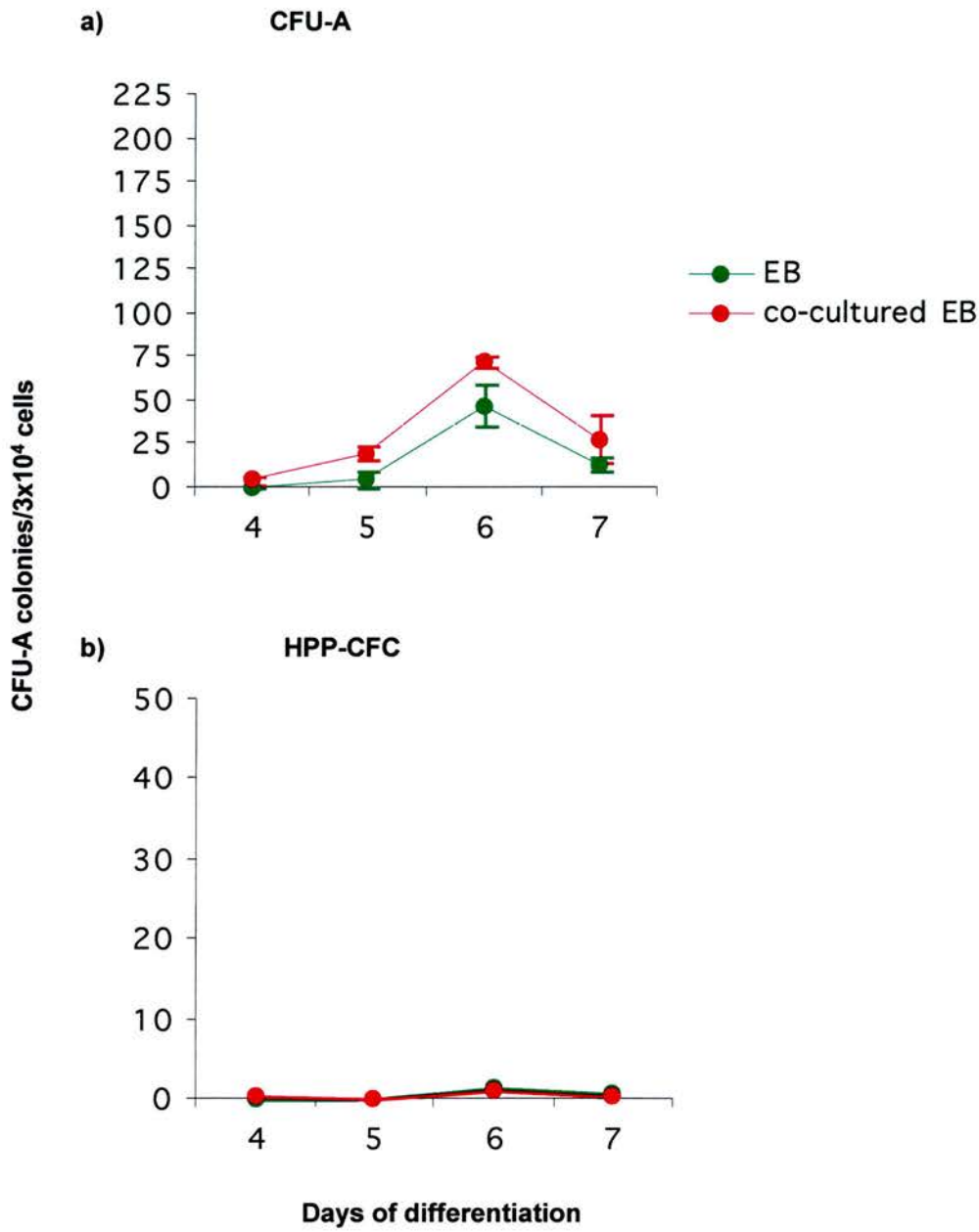


Figure A.2 Time course of CFU-A and HPP-CFC activity in day 3 embryoid bodies after co-culture with AGM region. Numbers of (a) CFU-A and (b) HPP-CFC colonies/3x10^4 cells in EBs cultured alone (●) and co-cultured (●) with E10.5 AGM region for up to 7 days. Error bars represent the standard deviations of the mean of triplicate plates.

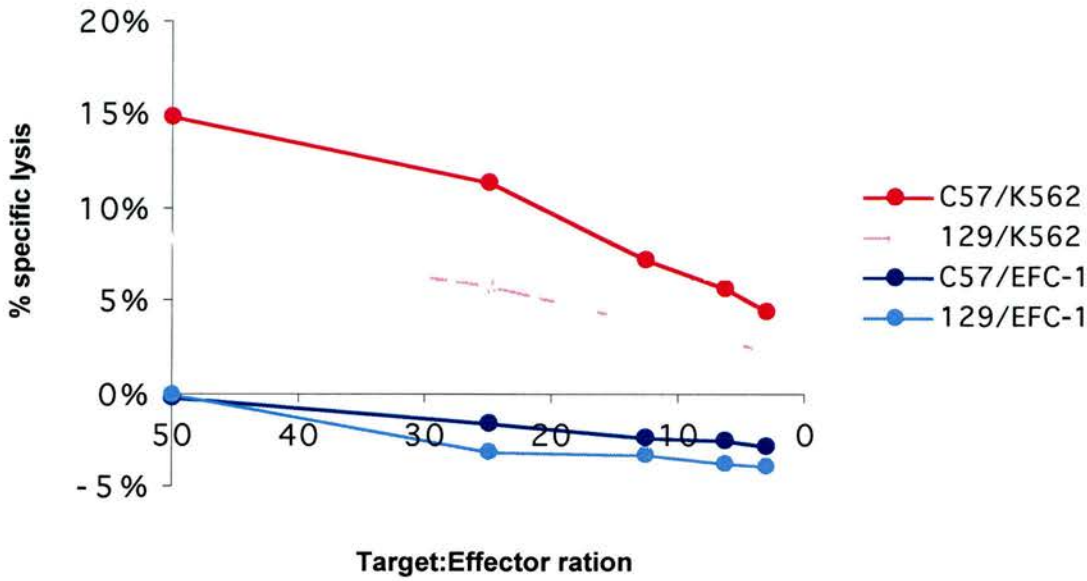


Figure A.3 NK cell activity against ES cells measured by a chromium-release assay. NK cell activity against ES cells (EFC-1) was measured and compared to a control NK sensitive cell line (K-562) by determining the ability of spleen mononuclear cells to lyse the target cells. Target cells were loaded with radioactive chromium (^{51}Cr) and added to a dilution series of spleen mononuclear cells. After an incubation period, the supernatants were harvested and the amount of intracellular ^{51}Cr released into the supernatant was measured with a gamma counter. The amount of ^{51}Cr released is proportional to the lytic activity of the NK cells against the target cells. The mean of the triplicate counts per minute (cpm) values were determined for each effector to target (E:T) ratio and for the spontaneous and maximum release wells. Percent specific target cell lysis is calculated for each E:T ratio as follows:

$$\% \text{ specific lysis} = \frac{\text{experimental release cpm - spontaneous release cpm}}{\text{maximum release cpm - spontaneous release cpm}} \times 100$$

The NK assay was set up and analysed by Dr Martin Waterfall.

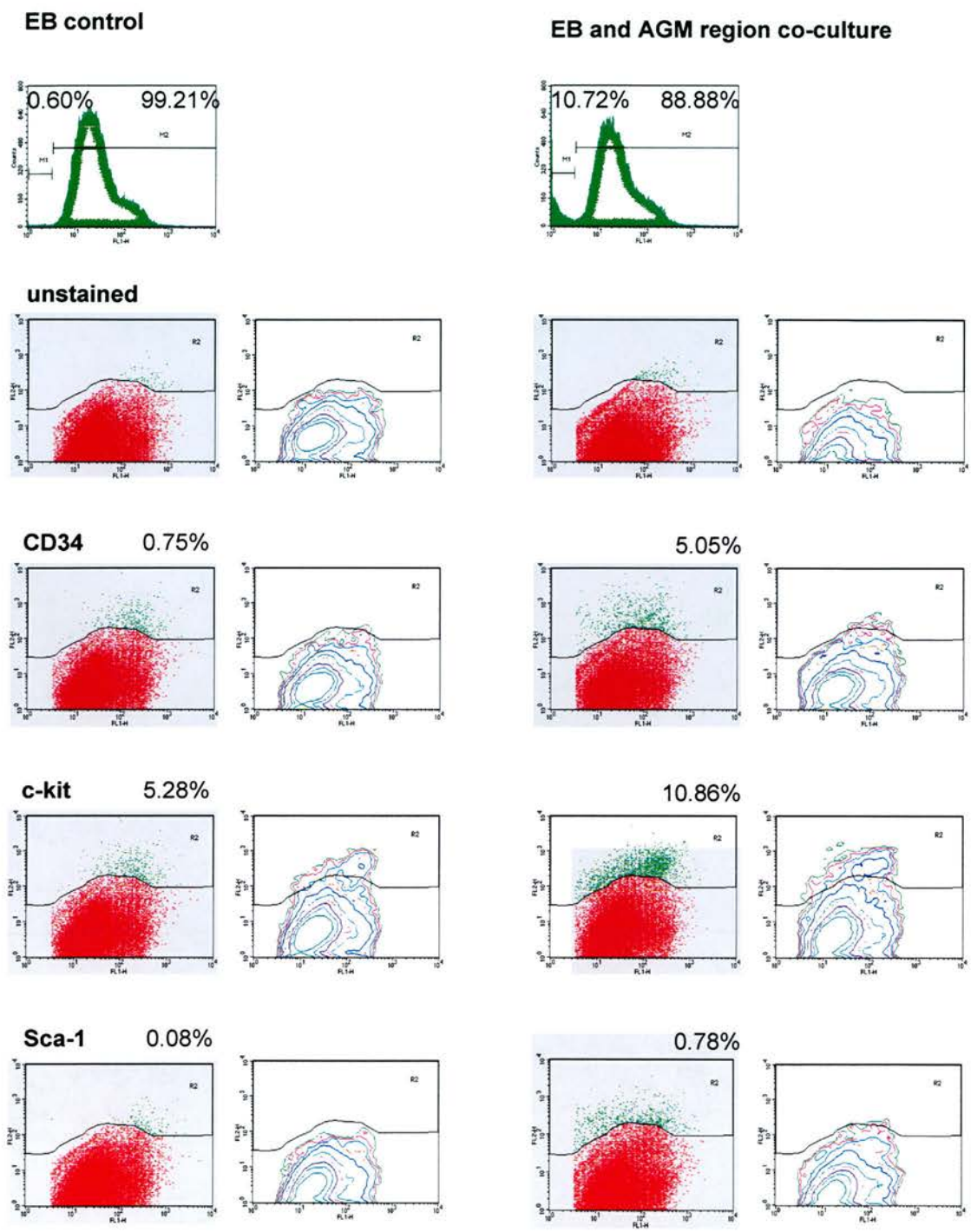
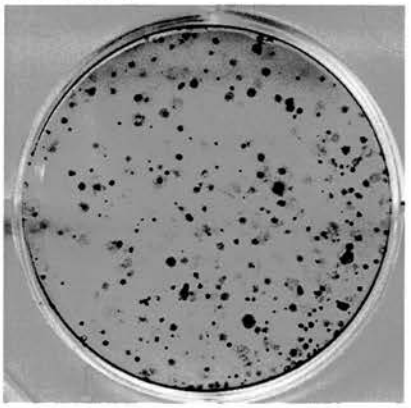
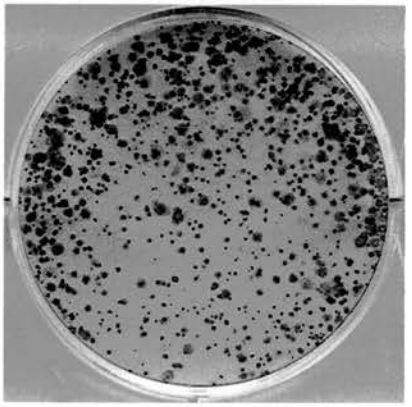


Figure A.4 Flow cytometric analysis of surface antigens associated with HSCs on embryoid body cells after co-culture with embryonic AGM region. Histograms in upper panels show the percentage of GFP$^+$ EB-derived cells. Cells gated in M2 were analysed for surface staining with antibodies against CD34, c-kit and Sca-1. The R2 gate includes all positive cells. Unstained cells in R2 gate have been subtracted from the % of +ve cells given.

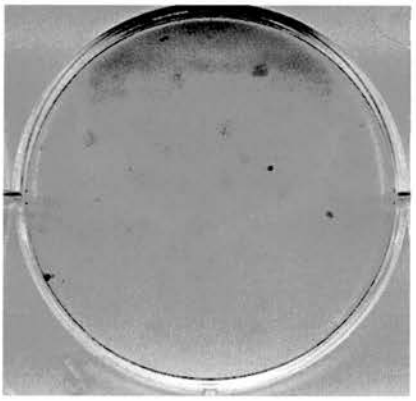
AM20.1B4



gelatin -LIF



UG26.1B6



EL08.1D2

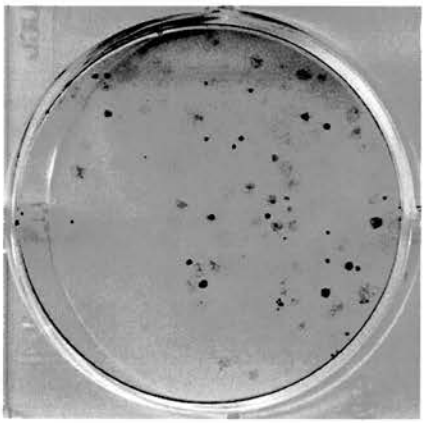


Figure A.5 Enlargement of Figure 5.8b.