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# Haematopoietic differentiation of murine embryonic stem cells

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**PhD**  
**The University of Edinburgh**  
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## **Declaration**

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I declare that this thesis was composed by myself and that the work contained therein is my own, except where explicitly stated otherwise in the text.

*Sabrina Gordon-Keylock*



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## Abstract

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Haematopoietic stem cells (HSCs) are routinely used to treat haematological disorders, as they can engraft into the bone marrow of immuno-compromised recipients where they undergo self-renewal and multilineage differentiation to provide long-term reconstitution of the blood system. Identification of novel factors able to regulate or expand HSCs would have a significant impact in a clinical setting. Mouse embryonic stem (ES) cells can be used as a model system to investigate haematopoietic regulation, since these pluripotent cells are amenable to large-scale culture and have the capacity to differentiate into a variety of cell types *in vitro*, including cells of haematopoietic lineages. Mature blood cells can be generated relatively easily from ES cells; however, HSCs are generated at relatively low frequencies and there has been only limited success in the contribution of these cells to the adult haematopoietic system *in vivo*. Previous work demonstrated that the frequency of haematopoietic progenitors was significantly increased when ES cells were co-cultured with primary E10.5 aorta-gonad-mesonephros (AGM) tissue explants, a region which is able to give rise to HSCs *in vivo*. Therefore, the AGM region is a potent source of haematopoietic inductive signals both *in vivo* and for ES cells *in vitro*.

This project aimed to determine which subregion(s) of the AGM were responsible for the haematopoietic enhancing effects that primary AGM explants had on differentiating ES cells. To this end, a novel co-culture system has been established to test the enhancing effects of a panel of clonal stromal cell lines derived from different subregions of the midgestational AGM. It was found that three clonal stromal cell lines derived from the dorsal aorta and surrounding mesenchyme (AM) subregion of the AGM were able to significantly enhance the frequency of ES cell derived multipotent haematopoietic progenitors, as measured by *in vitro* colony assays and flow cytometry. By contrast, two stromal cell lines derived from the

urogenital ridges (UG) of the AGM did not enhance haematopoietic differentiation of ES cells. Interestingly, the enhancing capacity of the AM-derived stroma was comparable with that of the bone marrow derived OP9 stromal cell line, which has been widely used in the literature to promote haematopoietic differentiation of ES cells. Further investigation revealed that the enhancing capacity is not retained by extracellular matrices isolated from the AM stromal cell layers and the effects were dependent on direct ES cell-stromal cell contact. Co-culture of an ES cell line carrying a mesoderm specific *Brachyury*-eGFP reporter gene demonstrated that the stromal lines mediated their effects post- *Brachyury* (mesoderm) induction in the ES cells. In addition, co-culture of sorted ES cell populations confirmed that *Brachyury*<sup>+</sup>, but not *Brachyury*<sup>-</sup>, cells gave rise to haematopoietic progenitors in AM stromal co-culture, supporting the notion that ES cell differentiation recapitulated the *in vivo* pattern of lineage specification. Transplantation of co-cultured ES cells into irradiated adult NOD/SCID mouse recipients led to low levels of donor cell engraftment in the spleen and bone marrow, which expanded upon serial transplantation; but full repopulation of the recipient haematopoietic system was not confirmed. Adult bone marrow cells were found to achieve repopulation more readily in the NOD/SCID animal model when transplanted intra-splenically, as compared to intra-venous injection. This suggests that transplantation of ES-derived haematopoietic cells directly into the haematopoietic niche, by intra-splenic or intra-femoral injection, could facilitate repopulation.

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## List of Abbreviations

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AGM	- aorta-gonad-mesonephros region
AM	- aorta and surrounding mesenchyme of AGM region
BFU-E	- erythroid burst forming unit
BL-CFC	- blast colony forming cell (haemangioblast)
BM	- bone marrow
BMP	- bone morphogenic protein
Bry	- Brachyury gene
CAFC	- cobblestone-area forming cell
CFC	- colony forming cell
CFU	- colony forming unit
CFU-A	- colony forming unit A
CFU-E	- erythroid colony forming unit
CFU-GM	- granulocyte/macrophage colony forming unit
CFU-Mix	- granulocyte, erythroid, macrophage and/or megakaryocyte CFU
CFU-M	- macrophage colony forming unit
CSF-1	- colony stimulating factor 1
EB	- embryoid body
ECM	- extracellular matrix
eGFP	- enhanced green fluorescent protein
EL	- embryonic liver
EPO	- erythropoietin
Ery/Mac	- erythroid/macrophage colony
ESC	- embryonic stem cell
FACS	- fluorescence activated cell sorting
FCS	- foetal calf serum
FDG	- fluorescein-di-beta-galactosidase
FGF	- fibroblast growth factor
GM-CSF	- granulocyte/macrophage colony stimulating factor
H.d.	- hanging drop
H&E	- haematoxylin and eosin stain
HPC	- haematopoietic progenitor cell
HPP	- high proliferative potential
HSC	- haematopoietic stem cell
ICM	- inner cell mass
IL	- interleukin
JHBL	- John Hughes Bennett Laboratory, QMRI Edinburgh
KTLS	- cKit, Thy1, Lineage, Sca-1
LIF	- leukaemia inhibitory factor
LTC-IC	- long term culture -initiating cell
LTR	- long term repopulating
M-CSF	- macrophage colony stimulating factor

MEF	- mouse embryonic fibroblasts
MHC	- major histocompatibility complex
MIP-1 $\alpha$	- macrophage inflammatory factor -1 $\alpha$
NOD	- non obese diabetic
OP9	- stromal cell line derived from op-/- mice
PBL	- peripheral blood
PCR	- polymerase chain reaction
PEC	- peritoneal exudate cells
P-Sp	- para-aortic splanchnopleura region
RT	- reverse transcriptase
SCF	- stem cell factor
SCID	- severe combined immuno-deficient
SDF-1	- stromal derived factor 1
SLAM	- signalling lymphocyte activation molecule
SMA	- smooth muscle actin
SP	- side population
SPL	- spleen
STR	- short term repopulating
TGF	- transforming growth factor
TPO	- thrombopoietin
UG	- urogenital ridges of AGM region
VCAM	- vascular cell adhesion molecule
VEGF	- vascular endothelial growth factor
VLA	- very late antigen
VSMC	- vascular smooth muscle cell

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Chapter One  
**Introduction**

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## 1.1 Introduction

Haematopoietic differentiation of embryonic stem (ES) cells *in vitro* has thus far been a valuable model system in which to study induction and regulation of haematopoietic cells; however, some limitations remain. For instance, haematopoietic stem and progenitor cells which have multilineage potential *in vivo* are generated at relatively low frequencies and the ES cell derived populations are highly heterogenous. This project aimed to optimise the culture conditions for directing haematopoietic differentiation of mouse ES cells. A novel differentiation system has been established using embryo-derived stromal cell lines to enhance the frequency of early multipotent haematopoietic progenitors generated in culture. Therefore, this introduction will focus on the murine system to review the properties of adult haematopoietic stem cells, their induction during embryogenesis and the haematopoietic differentiation of mouse embryonic stem cells.

## 1.2 Adult haematopoietic stem cells (HSCs)

Haematopoiesis is the process whereby a limited number of haematopoietic stem cells (HSCs) undergo multilineage differentiation to give rise to the mature cells of the blood and immune system (Figure 1.1). This includes terminally differentiated cells of erythroid, myeloid and lymphoid lineages. These mature haematopoietic cells are short-lived and therefore need to be continually replaced from a pool of stem cells. In the adult, long-term repopulating (LTR) HSCs residing in the bone marrow (BM) are at the top of the haematopoietic hierarchy. These stem cells are characterised by their high self-renewal and proliferative potential, as well as their multilineage differentiation capacity.

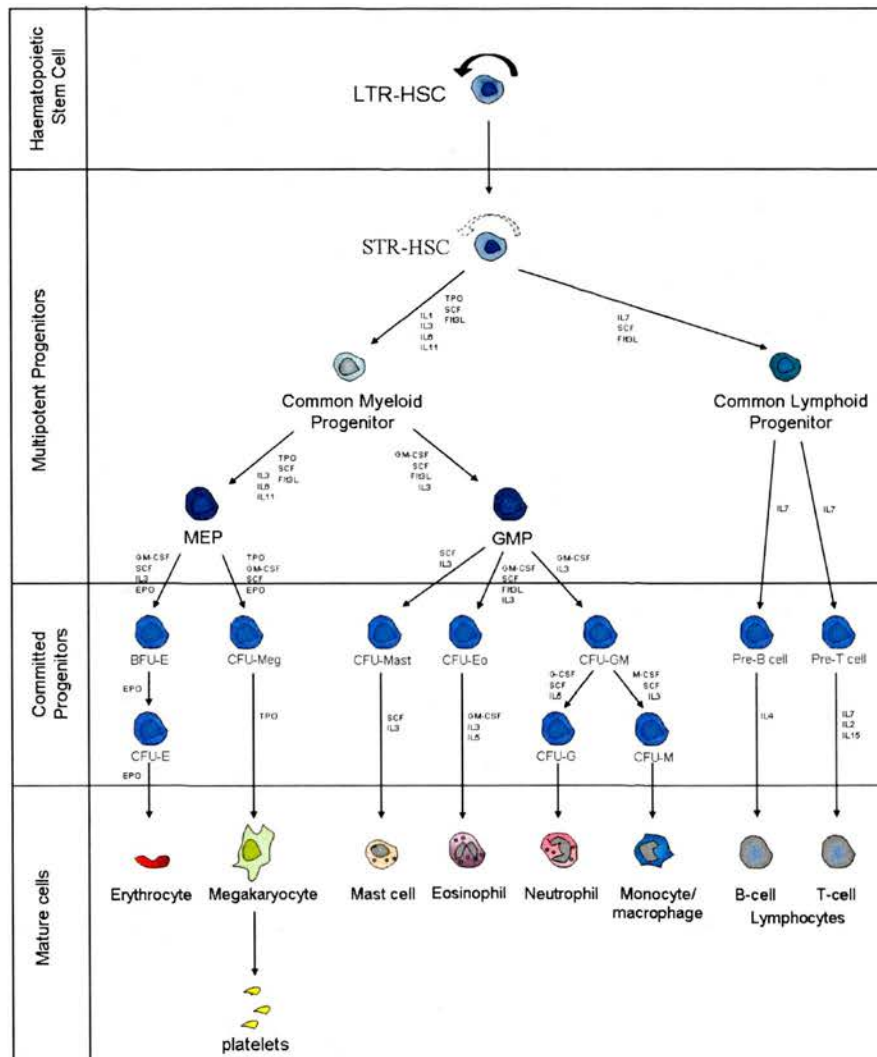


Figure 1.1 The haematopoietic hierarchy (simplified). The diagram illustrates the differentiation of HSCs into mature cell types of the adult haematopoietic system. Included are the cytokine combinations to which progenitors are responsive. Diagram adapted from figures in reviews by Orkin and Zon (2008) and Robb (2007).



Bone marrow HSCs are a valuable resource for the treatment of haematological disorders, as they can provide stable and long term repopulation of the host haematopoietic system (for more than 6 months in mice) when transplanted into immuno-compromised irradiated adult recipients (Morrison and Weissman, 1994). HSCs are a rare population in adult bone marrow (1 to 2 per 100,000 cells) (Micklem et al., 1987; Harrison et al., 1990) and they are largely quiescent (Jordan and Lemishka, 1990). However, it has been shown that single HSCs are able to achieve full multilineage and long-term reconstitution of a recipient (Keller et al., 1985; Lemischka et al., 1986; Osawa et al., 1996). This was shown by transplanting retrovirally marked bone marrow cell populations into irradiated adult mice. Unique proviral integration sites, which stably marked HSCs and their progeny, were used to demonstrate that a single HSC clone could contribute to both lymphoid and myeloid lineages in recipients. More recently, Krause et al (2001) have also shown that a single HSC, selected on the basis of its ability to home to the adult bone marrow, can be serially transplanted without loss of repopulating ability, thus demonstrating the high self-renewal potential of these cells.

Differentiation of HSCs occurs via a series of defined steps through which the cells become sequentially more lineage restricted, lose proliferative potential and progressively acquire characteristics of mature blood cells until they terminally differentiate. Compared to LTR-HSCs, short-term repopulating (STR)-HSCs are more limited in their potential, in that they can only transiently repopulate an irradiated adult recipient by contributing to lymphoid and myeloid cell lineages (Adolfsson et al., 2001; Christensen and Weissman, 2001). The quiescent nature of LTR-HSCs and this hierarchy of stem cells and progenitors with increasingly limited self-renewal potential is believed to protect against genetic mutations arising in the highly potent stem cells.

### ***1.2.1 Phenotype of HSCs***

The haematopoietic compartment in bone marrow is highly heterogenous and no single surface antigen exclusively expressed on HSCs has been identified, as many haematopoietic surface markers are also expressed by cells of non-haematopoietic

lineages. To date it is not possible to isolate a pure population comprising only LTR-HSCs; however, the expression of particular combinations of surface antigens can be used to enrich for HSC activity in a given cell population. LTR-HSCs are highly enriched in the population of bone marrow cells which co-express c-kit, stem cell antigen (Sca)-1 and Thy1.1<sup>low</sup>, but do not express lineage specific markers (such as myeloid markers Mac1 and Gr1, erythroid marker Ter119 and lymphoid markers B220, CD4 or CD8). These c-Kit<sup>+</sup>Thy1.1<sup>low</sup>Lin<sup>-</sup>Sca-1<sup>+</sup> cells are known as the KTLS population (Okada et al., 1991; Spangrude et al., 1988).

Kiel and colleagues (2005) reported that the differential expression pattern of signalling lymphocyte activation molecule (SLAM) family receptors on LTR-HSCs, STR-HSCs and restricted haematopoietic progenitors correlates with the potency of the cells. CD150 was detected in the KTLS HSC population derived from adult mouse bone marrow, but was not detected in the Thy-1<sup>lo</sup>Sca-1<sup>+</sup>Mac-1<sup>lo</sup>CD4<sup>lo</sup>B220<sup>-</sup> progenitor population nor in restricted haematopoietic populations. The authors reported that bone marrow LTR-HSCs were CD150<sup>+</sup>CD244<sup>-</sup>CD48<sup>-</sup>, whereas multipotent haematopoietic progenitors (STR-HSCs) were CD150<sup>-</sup>CD244<sup>+</sup>CD48<sup>-</sup> and some more restricted haematopoietic cells were CD150<sup>-</sup>CD244<sup>+</sup>CD48<sup>+</sup>. Thus, the expression of CD150 and other SLAM receptors could be used to predict the developmental potential of haematopoietic cells and to enrich for LTR-HSCs in a population. In an important development, the use of combinations of SLAM receptors simplified the identification of HSCs, as compared to KTLS markers, thus allowing HSCs to be readily detected in tissue sections and thereby facilitating the examination of HSC localisation within the bone marrow niche *in vivo* (Kiel et al., 2005)(see section 1.4.2). Further to this study, Kim et al (2006) reported that LTR-HSC activity is highly enriched in the CD150<sup>+</sup>CD48<sup>-</sup> fraction of E14.5 foetal liver cells; demonstrating that these markers can be used to isolate and detect both adult bone marrow and foetal HSCs.

HSCs can be enriched in a cell population by a number of methods. For instance, the treatment of cells with the cytotoxic drug 5-Fluorouracil enriches for quiescent HSCs as it is toxic to actively cycling progenitors (Hodgson and Bradley, 1979).

Haematopoietic populations can also be separated by fluorescence activated cell sorting (FACS) based on their exclusion of fluorescent dyes. In adult bone marrow, a small subset or “side population” of cells (0.1%) are able to efficiently efflux Hoechst 33342 fluorescent (DNA-binding) dye and therefore exhibit a distinct pattern of fluorescence when analysed by flow cytometry. This side population (SP) comprises of quiescent  $\text{lin}^{-\text{lo}}\text{Sca-1}^+$  cells and is highly enriched for multilineage LTR-HSC activity (Goodell et al., 1996). This distinct fluorescence profile could be conferred by the high expression of p-glycoprotein (CD44) or multidrug resistance protein (mdr) on HSCs, a membrane pump that effluxes the dye. In support of this, Hoechst staining of whole bone marrow in the presence of verapamil, which inhibits p-glycoprotein, eliminates the SP cells from the fluorescence profile (Goodell et al., 1996). P-glycoprotein is a member of the ATP-binding cassette (ABC) transporter superfamily and, more recently, another member of this family, *Abcg2* (or *Bcrp1*), has been implicated in the active efflux of Hoechst from SP cells (Zhou et al., 2001). HSCs can also be identified (using flow cytometric analysis) by efflux of Rhodamine-123 (mitochondrial binding) dye, as the highly quiescent LTR-HSC population is Rhodamine-123<sup>low</sup> or dull, whereas STR haematopoietic cells are Rhodamine-123<sup>medium/high</sup> or bright (Spangrude et al., 1990; Zijlmans et al., 1995).

### ***1.2.2 In vivo repopulating assays and in vitro colony assays***

*In vivo* repopulating assays and *in vitro* colony assays can be used to retrospectively demonstrate the presence of HSCs and their progenitors in a given population of cells. The only way to definitively demonstrate that LTR-HSCs are present in a test cell population is to show that the cells can achieve full and long-term reconstitution of all the haematopoietic lineages for more than 6 months after (serial) transplantation into irradiated (or immuno-compromised) adult recipients. Limiting dilution or competitive long-term repopulating assays have been used to estimate the number LTR-HSCs in a test cell population. This involves injecting limiting doses of test donor cells along with a known defined number of competing bone marrow

derived cells. The number of recipients that are not reconstituted in each test cell dose is used to calculate the number of HSCs present (by poisson statistics)(Szilvassy et al., 1989, 1990).

The first quantitative *in vivo* assay for short-term repopulating haematopoietic progenitor cells was designed by Till and McCulloch (1961). Bone marrow cells were injected into irradiated recipient mice and the macroscopic colonies (nodules) which formed on the spleens 8 to 12 days later were termed spleen colony forming units (CFU-S). At first, these were thought to represent LTR-HSCs, as the frequency of CFU-S correlated with the numbers of bone marrow cells injected; however, secondary transplantation of cells excised from CFU-S colonies demonstrated that they comprised of multipotent progenitors of erythroid and myeloid lineages, but they had limited self-renewal capacity and were therefore likely to represent short-term repopulating HSCs (Siminovitch et al., 1963; Jones et al., 1990). In support of this, CFU-S content of 5-Fluorouracil treated bone marrow was not representative of LTR-HSC activity (Hodgson and Bradley, 1979). It was determined that some day 12-13 CFU-S colonies had radioprotective properties when injected into irradiated recipients, as they could rapidly induce transient haematopoietic repopulation, but this only persisted for 3 to 4 months (Jones et al., 1990).

*In vitro* colony forming assays can be used to assess the differentiation potential of haematopoietic stem and progenitor cells prior to *in vivo* transplantation. For instance, cells can be placed onto stromal layers for long-term *in vitro* culture. Cobblestone-areas (CA) are formed by actively proliferating early haematopoietic progenitor cells which preferentially grow beneath the adherent stromal layer (Dexter et al., 1976, 1984). As the progeny of these cells differentiate, they migrate to the surface of the stromal layer and shed into the growth medium. These cells can be harvested and analysed to determine which terminally differentiated cell types are present. Analysis of cobblestone-area forming cells (CAFC) at different time points can reveal the differentiation and self-renewal potential of subsets of HSCs. For example, CAFCs appearing 10 days after bone marrow cells are seeded onto the stromal layers have been correlated with CFU-S<sub>12</sub> content, whereas 28 day CAFCs

correlate with cells displaying LTR activity (Ploemacher et al., 1989, 1991). In long-term cultures, the least potent haematopoietic cells appear to exhaust their self-renewal potential, giving rise to terminally differentiated haematopoietic cells. By contrast, HSCs with higher self-renewal potential are maintained in culture for longer before generating differentiated cell types. This type of assay (long term culture initiating cell assay, LTC-IC) can be used to detect HSCs with high self-renewal and differentiation potential. A limiting dilution strategy can be used to calculate the frequency of CAFC in a test population (by poisson statistics), thus providing an estimate of the number of HSCs present. This is also a useful assay for assessing the effects of exogenous factors or stromal layers on the *in vitro* maintenance of HSCs.

Test cell populations can also be seeded directly into semi-solid medium supplemented with specific combinations of cytokines to which haematopoietic progenitor cells respond by differentiating and forming colonies. These colonies can comprise of erythroid and/or myeloid or lymphoid cells depending on the potential of the progenitor cell from which the colony originated. Thus, colony assays can reliably detect haematopoietic progenitors with multilineage and unilineage potential *in vitro*. Each colony represents a single progenitor cell or colony forming unit/cell (CFU/CFC), thereby allowing enumeration of the progenitors present in a given population.

The colony forming unit-A (CFU-A) and the HPP-CFC (high proliferative potential-colony forming cell) assays are believed to detect amongst the earliest haematopoietic progenitors measurable by *in vitro* colony assay. Replating strategies have shown that CFU-A and HPP-CFC progenitor cells have self-renewal potential *in vitro*. The CFU-A assay uses medium conditioned on L929 cells (a mouse lung fibroblast cell line) and AF1.19T cells (a rat fibroblast cell line) as sources of colony stimulating factor (CSF)-1 and granulocyte/macrophage (GM)-CSF, respectively (Pragnell et al., 1988). CFU-A are thought to share properties with cells forming CFU-S<sub>12</sub> *in vivo* (Lorimore et al., 1990). Furthermore, intra-venous injection of bone marrow derived CFU-A colonies (picked from assays after 5 days and pooled)

resulted in CFU-S<sub>12</sub> formation in recipient mice (Pragnell et al., 1988). Despite this, it is possible that the CFU-A could detect more mature cells, such as macrophage and granulocytic progenitors, which also respond to CSF-1 and GM-CSF.

CFU-A are thought to be included in the HPP-CFC producing population, which are myeloid cells with high proliferative potential (McNiece et al., 1986). HPP-CFC(1) progenitors respond to CSF-1, interleukin (IL)-3 and IL-1. These cells are Lin<sup>-</sup> Sca-1<sup>+</sup> and Rhodamine-123<sup>dull</sup> and are proposed to be the most immature subpopulation as they have the least restricted differentiation capacity. HPP-CFC(2) respond to CSF-1 and IL-3, are Rhodamine-123<sup>bright</sup> and can produce macrophage progenitors (CFU-M). HPP-CFC(3) respond to CSF-1 only and are present in higher numbers in the bone marrow compared to HPP-CFC(1) and (2), suggesting that they represent a more mature haematopoietic cell (Freshney, Pragnell and Freshney, 1994).

Methylcellulose-based colony assays supplemented with defined concentrations of recombinant cytokines are routinely used to detect more mature haematopoietic progenitor cells. A combination of IL-3, IL-6, stem cell factor (SCF) and erythropoietin (Epo) is routinely used to detect erythroid progenitors (BFU-E or CFU-E), as well as macrophage (CFU-M) and granulocyte-macrophage (CFU-GM) progenitors. Multilineage CFU-GEMM (-Mix) progenitors that have granulocyte, erythroid, macrophage and megakaryocyte potential *in vitro* can also be detected.

### **1.3 Emergence of definitive HSCs in the developing embryo**

During mammalian embryogenesis, haematopoiesis is thought to be established in two waves. The first wave is initiated extra-embryonically; this transient “primitive” haematopoiesis is believed to meet the immediate needs of the embryo once circulation is established. The second wave of haematopoiesis, known as “definitive”, takes place in the intra-embryonic tissues and gives rise to adult HSCs.

Primitive haematopoiesis originates in the yolk sac and is marked by the formation of blood islands at E7 (to E8.5). These contain macrophages, which develop before monocytes are detectable (Naito et al., 1996). Both nucleated and enucleated



primitive erythrocytes expressing  $\gamma$ H1 embryonic globin are also present (Kingsley et al., 2004). Primitive megakaryocytes and their progenitors have also been detected in the pre-circulation yolk sac and platelets are detectable in circulation before definitive haematopoiesis is established (Tober et al., 2007). These committed yolk sac derived haematopoietic cells with uni- or bi-lineage potential enter the circulation when the circulation between the yolk sac and embryo proper is established at E8.5 (Palis and Yoder, 2001). These cells expand and differentiate until E13.

At first, yolk sac blood islands were believed to be the source of adult-type HSCs in the embryo (Moore and Metcalf, 1970). This hypothesis was contested when Dieterlen-Lievre (1975) demonstrated, by means of quail-chick (embryo proper-yolk sac) chimaeras, that yolk sac haematopoietic progenitors have limited self-renewal capacity and that definitive long-term haematopoiesis has intra-embryonic origins. In these experiments, chick extra-embryonic tissue was grafted onto quail embryo bodies before circulation was established. Quail and chick derived cells were distinguished by visualising cell nucleoli. This study created a significant shift in the field of haematopoietic research by bringing into focus the role of the embryo proper in establishing haematopoiesis.

It is now widely accepted that definitive haematopoiesis in the mouse is initiated in the embryo proper in the para-aortic splanchnopleura (P-Sp, E7.5 to E9), which goes on to become the aorta, gonad and mesonephros (AGM) region (from E9). These sites were found to harbour potent haematopoietic activity (Godin et al., 1993; Medvinsky et al., 1993) and HSCs capable of long-term multilineage repopulation of irradiated adult recipients (E10.5-11) (Muller et al., 1994). It was found that the pre-circulation P-Sp region is the first site in which multipotent lymphoid and myeloid haematopoietic progenitors are detectable (Cumano et al., 1996). The establishment of circulation between the embryo proper and yolk sac at E8.5 facilitates the migration of haematopoietic cells between these tissues. This complicates experiments aimed at identifying the origins of LTR-HSCs. In order to overcome this, Medvinsky and Dzierzak (1996) carried out *in vitro* organ culture of E8 to E11

yolk sac, P-Sp and foetal liver explants to allow haematopoietic cell induction and expansion to take place while preventing migration between sites. It was found that E8-9 P-Sp explants do not contain cells with the capacity to repopulate adult recipients, but long term multilineage repopulating HSCs were present in E10 AGM explants after 2 days of *in vitro* organ culture. Only the P-Sp/AGM was able to initiate and proliferate LTR-HSCs during the organ culture period; E8-9 yolk sac explants did not develop HSC activity. Therefore, the E10 AGM region is believed to be the earliest site in the embryo proper capable of autonomously generating definitive LTR-HSCs with the ability to achieve long-term reconstitution of irradiated adult recipients (Medvinsky and Dzierzak, 1996). The HSCs which emerge in the AGM undergo expansion and are proposed to be the founders of adult definitive haematopoiesis (Cumano et al., 2000).

Cumano and colleagues (2001) demonstrated that E7.5 P-Sp, isolated prior to circulation and cultured *in vitro*, were able to achieve multilineage repopulation for 6-8 months in adult  $Rag2\gamma c^{-/-}$  mice, which are devoid of natural killer cells and depleted of B and T lymphoid cells. By contrast, cultured E7.5 yolk sac cells only contributed transiently to erythroid and myeloid lineages (Cumano et al., 2001).  $Rag2\gamma c^{-/-}$  (recombination activating gene 2 common  $\gamma$  chain) mice are believed to be suitable recipients of embryo-derived cells, as the lack of MHC class I expression on embryonic cells prior to E10.5 could lead to these cells being targeted by natural killer cells. This report supports the P-Sp/AGM origin of LTR-HSCs, as tissues were removed prior to the establishment of circulation between embryo proper and yolk sac. Before E11-11.5, the yolk sac does not contain HSCs able to engraft adult recipients (Cumano et al., 1996; Palis and Yoder, 2001; Palis et al., 1999, 2001), whereas the AGM first shows this ability by E10-10.5 (de Bruijn et al., 2000a, 2000b; Muller et al., 1994), suggesting that AGM HSCs enter the yolk sac through the circulatory system. Interestingly,  $CD34^{+}c-kit^{+}$  yolk sac cells isolated from E9-10 embryos are capable of long-term reconstitution in (busulfan conditioned) newborn, but not adult, mice (Yoder and Hiatt, 1997; Yoder et al., 1997a, 1997b). This alludes to the presence of an immature population of pre-LTR-HSCs in the E9-10 yolk sac, though these cells could have migrated there from intra-embryonic haematopoietic



sites. These studies also bring into question whether it is reasonable to expect an embryonic HSC to achieve reconstitution in adult recipients, as these cells may not yet have the ability to home to, respond and/or function in the adult haematopoietic microenvironment.

A summary of the emergence of definitive HSCs during embryogenesis is depicted in Figure 1.2. After circulation is established, adult repopulating LTR-HSCs can be detected in the yolk sac as well as the embryo proper (Kumaravelu et al., 2002). Pools of definitive HSCs have also been identified in the umbilical and vitelline arteries (de Bruijn et al., 2000a) and in the placenta from E10.5-11 (Gekas et al., 2005; Ottersbach and Dzierzak, 2005); however, it is unclear whether the HSCs are generated *de novo* in these tissues or whether they migrate there from other haematopoietic sites to undergo expansion. In the AGM, HSC and progenitor numbers peak at E11-12 (Muller et al., 1994; Godin et al., 1999; Kumaravelu et al., 2002). At E11.5, an increase in HSCs in the blood circulation coincides with their colonisation of the foetal liver (Kumaravelu et al., 2002, Christensen et al., 2004, Ema and Nakauchi, 2000; Morrison et al., 1995). By E12.5, definitive HSCs from different haematopoietic tissues in the embryo (AGM, yolk sac and placenta) migrate to the foetal liver, which serves as the major haematopoietic site for the remainder of foetal development (Kumaravelu et al., 2002). Here, HSCs undergo significant expansion in numbers prior to colonising the bone marrow at E16, where they will reside in a highly regulated stem cell niche throughout the adult lifespan.

The E10.5 AGM region is widely viewed as the principal site of HSC induction in the embryo, while other embryonic sites are thought to mediate proliferation of these cells. A recent publication by Samokhvalov and colleagues (2007) rekindled the debate over the exact site of HSC emergence during mammalian embryogenesis. In attempt to resolve the developmental relationship between AGM and yolk sac, the authors carried out *in vivo* lineage tracing of yolk sac cells by using *Runx1<sup>cre/+</sup>* transgenic mice in which a Rosa26-Flox-LacZ reporter gene was conditionally activated by administration of tamoxifen to pregnant female mice, resulting in LacZ marking cells that expressed *Runx1*.

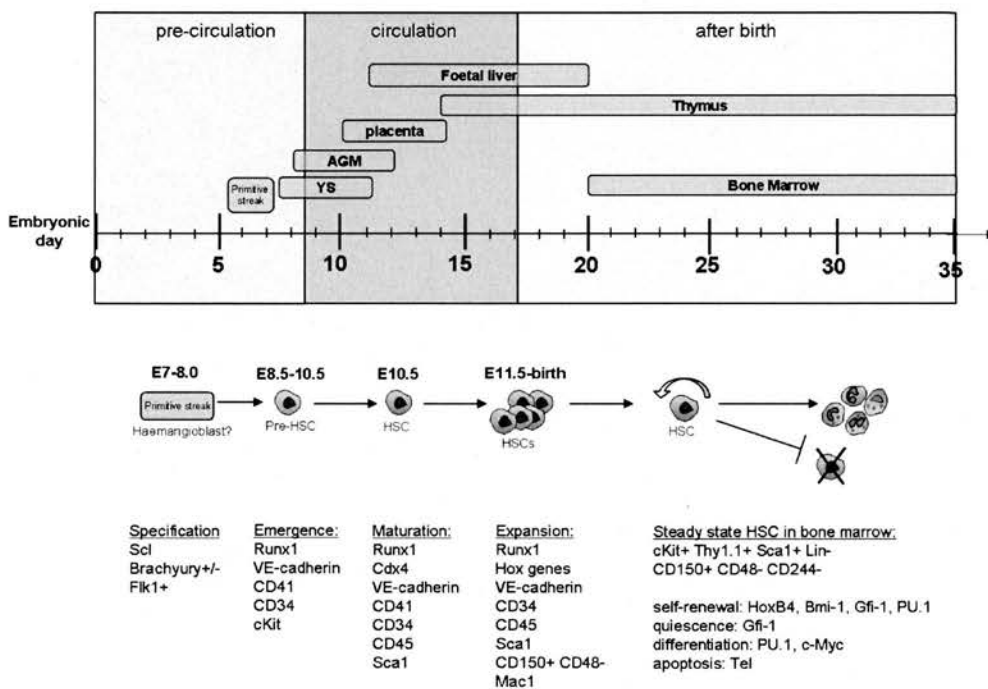


Figure 1.2 Emergence of HSCs in the mouse embryo. Shown are the haematopoietic sites in the embryo and some surface markers and genes associated with HSCs at different stages of development. This diagram was adapted from figures in the following review articles: Mikkola and Orkin (2006), Teitell and Mikkola (2006), Orkin and Zon (2008).

Runx1 is required for definitive haematopoiesis. When the embryos were treated at E7.5, a time when yolk sac appears to be the only haematopoietic site, the authors reported that Runx1 (LacZ<sup>+</sup>) yolk sac derived cells contributed to the definitive HSC pool in the E12.5 foetal liver and to adult bone marrow LSK cells, leading them to conclude that yolk sac can contribute to definitive haematopoiesis. However, Runx1 haplo-insufficiency is known to affect the temporal and spatial emergence of HSCs, by causing an acceleration in the timing of HSC induction (Cai et al., 2000; North et al., 1999; North et al., 2002). Thus, these experiments did not reflect the normal *in vivo* development of HSCs. This study has been the subject of heated debate; but the two-wave model of haematopoiesis described here remains the prevalent view.

### ***1.3.1 Pre-HSC population***

When AGM regions were sub-dissected and disaggregated cells transplanted, it was found that HSCs arise *de novo* in the dorsal aorta and surrounding mesenchyme (AM) subregion of the AGM (de Bruijn et al., 2000a, 2000b; de Bruijn et al., 2002). Repopulating LTR-HSC activity at E10-10.5 correlates with the formation of rounded cell clusters on the ventral floor of the dorsal aorta, from which HSCs are believed to bud (Medvinsky and Dzierzak, 1996; North et al., 2002; North et al., 1999; de Bruijn et al., 2002). HSCs are in close association with aortic endothelium and are localised to the ventral portion of the E10.5 dorsal aorta (Taoudi et al., 2007). Similar rounded cell clusters have also been identified in the umbilical and vitelline arteries of the embryo (Garcia-Porrero et al., 1995). It is thought that HSCs which emerge from the AM subregion of the AGM subsequently move to the urogenital ridges at E11 where they undergo proliferation (de Bruijn et al., 2000a, 2000b, 2002). The urogenital ridge, the UG subregion of the AGM, appears to be the only site in the embryo where HSC proliferation occurs with limited differentiation.

Characterisation of the intra-aortic clusters in the AM subregion has revealed that the cells express both haematopoietic and endothelial markers (CD45<sup>+</sup>, CD34<sup>+</sup>, CD31<sup>+</sup>)(Taoudi et al., 2007). The cells also express endothelial-specific vascular endothelial (VE)-cadherin (Yao et al., 2007; Taoudi et al., 2005). Furthermore, the definitive haematopoietic transcription factor Runx1 is expressed in these clusters at

E10 (North et al., 1999, 2002) and genetic markers that are common to endothelial and haematopoietic cells have also been detected (Yao et al., 2007). For these reasons, the rounded cell clusters are thought to represent haemogenic endothelium. Though, it is not clear whether HSCs have endothelial origins or whether they emerge from a precursor in the mesenchyme (in sub-aortic patches) and migrate through the aorta wall before being released into the vessel (Bertrand et al., 2005; North et al., 2002; Taoudi et al., 2008).

It has been proposed that the direct precursor of the HSC is the “haemangioblast”, a common bi-potent endothelial and haematopoietic precursor. In the yolk sac, the close association between the vasculature and haematopoietic progenitors fuelled this theory. While the existence of the haemangioblast *in vivo* has not been proven conclusively, there is some evidence supporting this hypothesis. Yao et al (2007) have shown that a bi-potent cell, which can give rise to cells of both endothelial and haematopoietic lineages, is present in the P-Sp at E8.5 and further develops in the AGM at E10.5-12.5. However, the bi-potency of the cells was demonstrated by *in vitro* colony assay after removing the cells from the *in vivo* microenvironment. Thus, it remains unclear whether cells with haemangioblast potential indeed give rise to both these lineages *in vivo*. Although, the absence of both endothelial and haematopoietic lineages in Flk-1<sup>-/-</sup> mice, which die at E8-9 with no blood island formation, seems to point towards the existence of the haemangioblast *in vivo* (Shalaby et al., 1997; Shalaby et al., 1995).

Huber and colleagues (2004) demonstrated that a population of cells with haemangioblast potential exists in the posterior primitive streak at E7 to 7.5. These cells migrate to the yolk sac and could therefore represent the progenitors of yolk sac (primitive) haematopoiesis. Again, the bi-potency of these cells was demonstrated by means of colony assay after removal from the *in vivo* microenvironment, therefore these cells do not necessarily give rise to both endothelial and haematopoietic progenitors in the embryo. Ueno and Weissman (2006) have shown that yolk sac blood islands might not have clonal origins, which appears to contradict the haemangioblast theory. In their study, three distinguishable marked ES cell lines

were co-injected into blastocysts and subsequent analysis of individual yolk sac blood islands in E7.5 chimaeras revealed the contribution of more than one ES cell, suggesting that blood and vascular cells did not arise from a single (haemangioblast) progenitor. It has been proposed that the polyclonal nature of the blood islands is a reflection of the transience of haemangioblast cells (Pearson et al., 2008), as they are first detected at E7 in the primitive streak (Huber et al., 2004) and could have already differentiated to endothelial and haematopoietic progenitor cell types on reaching the yolk sac, thereby generating non-clonal blood islands.

### ***1.3.2 Surface phenotype of embryo-derived HSCs***

A number of transplantation studies have shown that within the E11.5 AGM region, LTR-HSCs fall within the fraction of cells that express the following markers: cKit, CD34, Sca-1, VE-cadherin, CD45 and CD49d (alpha4 integrin) (Sanchez et al., 1996; Taoudi et al., 2005; Gribi et al., 2006; Taoudi et al., 2008). E11 AGM-derived HSCs are reported to be cKit<sup>+</sup>CD34<sup>+</sup> (Sanchez et al., 1996) and Gribi et al (2006) showed that 30% of cKit<sup>+</sup> cells within the E11.5 AGM co-express CD49d. Gribi and colleagues (2006) identified a rare CD34<sup>+</sup>CD49d<sup>+</sup> co-expressing population within the E11.5 AGM region and also found that CD49d was expressed on most of the cells within the VE-cadherin<sup>+</sup>CD45<sup>+</sup> fraction of the E11.5 AGM, which is a population highly enriched for LTR-HSC (Taoudi et al., 2005). Transplantation experiments demonstrated that the repopulating activity of E11.5 AGM cells was only found in the CD49d<sup>+</sup> cell fraction. Gribi et al (2006) proposed that CD49d expression on LTR-HSCs mediates their interaction with the foetal/neonatal microenvironment. Other reports suggest a role for CD49d in haematopoietic homing and differentiation, as it may mediate interaction of these cells with haematopoietic stroma (Arroyo et al., 1996; Arroyo et al., 1999).

Taoudi and colleagues (2005) also reported that in the E12.5 yolk sac, HSCs capable of adult repopulation were also present in the VE-cadherin<sup>+</sup>CD45<sup>+</sup> co-expressing fraction, similar to those in the E11.5 AGM. VE-cadherin expression was lost as HSCs matured, since VE-cadherin positive and negative LTR-HSCs were present in

E12.5 AGM, placenta and E13.5 foetal liver. Finally, LTR-HSC in adult BM were VE-cadherin<sup>-</sup>.

It has been reported that CD41 could be expressed on embryonic HSCs, as CD41<sup>+</sup> cells were found to co-express markers of definitive haematopoiesis (CD45) and markers of repopulating cells (cKit and CD34) (Mikkola et al., 2003). In support of this, CD41<sup>+</sup> cells are present in the haematopoietic clusters of the dorsal aorta and vitelline and umbilical vessels (Muller et al., 1994; Medvinsky and Dziezak, 1996; Cumano et al., 1996; Sanchez et al., 1996; Cumano et al., 2001). Mikkola et al (2003) sorted cKit<sup>+</sup>CD41<sup>+</sup>CD45<sup>+</sup> and cKit<sup>+</sup>CD41<sup>+</sup>CD45<sup>-</sup> cells from the E8.5, E9.5 and E10.5 yolk sac and found that these cells could generate CFU-Mix haematopoietic colonies in assays. CD45 is a pan-haematopoietic cell marker, yet CD45 expression did not enrich for progenitor activity, suggesting that CD41 is an earlier haematopoietic marker than CD45. The authors also found that CD41 was expressed by CD34<sup>+</sup>cKit<sup>+</sup> cells in the E9 yolk sac, a population which has been reported to be able to repopulate newborn but not adult recipients (Yoder and Hiatt, 1997; Yoder et al., 1997a, 1997b). One caveat of this study was that the data were based on *in vitro* colony assays and the repopulating potential of CD41<sup>+</sup> cells in the embryo is yet to be assessed by *in vivo* assay.

## **1.4 Regulation of embryonic and adult haematopoiesis**

The induction and subsequent regulation of haematopoiesis in the embryo and adult is achieved by an intricate network of factors that activate certain signalling pathways and act in concert with those that suppress other pathways. Signalling from the *in vivo* microenvironment has a dynamic influence on these processes. The following is a brief overview of the key regulators involved in haematopoietic induction in the embryo, regulation of bone marrow HSCs in the adult and the role of the adult bone marrow niche in maintaining haematopoietic homeostasis.

### ***1.4.1 Regulation of HSC induction in the embryo***

A number of transcription factors have been implicated in the induction and regulation of embryonic haematopoiesis and many of these genes were first



recognized in chromosomal translocations associated with haematopoietic malignancies. These factors can be classified according to their roles in haematopoiesis; for instance, some are important in establishing or regulating both primitive and definitive haematopoiesis (such as *Scl* and *Lmo2*), whereas others are required only for definitive haematopoiesis (as is the case for *Runx1*).

### *Scl/TALI*

The transcription factor stem cell leukemia (*Scl*) was originally identified as a target of chromosomal rearrangements which leads to childhood T cell acute leukaemia. *Scl* is expressed in HSCs, haemangioblasts and some mature haematopoietic cells and endothelium. *Scl* is also expressed in the E11.5 dorsal aorta where HSCs are specified (Pimanda et al., 2007). Work carried out by Robb et al (1995) and Shivdasani et al (1995) has shown that *Scl*<sup>-/-</sup> knock out mice die at E8.5-10.5 due to a complete lack of yolk sac primitive haematopoiesis which results in severe anaemia. It has also been reported that *Scl*<sup>-/-</sup> ES cells do not contribute to primitive or definitive haematopoiesis in chimaeras (Robb et al., 1996; Porcher et al., 1996). In further studies, Mikkola and colleagues (2003) found that adult bone marrow HSCs in which *Scl* was conditionally inactivated, did have multilineage repopulating potential in serial recipients. Therefore, *Scl* is dispensable for long-term HSC self-renewal and differentiation in adults, but is required for their induction during embryogenesis. Though, terminal erythroid and megakaryocyte differentiation and short-term repopulation activity of *Scl*<sup>-/-</sup> HSCs is affected (Curtis et al., 2004; Hall et al., 2005). These data led to the conclusion that *Scl* is required for the establishment of primitive and definitive haematopoiesis in the embryo, but that its continued expression is not necessary for the maintenance of LTR-HSCs in adult bone marrow.

### *Lmo2*

Lim finger protein (*Lmo2*) knockout mice die at E10.5 due to anaemia, which is caused by a lack of primitive yolk sac erythropoiesis (yolk sac macrophages were unaffected) (Warren et al., 1994). Knockout mice died before establishment of definitive haematopoiesis, but Yamada and colleagues (1998) generated *Lmo2*<sup>-/-</sup> ES cells and reported that these do not contribute to haematopoiesis in chimaeras,

including definitive myeloid or lymphoid lineages, demonstrating a role in definitive haematopoiesis. Therefore, Lmo2 is considered to be important for primitive erythropoiesis, as well as establishment of definitive haematopoiesis. However, the adult HSC compartment has not yet been assessed in a conditional *Lmo2*<sup>-/-</sup> knockout model, therefore it is unknown whether continued expression of *Lmo2* is required for maintenance of adult HSCs after they have been induced. Though, Lmo2 is expressed in mature definitive haematopoietic cell types and it is believed to interact with Scl in a multiprotein complex such that definitive haematopoiesis can occur (Lecuyer et al., 2007). Lmo2-Scl interactions are thought to be important for haematopoietic fate specification, which explains the phenotypic similarity between *Scl*<sup>-/-</sup> and *Lmo2*<sup>-/-</sup> embryos (Schlaeger et al., 2004; Patterson et al., 2007).

#### *Runx1/AML1*

*Runx-1* (*AML-1* or *Cbfa2*) gene encodes the alpha DNA-binding subunit of core binding factor. This was the first transcription factor found to be exclusively required for definitive haematopoiesis in the embryo, while being dispensable for primitive haematopoiesis. *Runx1*<sup>-/-</sup> knockout mice die between E12.5 and E13.5 due to internal bleeding (Okuda et al., 1996; Wang et al., 1996a). There was complete absence of blood cell emergence from the dorsal aorta, umbilical and vitelline arteries (North et al., 1999); while, primitive erythropoiesis was unaffected. It has been reported that the expression of Runx-1 is restricted to intra-aortic clusters and, prior to their formation, expression is restricted to some cells in the ventral floor of the dorsal aorta (North et al., 1999). Intra-aortic clusters are absent in *Runx1* deficient embryos; therefore, it has been postulated that this factor plays a role in the specification of definitive HSCs from a pre-HSC population in the haemogenic endothelium (North et al., 1999; North et al., 2002). *Runx1* has been found to act in a dose-dependent manner. In *Runx1*<sup>lacZ/+</sup> mice, LTR-HSC emergence in the AGM is accelerated by 1 day (Cai et al., 2000; North et al., 2002). Furthermore, Mukoyama and colleagues (2000) reported that *Runx1*<sup>+/-</sup> mice have a reduction in colony formation from E9.5 P-Sp and E11.5 AGM. Nottingham et al (2007) have shown that the transcription factors Gata-2 and Ets are direct upstream regulators of *Runx1*. Landry and colleagues (2008) have recently shown that in the yolk sac and foetal



liver, a Scl/Lmo-2/Gata-2 protein complex directly binds to and regulates *Runx1* (and *Runx3*). Ichikawa and others (2004) demonstrated that, in conditional knockout mice, continued expression of *Runx1* is not required to maintain adult bone marrow HSCs; but it is required for megakaryocyte development and for B- and T-cell differentiation. In the bone marrow, *Runx1* is expressed at relatively constant levels in all haematopoietic lineages, except the erythroid lineage (North et al., 2004; de Bruijn and Speck, 2004).

#### ***1.4.2 Regulation of HSCs in the adult bone marrow niche***

In the adult, definitive HSCs reside primarily in the bone marrow, where they are supported by a scaffold of stromal (mesenchymal) cells, which produce a plethora of stimulatory and inhibitory factors that enable this organised stem cell compartment to regulate the cell cycle status, self-renewal, apoptosis and differentiation of LTR-HSCs and their progeny. Adult HSCs are predominantly quiescent, with a turn over of 30 days; this may be important in safeguarding against excess differentiation and exhaustion of the HSC pool and also to limit the possibility of acquiring genetic mutations. A combination of intrinsic and extrinsic factors has been implicated in the maintenance of adult haematopoietic homeostasis. Here, the focus is on extrinsic regulation of HSCs by the bone marrow niche; in which growth factors, chemokines, accessory cells and extracellular matrix proteins play a role.

HSCs interact closely and reciprocally with stromal elements of the bone marrow niche, these include osteoblast, osteocyte, adipocyte and endothelial cells. The most potent HSCs are located in the endosteal marrow (Gong 1978; Lord et al., 1990; Nilsson et al., 2001). Osteoblasts, which line the endosteal surfaces, have been found to secrete cytokines that could modulate HSCs and their progeny and are thought to regulate HSC number. Calvi et al (2003) observed that when parathyroid hormone (PTH) was constitutively expressed or administered, an increase in osteoblasts was accompanied by an increase in HSCs. The authors proposed that the increase in HSC number occurred via Notch activation, since the Notch ligand Jagged-1 was up-regulated by osteoblasts in their system. Activated Notch (NotchIC) was shown to increase the HSC pool by promoting self-renewal (Stier et

al., 2002; Varnum-Finney et al., 2000). Zhang et al (2003) reported that conditional inactivation of the BMP receptor type 1A in bone marrow resulted in a significant increase in the number of functional (repopulating) HSCs, which was associated with an increase in the number of specialised spindle-shaped N-cadherin expressing osteoblasts (SNO cells) on the endosteal surfaces. There was no increase in numbers of differentiated haematopoietic progenitors. N-cadherin was proposed to be critical for HSC anchorage to osteoblasts and it was suggested that normal BMP signalling in osteoblasts promotes HSC quiescence. Further to this, Nilsson et al (2005) demonstrated that osteopontin, which is synthesised by osteoblasts, negatively regulates proliferation of HSCs both *in vivo* and *in vitro*. Recent findings have suggested that thrombopoietin-producing osteoblasts play a role in maintaining HSCs in their quiescent state (Yoshihara et al., 2007). A number of other factors have also been implicated in the regulation of HSCs in the endosteal niche. These include membrane-bound stem cell factor (SCF)/cKit interactions, implicated in HSC lodgement in bone marrow (Driessen et al., 2003); angiopoietin-1/Tie2 interactions, whereby quiescent Tie2<sup>+</sup> HSCs attach to angiopoietin-1 expressed on the surface of osteoblast cells (Arai et al., 2004) and calcium sensing receptors, which have been implicated in HSC localisation and retention in bone marrow (Adams et al., 2006). Conditional knockout of c-Myc has revealed it may control the balance between HSC differentiation and self-renewal in the endosteal niche (Wilson et al., 2004).

*Ex vivo*, expansion of repopulating HSCs can be facilitated by transduction of primary bone marrow cells with HoxB4, which results in an increase in LTC-IC and LTR-HSC activity (Antonchuk et al., 2002; Schiedlmeier et al., 2007; Schmittwolf et al., 2005; Thorsteinsdottir et al., 1999). In a recent study in Nature, North and colleagues (2007) demonstrated that *ex vivo* exposure of cKit<sup>+</sup>Sca-1<sup>+</sup>Lin<sup>-</sup> mouse bone marrow cells to a stabilised form of prostaglandin E2 (PGE2) significantly increased the frequency of LTR-HSCs, as assessed by competitive repopulation assays.

In addition to the endosteal niche, HSCs have been found to reside in the bone marrow vasculature, as visualised by SLAM expression in wild-type bone marrow tissue sections (Kiel et al., 2005). Recent publications have proposed that the

vasculature niche does not simply act as a means for nutrient supply and a conduit for HSC/HPC migration, but that it represents a second niche which mediates homing and engraftment via endothelial specific factors and interactions. Endothelial cells are located at the juncture between blood vessel lumen and bone marrow. In accordance with this proposed role, circulating HSCs home to and engraft in the vasculature endothelium of the bone marrow. This niche is characterised by distinct areas where endothelial cells express high levels of chemokines and specific cell adhesion molecules, including stromal derived factor (SDF)-1, integrins, endothelial cell (E)-selectin and vascular cell adhesion molecule (VCAM)-1 (Sipkins et al., 2005; Kiel et al., 2005). These factors are believed to be involved in HSC homing and engraftment. SDF-1 is a well documented chemokine, also known as CXC-chemokine ligand 12 (CXCL12). SDF-1 is highly expressed by reticular cells around bone marrow sinusoids (vasculature) to which HSCs localise (Sugiyama et al., 2006; Kiel and Morrison, 2006). The receptor for CXCL12/SDF-1 is CXCR4, which is widely expressed on haematopoietic cells and also by HSCs. Knockout strategies have demonstrated that SDF-1/CXCR4 interactions mediate HSC homing to the bone marrow and are important for HSC retention and maintenance (Ma et al., 1998). In addition, leukaemic tumour cells have also been shown to home to and engraft in the vascular endothelial bone marrow niche in an SDF-1 dependent manner (Sipkins et al., 2005) and it has been proposed that tumour cells could utilise the same signalling cues as normal HSCs.

### **1.5 Stromal cell lines as microenvironments for the support of HSCs *in vitro***

Stromal cell lines have been generated from various haematopoietic niches, including adult bone marrow, foetal liver and AGM tissues, in order to test their ability to support or expand HSCs *in vitro*, with a view to identifying novel regulatory factors (Moore et al., 1997; Yoder et al., 1994; Ohneda et al., 1998; Xu et al., 1998; Oostendorp et al., 2002b).

To investigate the early HSC niche, Ohneda and colleagues (1998) derived endothelial CD34<sup>+</sup> cell lines from the E11 AGM region. It was demonstrated that the endothelial cell line, DAS104-4, was capable of inducing a 3 to 5 fold expansion of lin<sup>-</sup>CD34<sup>+</sup>Sca-1<sup>+</sup>cKit<sup>+</sup> murine foetal liver derived HSCs after 7 days of co-culture, without loss of repopulating potential. This supported a role for AGM endothelium in the regulation of embryonic HSCs. Indeed, a number of studies have generated and identified AGM derived cell lines that are highly supportive to embryonic and adult HSC/HPCs from both murine and human tissues. Several of these stromal cell lines display a surface phenotype consistent with cells on a vascular smooth muscle differentiation pathway (Charbord et al., 2002) and many are able to differentiate into mesenchymal cell types such as osteoblasts, adipocytes and chondrocytes (Durand et al., 2006).

Weisel and others (2006) isolated 106 stromal cell lines from mouse E10.5 AGM tissue. Most of these stromal lines could support adult murine BM haematopoietic progenitor cells (CFU and CAFC) for up to 3 weeks in co-culture. Some of these lines could also maintain human cord blood CD34<sup>+</sup> cells in culture without the addition of exogenous cytokines (aside from those present in foetal calf serum) and 1 cell line was capable of promoting haematopoietic differentiation of mouse embryonic stem cells. In an earlier study, Xu and colleagues (1998) derived 17 stromal cell lines (non-clonal) from the E10.5 AGM region and 3 of these lines supported haematopoiesis. In particular, the AGM-S3 stromal line could promote production of CFU-S and haematopoietic progenitors from Sca-1<sup>+</sup>cKit<sup>+</sup>Lin<sup>-</sup> murine adult BM cells and human cord blood CD34<sup>+</sup>CD38<sup>-</sup> cells, respectively. A significant finding was that AGM-S3 supported repopulating activity of human cord blood LTR-HSCs even after 4 weeks of *in vitro* co-culture. Furthermore, Matsuoka et al (2001) demonstrated that culture of E8.5 yolk sac and P-Sp cells on the AGM-S3 stromal line supported the generation of CFU-S and LTR-HSC activity from these embryonic populations. AGM-S3 were found to express SCF, IL11 and Oncostatin M, but culture of P-Sp cells in these cytokines alone did not result in CFU-S or LTR-HSC activity, suggesting the involvement of additional interactions or factors.

An important finding was that when E8 yolk sac-derived neonatal repopulating pre-HSCs were co-cultured with AGM-S3 for 4 days in the absence of exogenous cytokines, the cells could subsequently achieve long-term repopulation of irradiated adult recipients (Matsuoka et al., 2001). Therefore, AGM-S3 could support or induce the generation of definitive HSCs from a pre-HSC yolk sac population *in vitro*. These observations suggest that pre-HSCs from the yolk sac require an additional, perhaps AGM-specific, maturation step in order to achieve repopulation in an adult microenvironment.

Oostendorp and colleagues (2002b) derived over 100 clonal stromal cell lines from E11 foetal livers (EL stromal clones) or from sub-dissections of E10-11 AGM regions; namely, the aorta and surrounding mesenchyme (AM) region and the urogenital ridges (UG). The authors reported that many of the stromal clones supported CFU production and repopulating activity of adult BM and human CD34<sup>+</sup> cord blood cells (Oostendorp et al., 2002a; 2002b). Both the UG and AM subregions of the E10-11 AGM produced supportive stromal clones, but fewer supportive cell lines were derived from the E11 foetal liver, suggesting that the mid-gestational AGM region is a potent haematopoietic microenvironment. Furthermore, several stromal clones were comparable or more highly supportive than control BM-derived stroma. In long term cultures, UG26.1B6 and EL08.1D2 stroma were particularly potent supporters of repopulating HSCs isolated from murine adult BM (CD31<sup>+</sup>cKit<sup>+</sup>Ly6C<sup>-</sup> cells), as well as human CD34<sup>+</sup> cord blood HSCs (Oostendorp et al., 2002a). Interestingly, UG26.1B6 or EL08.1D2 were also able to support repopulating activity of CD34<sup>+</sup>c-kit<sup>+</sup> cells sorted from E11 AGM and yolk sac tissues. However, yolk sac CD34<sup>+</sup>c-kit<sup>+</sup> pre-HSCs, previously shown to repopulate newborn but not adult recipients, failed to repopulate adult recipients after co-culture with these stromal clones. Therefore, contrary to AGM-S3 (Matsuoka et al., 2001), UG26.1B6 and EL08.1D2 stroma were unable to support or induce pre-HSCs to acquire definitive long term repopulating capabilities.

Harvey and Dzierzak (2004) co-cultured E11 AM or UG-derived cells on UG26.1B6 and found that direct contact with stromal cells was required to maintain the

repopulating activity of the embryonic HSCs in these tissues. Interestingly, UG26.1B6 and EL08.1D2 lines supported adult BM-derived HSCs in non-contact cultures, suggesting that secreted soluble factors might play a role in their effect on adult HSCs (Oostendorp et al., 2005).

In the studies described above, characterisation of the supportive stromal cell lines revealed that they express different combinations of known haematopoietic cytokines; such as monocyte-colony stimulating factor (M-CSF), interleukins, LIF, thrombopoietin, transforming growth factor  $\beta$  (TGF $\beta$ ), stem cell factor (SCF), Flt3-ligand and bone morphogenic protein 4 (BMP4). However, even when combinations of known haematopoietic factors are added to *in vitro* cultures, it is very difficult to maintain self-renewal or promote expansion of repopulating HSCs or HPCs in the absence of stromal cell layers. This suggests that additional interactions, such as those mediated by cell adhesion molecules and extracellular matrix proteins, play important roles in haematopoietic regulation *in vitro*.

Durand and others (2007) found that UG26.1B6 stromal cells expressed high levels of  $\beta$ -NGF (neurotrophic growth factor) and BMP4 (bone morphogenic protein) compared to the non-supportive UG26.3B5 clone, which instead expressed high levels of MIP-1 $\gamma$  (a member of the c-c chemokine family). Addition of exogenous  $\gamma$ -NGF, MIP-1 $\gamma$  and BMP4 to E11 AGM explant cultures all enhanced the repopulating activity of AGM-HSCs. The activity of E11 AGM-HSCs, which express BMP receptors, was inhibited when cultured with a BMP antagonist. Furthermore, localised BMP4 expression was detected in the mesenchyme underlying the haematopoietic aortic clusters at E11, which is in support of a role for BMP4 in the regulation of HSC emergence in the AGM. This study is an elegant example of how investigation of stromal support *in vitro* can be used to identify haematopoietic regulators that play a role *in vivo*.



## 1.6 Embryonic stem (ES) cells

Embryonic stem (ES) cells were first derived from the pre-implantation (E3.5) mouse blastocyst in 1981 by Professor Sir Martin Evans and colleagues (Evans and Kaufman, 1981; Martin, 1981). These pluripotent cells have since become a powerful tool in the study of mammalian developmental biology, as they can be readily genetically modified *in vitro* and are able to contribute to all three germ layers and the germ line when re-introduced into host blastocysts (Bradley et al., 1984). ES cells can be cultured indefinitely in their undifferentiated, pluripotent state *in vitro* by maintaining them on murine embryonic fibroblasts (MEF) or in the presence of optimal concentrations of exogenous leukaemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988; Nichols et al., 1990). Importantly, ES cells retain a normal karyotype in culture. *In vitro*, withdrawal of LIF causes mouse ES cells to spontaneously differentiate into a broad spectrum of cell types, including those of ectodermal, mesodermal and endodermal germ layers. Under appropriate culture conditions, they can be directed to differentiate into lineages of interest, making this a valuable system in which to study tissue-specific differentiation and possibly to generate cells for drug screening. When human ES cells were isolated by Thomson and colleagues (1998) this presented the possibility of using differentiated cells for autologous cell replacement therapies; which would involve deriving ES cells from embryos generated by somatic cell nuclear transfer. Even though there are ethical considerations and technical limitations still to address before ES-derived cells can be used in humans, they represent an important and highly accessible system for *in vitro* studies.

There is accumulating evidence that haematopoietic differentiation of ES cells *in vitro* recapitulates the *in vivo* pattern of early haematopoietic specification and that functional ES-derived HSCs can be generated (section 1.6.1, 1.6.2). Thus, ES cells can serve as an alternative source of cells in which to study the specification, maintenance and regulation of HSC/HPCs. Identification of novel factors able to regulate or expand HSCs *ex vivo* would have a significant impact in a clinical setting, as it can be difficult to obtain suitable tissue-matched donor bone marrow and it has proven difficult to maintain or expand HSCs for extended times in culture.



### ***1.6.1 Haematopoietic differentiation of murine ES cells***

Haematopoietic differentiation of ES cells is routinely initiated by the formation of ES cell aggregates. These semi-organised 3-dimensional structures, known as embryoid bodies (EBs), can support differentiation into a number of lineages, including haematopoietic cells (Keller et al., 2005). EBs of a uniform size can be generated by preparing ES cells in hanging drops on the underside of a petri dish. Alternatively, high numbers of ES cells can be cultured in suspension, which causes spontaneous aggregation of the cells, thereby forming EBs of variable sizes. Seeding ES cells directly into semi-solid medium such as methylcellulose facilitates the formation of clonal EBs (Dang et al., 2002; Wiles and Keller, 1991). Initial observations of haemoglobinised cells (blood islands) in EBs were made by Doetschman and colleagues (1985) and it is now well-documented that mature haematopoietic cells can be generated from ES cells, including those of erythroid, myeloid and lymphoid lineages (Wiles and Keller, 1991; Fujimoto et al., 2003; Nakano et al., 1994; Potocnik et al., 1994).

Cells within EBs can undergo haematopoietic differentiation in the presence of foetal calf serum alone, without supplementing with exogenous cytokines, and some have proposed that this indicates that EBs inherently express haematopoietic cytokines and the corresponding receptors that facilitate differentiation (Hole et al., 1996; Keller et al., 1993; Kennedy et al., 1997). Under serum-free conditions, mouse ES cells can be induced to mesodermal and haematopoietic lineages by addition of BMP4 and vascular endothelial growth factor (VEGF) (Nakayama et al., 2000; Park et al., 2004; Ng et al., 2004). Recently, Pearson and colleagues (2008) have reported that under serum-free conditions, step-wise addition of BMP4 (at 2.5 days), Activin A, bFGF and VEGF (from 4 days) are sufficient to promote haematopoietic differentiation in mouse suspension EBs. Detailed analyses revealed that BMP4 enhanced mesoderm formation, bFGF and Activin A promoted the generation of haemangioblast cells and VEGF mediated maturation of the cells to committed haematopoietic progenitors. The authors reported robust production of haematopoietic progenitors. The absence of serum reduces variability in differentiation; since different batches of serum can contain varying levels of known

and unknown growth factors. Unless otherwise stated, the studies described below have used serum-containing media.

Addition of haematopoietic cytokines or culture of ES cells on specific stromal cell lines or extracellular matrices can also promote differentiation to particular haematopoietic lineages. For instance, the OP9 stromal cell line is known to enhance lymphoid differentiation of ES cells when they are seeded directly onto stromal layers for up to 14 days of differentiation. The OP9 cell line was derived from the bone marrow calvaria of newborn osteopetrotic *op/op* mice, which do not express functional M-CSF (Nakano et al., 1994). This property was thought to facilitate lymphoid differentiation, as M-CSF preferentially induces ES cells to monocytic/macrophage lineages. Addition of exogenous M-CSF to OP9/ES cell co-cultures significantly increased the frequency of macrophage progenitors and reduced the differentiation of ES cells to other haematopoietic lineages (Nakano et al., 1994). Direct comparison of the EB differentiation system and the OP9/ES co-culture system by Zhang and colleagues (2005) revealed that haematopoietic cells (CD45<sup>+</sup> and Ter119<sup>+</sup> cells) were more efficiently generated in the EB system. In their hands, the OP9 system supported endothelial differentiation (VE-cadherin<sup>+</sup> cells) rather than haematopoietic differentiation.

It has been proposed that haematopoietic commitment in EBs *in vitro* recapitulates that of embryonic yolk sac development *in vivo* (E6.5 to E7.5) (Keller et al., 1993; Keller, 2005). According to gene expression patterns and colony forming assays, suspension EBs generate primitive erythroid cells after 3.5 days of differentiation, marking the time after LIF withdrawal (Keller et al., 1993). This transient population peaks at day 4 of differentiation and reduces thereafter. Definitive haematopoietic progenitor cells are detected after these primitive cells have emerged; these include definitive erythrocytes (day 5), macrophage progenitors (day 6 onwards) and mast cell progenitors (day 10 to 14). Progenitors with granulocyte/macrophage potential (CFU-GM) and multipotent CFU-GEMM appear at day 6 and are present at maximum numbers at day 8 of differentiation, after which their numbers gradually decline. According to Keller et al (1993), the growth factor

responsiveness of these EB-derived CFUs is similar to that of haematopoietic precursors present in the E10 yolk sac and E12 foetal liver. This supports the notion that EB haematopoiesis parallels *in vivo* development.

Keller and colleagues have also demonstrated that bipotent haemangioblast progenitors, which can give rise to both endothelial and haematopoietic cells, are generated in EBs between 3.25 and 3.75 days of differentiation (Kennedy et al., 1997; Choi et al., 1998). Much of the evidence supporting the existence of the haemangioblast has come from the ES cell model. Haemangioblasts can be enumerated using an *in vitro* blast-colony or BL-CFC assay, which comprises methylcellulose supplemented with VEGF and SCF (Kennedy et al., 1997). After 4 days in the assay, resultant blast colonies express genes of endothelial and haematopoietic lineages and replating studies showed that clonal blast colonies indeed consist of cells with both endothelial and haematopoietic potential (primitive and definitive). Recently, BL-CFCs have also been detected in human ES cell cultures (Kennedy et al., 2007). In murine EBs, haemangioblast (BL-CFC) and haematopoietic progenitor numbers are positively regulated by bFGF, VEGF, BMP4, ActivinA (Nakayama et al., 2000; Faloon et al., 2000; Pearson et al., 2008; Park et al., 2004; Purpura et al., 2008). North et al (2007) reported that addition of prostaglandin E2 to ES cells and OP9/ES cultures increases CFU-GEMM formation. Further factors involved in haematopoietic differentiation of ES cells include: endoglin (Perlingeiro et al., 2007); Notch signalling (Cheng et al., 2008); Wnt signalling (Kim et al., 2008; Wang et al., 2007) and Ephrin/Eph (Wang et al., 2004).

In support of the haemangioblast origins of haematopoietic lineages in EBs, Nishikawa et al (1998) demonstrated that haematopoietic and endothelial cells arise from a precursor that co-expresses endothelial markers Flk1 and VE-cadherin. Furthermore, Faloon et al (2000) reported that BL-CFCs are generated by Flk1<sup>+</sup>, but not Flk1<sup>-</sup>, EB derived cells. This is perhaps not unexpected as the BL-CFC assay comprises VEGF, the ligand for Flk1 receptor. Consistent with this, Schuh et al (1999) reported that *Flk1*<sup>-/-</sup> EBs generate reduced numbers of BL-CFCs; however, their subsequent differentiation into haematopoietic and endothelial lineages was not

disrupted. This suggested that functional haemangioblast cells are specified independently of Flk1, but that Flk1 mediated signalling is required for proliferation of these cells (Schuh et al., 1999; Hidaka et al., 1999). This is in agreement with the phenotype of *Flk1*<sup>-/-</sup> embryos, which die between E8.5 and E9.5 with a lack of blood vessels and yolk sac blood islands and virtually no haematopoietic progenitors (Shalaby et al., 1995; Shalaby et al., 1997). Schuh et al (1999) have shown that even though *Flk1*<sup>-/-</sup> is embryonic lethal at E8.5, normal numbers of haematopoietic progenitors are present at E7.5, again suggesting that Flk1 is not required for haemangioblast specification, but is necessary for expansion of these cells and their progeny. It has been reported that in chimaeras, *Flk1*<sup>-/-</sup> ES cells do not contribute to the vasculature or primitive and definitive haematopoietic lineages (Shalaby et al., 1997). This is thought to be due to a defect in the migration of *Flk1*<sup>-/-</sup> cells from the posterior primitive streak to the yolk sac and possibly intra-embryonic sites, suggesting a role for Flk1 in mediating cell migration *in vivo*.

In later studies, it was reported that the precursor to BL-CFC haemangioblast cells expresses the early mesodermal gene, *Brachyury* (Robertson et al., 2000; Fehling et al., 2003). These pre-haemangioblast cells emerge in EBs one day earlier than BL-CFCs and give rise to blast-like colonies (or trans-CFC), which can in turn generate BL-CFC colonies upon replating. Pre-haemangioblast mesoderm was contained in the *Brachyury* expressing subpopulation of cells present in differentiating EBs at 2.5 to 3 days of differentiation (Fehling et al., 2003) and by using *Brachyury*-eGFP transgenic ES cells (serum-free), Kouskoff and colleagues demonstrated that as *Brachyury*<sup>+</sup> pre-haemangioblast cells mature, they show differential Flk-1 expression (Kouskoff et al., 2005). This is in agreement with the report by Huber and colleagues (2004), which showed that cells with BL-CFC potential isolated from the primitive streak of embryos co-expressed *Brachyury* and Flk1. Kouskoff et al (2005) reported that *Bry*<sup>+</sup>*Flk-1*<sup>+</sup> cells sorted from 3.25 day EBs expressed *Runx-1* and *Scl*, and that this fraction contained most of the BL-CFC activity. By contrast, *Bry*<sup>+</sup>*Flk-1*<sup>-</sup> ES-derived cells had little BL-CFC activity, but cardiac potential was restricted to this population.

Mikkola and colleagues (2003) demonstrated that during EB differentiation *in vitro*, CD41 is first expressed at day 4.25 to 4.75 of differentiation and increases to day 7. In their study, definitive haematopoietic progenitors (CFU) were enriched in CD41<sup>+</sup> EB cell fractions, which were also found to co-express neonatal reconstituting HSC markers CD34 and c-Kit. Sorting of CD41<sup>+</sup>cKit<sup>+</sup> co-expressing cells from EBs further enriched haematopoietic CFU activity. By contrast, endothelial cells, but few haematopoietic cells, developed from CD41<sup>-</sup> fractions. The authors also isolated populations from E8.5, E9.5 and E10.5 yolk sacs and found that haematopoietic CFU were present in CD41<sup>+</sup>CD45<sup>-</sup> and CD41<sup>+</sup>CD45<sup>+</sup> fractions; suggesting that CD41 is an earlier marker than CD45 for definitive haematopoiesis in both EBs and in yolk sac. In support of this proposed role, CD41 was found to be the earliest marker absent in differentiating *Scl*<sup>-/-</sup> EBs, which do not generate primitive or definitive haematopoietic CFU. Furthermore, the CD41<sup>+</sup>cKit<sup>+</sup> population was not present in Runx-1/AML-1<sup>-/-</sup> EBs lacking definitive haematopoietic potential. These data suggest that between days 5 and 7 of differentiation in EBs, CD41<sup>+</sup>cKit<sup>+</sup> expression marks the appearance of definitive haematopoiesis.

A number of other reports using gene-targeting strategies have confirmed that many of the genes involved in embryonic and adult haematopoiesis also play roles in haematopoietic differentiation of ES cells (Robb et al., 1996; Robertson et al., 2000; D'Souza et al., 2005; Ng et al., 2005; Hidaka et al., 1999; Chan et al., 2003; Lacaud et al., 2002). For example, D'Souza et al (2005) reported that *Scl*<sup>-/-</sup> ES cells can generate BL-CFCs (or trans-CFC) by day 3 of EB differentiation, but that the replated colonies only produce vascular smooth muscle and do not generate haematopoietic or endothelial lineages. This defect was rescued by exogenous expression of *Scl* (D'Souza et al., 2005), but not by ectopic expression of the closely related *Scl* paralog, *Lyl1*, which is expressed later in development (Chan et al., 2007). Elefanty and others (1997) assessed the gene expression patterns in differentiating *Scl*<sup>-/-</sup> EBs and found they had normal mesoderm and haemangioblast commitment, but expression of downstream haematopoietic genes was disrupted. These data are consistent with a role for *Scl* in the establishment of both primitive and definitive haematopoiesis.

It has also been shown that Runx1 is expressed in ES cell derived haematopoietic progenitors (Miller et al., 2001; Okuda et al., 1996; Lacaud et al., 2002; Lacaud et al., 2004; Cai et al., 2000; Okumura et al., 2007). In a gene-targeting study, Lacaud and colleagues (2004) demonstrated that Runx1, required for definitive haematopoiesis *in vivo*, is expressed in EBs at the haemangioblast stage and is important in the development of normal numbers of BL-CFCs. Furthermore, haplo-insufficiency in *Runx1*<sup>+/-</sup> ES cells resulted in accelerated mesodermal, haemangioblast and haematopoietic specification, in that *Brachyury*<sup>+</sup> cells, BL-CFCs, Flk1<sup>+</sup> and cKit<sup>+</sup> cells emerged approximately 12-24 hours earlier in heterozygous EBs compared to wild-type EBs. This is reminiscent of the Runx1 haplo-insufficiency phenotype observed *in vivo* (Cai et al., 2000; North et al., 1999, 2002). These studies validate the ES cell differentiation system as a model of developmental haematopoiesis and highlight the value of such an accessible system.

### ***1.6.2 Production of transplantable ES-HSCs***

Mature haematopoietic cell types are generated relatively easily from ES cells; however, multipotent haematopoietic stem and progenitor cells with *in vivo* repopulating potential are generated at low frequencies. As of yet, no specific EB-derived cell population has been shown to be LTR-HSCs and transplantation of ES-derived progeny into adult recipients has met with limited success. To illustrate, Muller and Dzierzak (1993) observed only 0.1-6% reconstitution in newborn severe combined immuno-deficient (SCID) mice for up to 6.5 months after injection of EB cells that had been differentiated in suspension cultures for 5 to 22 days. There was a peak in repopulating activity between days 11 and 13 of ES cell differentiation and no tumour formation was observed in any recipients of differentiated cells. However, the lineage differentiation potential of the cells was limited. Even though ES derived cells had myeloid CFU forming potential *in vitro*, only B and T lymphoid reconstitution was observed *in vivo* and donor macrophages and mast cells were not detected. This lymphoid restricted repopulation by ES derived haematopoietic cells was also observed by Gutierrez-Ramos and Palacios (1992). Later, Palacios et al (1995) reported that multilineage repopulating ES-HSCs could be generated by co-culture of ES cells on bone marrow-derived stromal cell layers (RP.0.10) in the



presence of exogenous recombinant IL3 and IL6 and conditioned medium from a foetal liver stromal line reported to contain Flt3 ligand, steel factor and an unknown cytokine able to support HSCs. ES cells were replated every 5-7 days and after 21 days, CD44<sup>+</sup>Lin<sup>-</sup> ES-derived cells were sorted from co-cultures and injected into sublethally irradiated SCID mice. Donor contribution to erythroid, myeloid and lymphoid haematopoiesis was detected for up to 6 months in primary and secondary recipients. However, the authors did not assess which time points were critical in HSC commitment *in vitro* and the roles of the exogenous cytokines were not elucidated. Subsequently, Hole and colleagues (1996) demonstrated that multilineage repopulating ES-HSCs were present in 4 day old EBs prepared by the hanging drop method and differentiated in suspension culture without exogenous cytokines. This study highlighted that HSCs can be generated in EBs in the absence of stromal cell layers and exogenous cytokines aside from those in foetal calf serum (FCS), though these cells may only be present for a short time *in vitro*.

It has also been demonstrated that Flk1<sup>+</sup> ES-derived cells arising in 4 day EBs generated in methylcellulose cultures are able to reconstitute the haematopoietic system of recipients (Miyagi et al., 2002). The Flk1<sup>+</sup> population was enriched for cKit<sup>+</sup> cells, indicative of haematopoietic cell types. After transplantation into irradiated SCID recipients, approximately 10% of the CD45<sup>+</sup> cells in bone marrow were donor derived and there was donor contribution to myeloid cells (granulocytes) and lymphocytes (CD3<sup>+</sup> and CD19<sup>+</sup> cells) in peripheral blood. Repopulation was not achieved with Flk1<sup>-</sup> sorted ES cell populations, suggesting that the differentiation of ES cells to HSCs includes a haemangioblast (Flk1<sup>+</sup>) stage. This supports the notion that ES cell differentiation *in vitro* could recapitulate *in vivo* HSC specification.

A number of explanations could account for the low levels of haematopoietic reconstitution achieved with ES-derived cells. ES-HSCs might share key characteristics with yolk sac progenitors which do not have LTR capacity in the adult microenvironment (Yoder and Hiatt, 1997; Cumano et al., 2001). Alternatively, ES-HSCs might not be produced in adequate numbers to facilitate reconstitution. It is possible that self-renewal of these cells is not supported in standard culture



conditions and that they are present for only a brief time period. Furthermore, ES-HSCs might have difficulty homing or responding to the adult bone marrow stem cell niche, thus leading to the low repopulation levels in recipients' haematopoietic compartments. Burt and colleagues investigated the efficiency with which ES cell-derived progenitors are able to home to the adult bone marrow by comparing intra-venous and intra-femoral injections of ES derived haematopoietic cells (Burt et al., 2004). In their experiments, ES cells were differentiated *in vitro* by cytokine-stimulation (IL-3, IL-6 and SCF) in methylcellulose culture for 7-10 days. cKit<sup>+</sup>CD45<sup>+</sup> cells that were isolated from resultant EBs comprised of Sca-1<sup>+</sup>Lin<sup>-</sup> cells, suggesting an enrichment of haematopoietic stem cells (KTLS). Interestingly, 10<sup>5</sup> sorted cKit<sup>+</sup>CD45<sup>+</sup> cells achieved particularly high levels of long-term reconstitution when transplanted directly into the intra-femoral cavity (with a range of 45.7-95.5% 20 weeks post-transplantation). This was significantly higher than that achieved by intra-femoral injection of non-sorted cells (2-12%) and intra-venous injection of 10<sup>6</sup> sorted cells (7.9-18.6%). These data indicate that ES-HSCs could be aberrant in their ability to home to the adult bone marrow niche and that intra-femoral injection can significantly improve repopulation activity. Furthermore, transplantation of sorted haematopoietic populations improved repopulating activity, by enriching for ES-HSCs. This suggests that transplantation of low frequencies of ES-HSCs could account for the limited repopulation previously observed.

To improve the self-renewal of ES-HSCs and thereby increase the numbers that are generated in culture, many studies have utilised genetically modified ES cell lines which over-express haematopoietic genes (Kyba et al., 2002; Kyba et al., 2003; Wang et al., 2005; Schuringa et al., 2004). For instance, Kyba and colleagues (2002) ectopically expressed a doxycycline inducible HoxB4 transgene in suspension EBs between days 4 and 6 of differentiation. Induced 6 day EBs were found to contain significantly higher frequencies of CFU-GEMM compared to their un-induced counterparts. HoxB4 induced 6 day old EBs were disaggregated and plated onto OP9 stromal layers in the presence of exogenous SCF, VEGF, TPO and Flt-3 ligand and doxycycline to maintain HoxB4 over-expression. When confluent, the cultures were replated onto fresh OP9 layers, so the OP9 co-culture duration was 10-14 days.

$2 \times 10^6$  differentiated ES cells were injected i.v. into lethally irradiated adult mice, resulting in 5 to 32% engraftment in primary recipient bone marrow and multilineage donor contribution in peripheral blood of secondary recipients for up to 5 months. More recently, Wang et al (2005) used the same differentiation protocol to compare the effects of ectopic expression of doxycycline inducible HoxB4 to those of Cdx4 induction. The authors reported that enforced expression of Cdx4, a modulator of Hox genes, in this differentiation system did not result in LTR-HSC activity. Though, a combination of Cdx4 and HoxB4 induction led to stable long term multilineage repopulation of primary and secondary recipients, with improved lymphoid contribution compared to ectopic expression of only HoxB4.

Many reports of transplantable ES-HSCs have used OP9 stromal co-culture in combination with over-expressing haematopoietic genes in ES cells. However, the role of OP9 stroma in these differentiation systems has not been directly assessed until recently. Ji and colleagues (2008) assessed the ability of OP9 cells to support haematopoietic precursors isolated from human EB cell populations. The authors found that OP9 co-culture supported proliferation of human haematopoietic precursors *in vitro*, but resultant cells were unable to repopulate irradiated recipients upon intra-femoral or intra-hepatic transplantation. Furthermore, in their hands, enforced expression of HoxB4 and/or Cdx4 in human ES cells in combination with OP9 co-culture did not generate cells with repopulating potential. Thus, the effects of OP9 co-culture and induction of HoxB4 and Cdx4 on human ES cells contrasts to their effects on murine ES cells, as reported by Kyba et al (2002) and Wang et al (2005). Though, the differentiation strategies differed in the exogenous cytokines used and co-culture timing was changed to accommodate the human ES cells. Nevertheless, it is possible that role of OP9 stroma in these co-culture systems is to support haematopoietic progenitors that arise, as opposed to inducing ES cells to haematopoietic fates. In support of this hypothesis, Matsuoka et al (2001) reported that culture of E8.5 PSp or yolk sac cells with OP9 did not support the generation of CFU-S or LTR-HSC activity. Taken together, these data suggest that OP9 stroma can support haematopoietic progenitors, but do not appear to support or induce LTR-HSCs derived from embryonic tissues or ES cell populations.

### ***1.6.3 Limitations of current haematopoietic differentiation strategies***

The systems to direct haematopoietic differentiation of ES cells are still subject to limitations, as multipotent HSC/HPCs are generated at relatively low frequencies and the differentiated ES cell populations are highly heterogenous. For instance, Hole et al (1996) reported that even though up to 40% of EBs had CFU-A activity over 8 days of differentiation, only 0.08% of total EB cells had CFU-A potential. In suspension EBs differentiated for 6 days in the presence of FCS but no additional growth factors, Keller (1993) reported that the EB population comprised of 0.5-1% haematopoietic CFUs. In recent studies, the total haematopoietic progenitor (CFU) output of EBs differentiated for 6 days in suspension was reported to be approximately 0.04-0.3% (un-induced EB cells of Wang et al., 2005; Kyba et al., 2003; Kyba et al., 2002). Transplantation of ES-derived haematopoietic cells, without over-expressing haematopoietic genes in culture, has led to only limited repopulation in immuno-compromised recipients (Muller and Dzierzak, 1993; Hole et al., 1996). Direct injection of cells into the femoral cavity led to the highest levels of repopulation reported thus far with non-genetically manipulated ES cells (Burt et al., 2004). As stated previously, these studies indicate that homing defects associated with ES-derived HSCs, as well as low HSC frequencies, could account for the previous difficulties in achieving repopulation. It is possible that standard culture conditions are not optimal for the maintenance of ES-HSCs in their self-renewing state. These cells may be inclined to differentiate in culture and might therefore only be present for a relatively short time period.

There is clearly a need to improve these differentiation protocols, since manipulation of haematopoietic genes in ES cells is not ideal in terms of the future clinical applications of this cell resource. Krassowska et al (2006) hypothesised that exposure of ES cells to AGM explants could enhance haematopoietic differentiation. The E10.5 AGM autonomously generates the first LTR-HSCs and these cells are able to home to different haematopoietic sites in the embryo, indicating that the AGM provides signals which lead to the generation of HSCs that have homing capabilities (Medvinsky and Dzierzak, 1996; Muller et al., 1994; Kumaravelu et al., 2002). In support of this strategy, *in vitro* culture of AGM tissue explants results in

the expansion of endogenous LTR-HSCs, suggesting that the elements responsible for maintaining and expanding these cells were retained during *in vitro* culture (Medvinsky and Dzierzak, 1996). In addition, others have identified AGM-derived stromal cell lines that are able to provide *in vitro* support to both adult and embryonic repopulating HSCs from human and mouse tissues (Xu et al., 1998; Ohneda et al., 1998; Matsuoka et al., 2001; Oostendorp et al., 2002a; Oostendorp et al., 2002b; Weisel et al., 2006). In light of these characteristics, Krassowska established a novel explant co-culture system whereby EBs were differentiated in contact with E10.5 AGM tissue *in vitro* at the air-surface interface. This strategy significantly increased ES cell -derived haematopoietic activity, with a 20-fold increase in CFU-A and a 50-fold increase HPP-CFC progenitors, as compared to EBs differentiated in the absence of AGM explants (Krassowska et al., 2006). The ES cells constitutively expressed GFP, allowing GFP<sup>+</sup> ES-derived CFU to be distinguished from GFP-AGM-derived colonies by fluorescence microscopy. This work demonstrated for the first time that primary AGM explants provide a potent source of signals that significantly enhance haematopoietic differentiation of ES cells.

## **1.7 Thesis Aims**

### ***1.7.1 Hypothesis***

The AGM microenvironment can provide the necessary signals to enhance haematopoietic differentiation of ES cells.

### ***1.7.2 Strategy***

To further the investigations of Krassowska et al (2006), the work presented in this thesis aimed to determine which subregion(s) of the AGM are responsible for the enhancing effects of primary AGM/EB co-culture. It was hypothesised that co-culture of EBs with stromal cell lines derived from different subregions of the E10/11 AGM might provide insight into which cells were responsible for the haematopoietic enhancing effects. The clonal stromal cell lines used in this project were kindly provided by Prof. Elaine Dzierzak, Prof. Alexander Medvinsky and colleagues (Oostendorp et al., 2002a; 2002b; 2005). Some of these stromal clones have already been shown to support both murine and human haematopoietic

progenitor and stem cells derived from embryonic and/or adult tissues (described in section 1.5). This work was carried out with a view to establishing a novel, reliable and highly efficient ES cell differentiation system using clonal AGM-derived stromal cell lines. The use of cell lines instead of primary AGM tissue explants lends itself to large scale investigation into the cellular interactions and molecular signalling underlying the haematopoietic enhancing effects of co-culture. In future, such a system would be extremely useful in the identification of novel haematopoietic regulatory factors.

### ***1.7.3 Experimental aims***

- To assess the haematopoietic inductive properties of AGM-derived clonal stromal cell lines on differentiating EBs:
  - Co-culture EBs on stromal cell layers and determine resultant haematopoietic activity in EBs by colony assays, flow cytometry and *in vivo* repopulating assays.
  
- To understand the mechanisms underlying the ability of stromal cell lines to promote haematopoietic differentiation of EBs:
  - Determine if the mechanism of action is dependent on cell-cell contact between EBs and stroma by culturing EBs in transwell inserts above the stroma to prevent direct contact.
  - Determine the role of extracellular matrices (ECMs) by culturing EBs directly on ECMs isolated from stromal cell layers.
  
- To assess the stage at which the stromal cells mediate their effects on differentiating ES cells:
  - Determine which ES-derived cell populations are responsive to haematopoietic signals from stromal cells by co-culturing defined populations of cells isolated from EBs by FACS.

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Chapter Two  
**Materials and Methods**

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## 2.1 Tissue culture

Cell culture was carried out using sterile technique in a specialised tissue culture facility fitted with Class II vertical laminar flow hoods or Biomat<sup>2</sup> Class II microbiological safety cabinets and in compliance with requirements for work with genetically modified cells. The media were not normally supplemented with antibiotics, unless otherwise stated. All cell lines used in this tissue culture facility were shown to be free of mycoplasma (using Cambio or Invitrogen detection kits) before use in experiments. Mycoplasma testing, preparation of tissue culture stock solutions, batch testing of serum and production of LIF, L929 and AF1 were routinely carried out by Helen Taylor, Julie Wilson and Aileen Leask.

### *2.1.1 Maintenance of embryonic stem (ES) cells*

Embryonic stem (ES) cell medium comprised of 1x Glasgow Minimal Essential Medium (Gibco) supplemented with 10% FCS (Sigma), 0.25% sodium bicarbonate (Gibco), 1% non-essential amino acids (Gibco), 4mM L-glutamine (Gibco), 2mM sodium pyruvate (Gibco) and 0.1mM 2-mercaptoethanol (Sigma). This was supplemented with 100U/ml Leukaemia Inhibitory Factor (LIF) as required. Unless otherwise stated, all experiments were carried out in serum-containing media.

LIF was acquired from medium conditioned on cos-7 cells which were transiently transfected with the murine LIF expression plasmid pCAGGSLIF-418. LIF concentration in the conditioned medium was determined by analysing the ability of serial dilutions to retain CP1 ES cells (Bradley et al., 1984) in a morphologically undifferentiated state. 1U of LIF was the lowest concentration required to maintain CP1 ES cells undifferentiated.

The ES cells were maintained in an undifferentiated state by passage every second day onto (feeder-free) gelatin (porcine)-coated 25cm<sup>2</sup> tissue culture flasks. To gelatinise flasks, 2-5ml 0.1% gelatin (Sigma) in PBS was applied to the flasks for approximately 5 minutes then aspirated prior to use. When passaging ES cells, the medium was first aspirated and the cells washed with 2-5ml PBS to remove



remaining serum. The cells were treated with 2ml trypsin solution (0.025% trypsin (Sigma), 0.1% chicken serum (Gibco) and 1.3mM EDTA (BDH) in PBS) for 2 minutes at 37°C in order to dissociate them. The flask was tapped sharply to obtain a single cell suspension, which was added to 8ml of ES medium to quench the trypsin activity and centrifuged at 100xg for 5 minutes (Mistral 1000 centrifuge, MSE). The supernatant was aspirated, the pellet resuspended in 10ml fresh ES medium and a cell count obtained (with a Neubauer haemocytometer).  $1 \times 10^6$  ES cells were seeded into a total of 10ml ES medium (plus 100U/ml LIF) in 25cm<sup>2</sup> tissue culture flasks (i.e.  $4 \times 10^4$  cells per cm<sup>2</sup>), which yielded approximately  $4\text{--}8 \times 10^6$  cells after two days. The cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, with the flask lid loosened slightly to allow gaseous exchange to take place.

ES cell lines used:

- 7a-GFP ES cells, which constitutively expresses eGFP (Gilchrist et al., 2003)
- Bry-201 ES cells, which express eGFP under the control of endogenous *Brachyury* promoter (Fehling et al., 2003)
- Wild-type E14 ES cells
- Wild-type CGR8 ES cells

### ***2.1.2 Freezing and thawing of cells***

To freeze cell stocks, cells were dissociated from flasks in trypsin, quenched in medium and centrifuged as during standard passage. The cell pellet was resuspended in freezing medium, which consisted of 10% dimethyl sulphoxide [DMSO] (Sigma) in ES medium or FCS. 1ml freezing medium was used per confluent 25cm<sup>2</sup> flask and this cell suspension was divided between 2 cryovials (each hold maximum 1ml). Cells were frozen slowly by placing them at -80°C overnight and then moving them to -140°C or liquid nitrogen for long-term storage.

To thaw cell stocks, cryovials were held in a 37°C water bath to thaw rapidly and then cell suspensions were immediately placed in 8ml of pre-warmed ES medium and centrifuged at 120xg for 3 minutes. After aspirating the medium, the pellet was resuspended in 10ml fresh ES medium (plus 100U/ml LIF) and transferred to a

gelatinised 25cm<sup>2</sup> flask. The medium was replaced approximately 8 hours later (or the following day) to remove dead cells and residual DMSO.

### 2.1.3 The stromal cell lines

The stromal cell lines were derived from haematopoietic tissues of mid-gestational mouse embryos and were kindly provided by Prof. Elaine Dzierzak, Prof Alexander Medvinsky and colleagues (Oostendorp et al., 2002a; Oostendorp et al., 2002b) (Figure 2.1). A summary of the clonal stromal cell lines used in this thesis is provided in Table 2.1. AM20.1B4 and AM20.1A4 stromal lines were derived from the dorsal aorta and surrounding mesenchyme (AM) of the AGM region from E10 *tsA58* transgenic embryos. UG26.1B6 and UG26.2D3 were derived from the urogenital ridges (UG) of the AGM region from E11 *tsA58* transgenic embryos. These lines were derived from transgenic mouse embryos carrying the SV40 T-antigen *Tag* (*tsA58*) immortalising gene, which is conditionally active at the permissive temperature of 33°C and is under the control of endogenous PGK or  $\beta$ -actin promoters to ensure constitutive and ubiquitous expression. The immortalising gene facilitated the isolation and establishment of clonal stromal cell lines from embryonic haematopoietic tissues. EL08.1D2 was derived from the foetal liver of a control BL1b E11 embryo. BL1b embryos carried a transgene whereby a *LacZ* reporter gene was under the control of *Ly-6E* (*Sca-1*). AM14.1C4 was derived from the AM subregion of AGM regions from E11 BL1b transgenic embryos.

#### 2.1.3.1 Maintenance of embryo-derived stromal cells

Stromal cell lines AM20.1B4, AM20.1A4, AM14.1C4, UG26.1B6, UG26.2D3 and EL08.1D2 were maintained as described (Oostendorp et al., 2002a; Oostendorp et al., 2002b) on gelatinised flasks in specialised stromal medium consisting of 50% MyeloCult long-term culture medium M5300 (Stem Cell Technologies), 40%  $\alpha$ -minimal essential medium (Invitrogen) and 10% FCS (Sigma) and an additional 1mM L-glutamine (Gibco) and 0.05mM 2-mercaptoethanol (Sigma) were added. At passage, this medium was supplemented with 10-20% 0.2 $\mu$ m-filtered conditioned medium obtained from the preceding passage. Sub-confluent cells were typically passaged (using trypsin solution) every 2-3 days after a 1:2 or 1:3 ratio split.

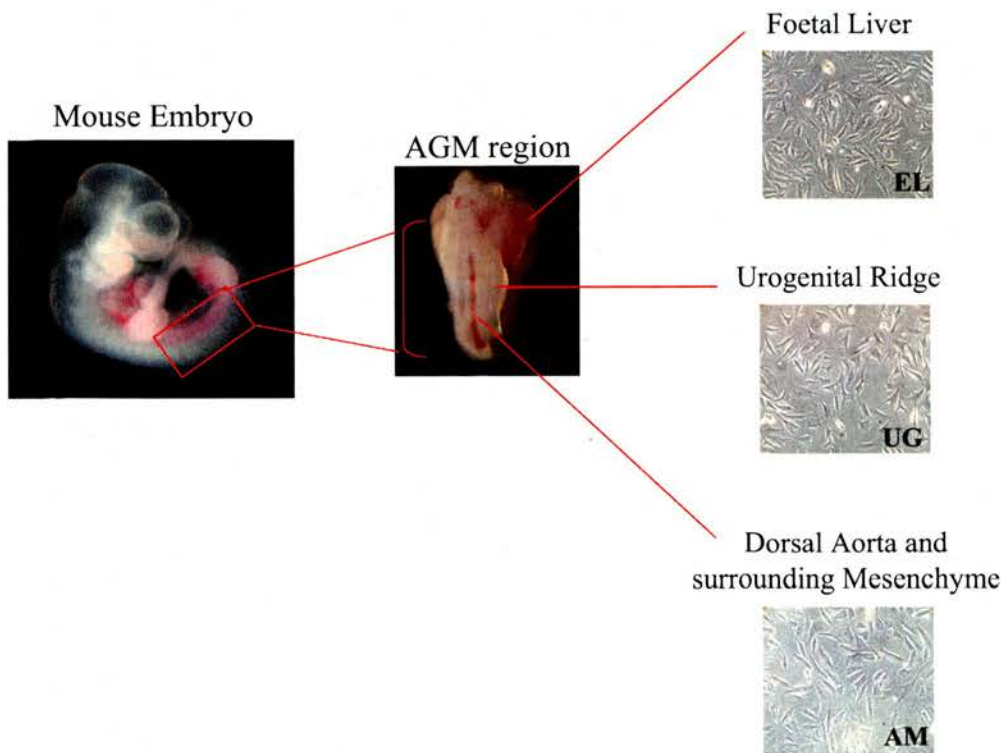


Figure 2.1 Stromal cell lines were derived from haematopoietic tissues of mid-gestational (E10 and E11) mouse embryos. These were kindly provided by Dzierzak and colleagues. Photographs of mouse embryo and AGM region kindly provided by Dr Anna Krassowska. AGM, aorta-gonad-mesenephros; AM, aorta-mesenchyme of the AGM; UG, urogenital ridge sub-region of AGM; EL, embryonic liver.

<b>Stromal cell line</b>	<b>Primary tissue</b>	<b>Embryonic day</b>	<b>Transgenic Embryo</b>
AM20.1B4	Aorta-mesenchyme of AGM	E10	<i>tsA58</i>
AM20.1A4	Aorta-mesenchyme of AGM	E10	<i>tsA58</i>
AM14.1C4	Aorta-mesenchyme of AGM	E11	BL1b
UG26.1B6	Urogenital ridges of AGM	E11	<i>tsA58</i>
UG26.2D3	Urogenital ridges of AGM	E11	<i>tsA58</i>
EL08.1D2	Foetal liver	E11	BL1b

Table 2.1 Summary of the clonal stromal cell lines. The *tsA58* Tag immortalising gene is temperature sensitive at 33°C and was expressed under the PGK or Beta-actin promoter. BL1b mice express the *LacZ* reporter gene under the control of *Ly6E* (Sca-1).

AM- and UG-derived stromal cell cultures were incubated at 33°C to activate the *tsA58* immortalising transgene. EL08.1D2 cells were maintained at 37°C. All the stromal lines were incubated in a humidified 5% CO<sub>2</sub> atmosphere.

### *2.1.3.2 Maintenance of bone marrow-derived OP9 stromal cells*

OP9 stromal cells are routinely used to promote haematopoietic differentiation of ES cells *in vitro*. The OP9 stromal cell line was derived from the BM of newborn osteopetrotic M-CSF<sup>-/-</sup> (macrophage colony stimulating factor) mice (Nakano et al., 1994). Therefore, OP9 stromal cells do not express functional M-CSF, which would preferentially promote ES cell differentiation into monocyte-macrophage lineages. In the absence of functional M-CSF, ES cells cultured on OP9 stromal cell layers reproducibly undergo lympho-haematopoietic differentiation (into erythroid, myeloid and B cell lineages). In the experiments presented in this thesis, OP9 cells were maintained at 37°C (in a humidified 5% CO<sub>2</sub> atmosphere). They were passaged, stored and maintained as described for the embryo-derived stromal lines, except that specialised OP9 medium was used for optimal growth. This consisted of 80%  $\alpha$ -minimal essential medium (Invitrogen) and 20% FCS, with an additional 2mM L-glutamine and 0.1mM 2-mercaptoethanol.

## **2.2 Differentiation of ES cells**

### *2.2.1 Embryoid body (EB) formation for ES cell Differentiation – hanging drop method*

Prior to differentiation, ES cells were prepared by the *hanging drop* method (Hole et al., 1996) to form embryoid bodies (EBs). This generated undifferentiated ES cell aggregates relatively uniform in size (Figure 2.2 a and b). ES cells were harvested by standard passage and resuspended at  $3 \times 10^4$  cells per ml in ES medium containing 100U/ml LIF (typically 20 ml). A multi-channelled pipette was used to aliquot 10 $\mu$ l droplets (300 cells per droplet) onto the bottom of an up-turned lid of a sterile bacteriological grade square petri dish.

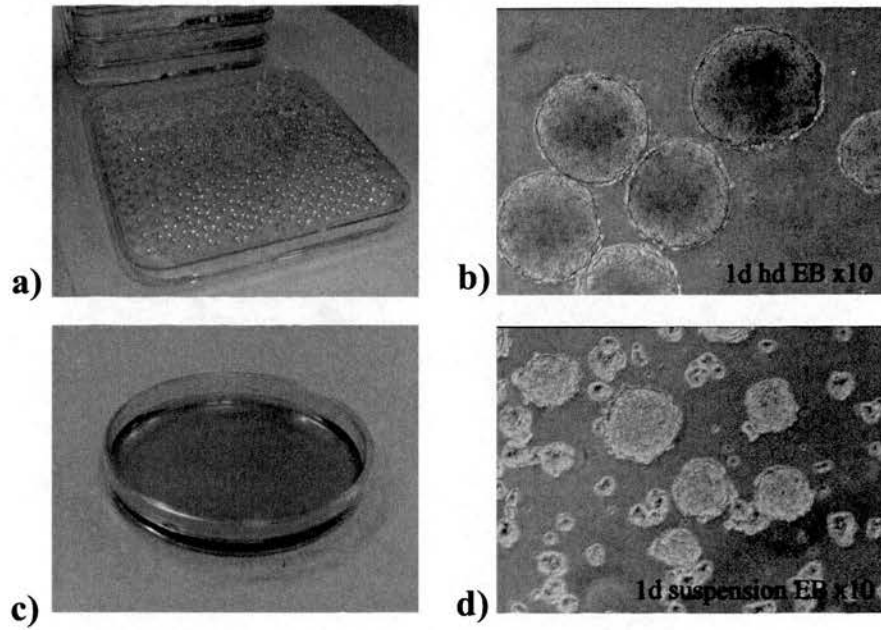


Figure 2.2 Embryoid body (EB) formation. (a) Hanging drop (hd) method generates EBs that are relatively uniform in size (b). (c) EBs formed in suspension are more variable in size as shown in (d).

Lids with droplets were carefully turned over and placed onto the dish bases, holding 8-10ml tissue culture grade water, intended to humidify the hanging droplets. The hanging drops were incubated at 37°C (humidified 5% CO<sub>2</sub> atmosphere) for 2 days. Subsequently, the undifferentiated ES cell aggregates were harvested from hanging drops by tapping the dish lid against the hood worktop and transferring the medium which collected at the edge to a fresh universal tube. The EB suspension was centrifuged gently at 80xg for 3 minutes. The supernatant was aspirated and the loose pellet of EBs resuspended in fresh ES medium in the absence of LIF to permit differentiation. These aggregates were known as 0 day EBs, marking the time elapsed from LIF withdrawal. Differentiating EBs were cultured in suspension in bacterial grade petri dishes to prevent them from adhering to the dish. Every 2 days, EBs were transferred to a new universal and allowed to settle slowly by gravity, then supernatant was aspirated. EBs were resuspended in fresh ES medium (no LIF) and transferred into a sterile bacterial grade petri dish. For the first 2 days after harvest, penicillin/streptomycin (Sigma, 2,000 units and 2mg, respectively) were added to the medium, as this stage was susceptible to bacterial contamination.

### ***2.2.2 Embryoid body (EB) formation for ES cell Differentiation – suspension method***

The *hanging drop* method of EB formation generated undifferentiated ES cell aggregates of relatively uniform size. However, ES cell aggregates can also be prepared in suspension. This relies on the ability of ES cells to spontaneously form aggregates when in suspension culture, producing EBs of many different sizes (Figure 2.2 c and d).

To generate suspension EBs, ES cells were harvested as during a normal passage and a suspension of  $3 \times 10^4$  cells per ml of ES medium containing no LIF was prepared (typically  $6 \times 10^5$  cells in 20 ml). The 20ml ES cell suspension was placed in a sterile bacteriological grade petri dish and incubated at 37°C (humidified 5% CO<sub>2</sub> atmosphere). At this point, LIF had been removed and the culture contained day 0 EB. Therefore, suspension EBs formed in the absence of LIF, in contrast to the *hanging drop* method, where EBs formed in the presence of LIF. Every 2 days, EBs



were transferred from the petri dish into a universal tube and allowed to settle by gravity, before aspirating the supernatant and resuspending the EBs in fresh ES medium (no LIF) and placing them into a sterile bacterial grade petri dish. Antibiotics were not used during this procedure.

### ***2.2.3 Co-culture of embryoid bodies with stromal cell lines to induce differentiation***

Mouse ES cells differentiate upon withdrawal of LIF. In these experiments, EBs were generated to initiate differentiation and these were co-cultured with stromal cell layers with a view to testing whether the stromal lines could promote haematopoietic differentiation of ES cells. The co-culture strategies used are outlined in Figure 2.3. For co-culture experiments, stromal cells were grown to confluence in tissue culture grade flasks or wells and  $\gamma$ -irradiated (30Gy) no more than 2 days prior to co-culture. Irradiation prevented further cell divisions. A GammaCell40 irradiator (Nordion) was used to irradiate cells, this irradiator uses caesium 137 as a source of unstable atoms that decay and emit beta and gamma radiation. The source is encapsulated such that only gamma radiation reaches the sample. After irradiation of the (adherent) stromal cells, the specialised stromal medium was aspirated, the stromal cell layers washed in PBS and then placed in ES medium (containing no LIF). EB differentiation was carried out in ES medium (-LIF) and with no additional cytokines, at 37°C (in a humidified 5% CO<sub>2</sub> atmosphere). Differentiation was allowed to proceed for up to 16 days in these experiments, with 50% of the medium being replaced every 2-3 days. Co-cultured cells were harvested and analysed at defined time points for ES cell -derived haematopoietic activity.

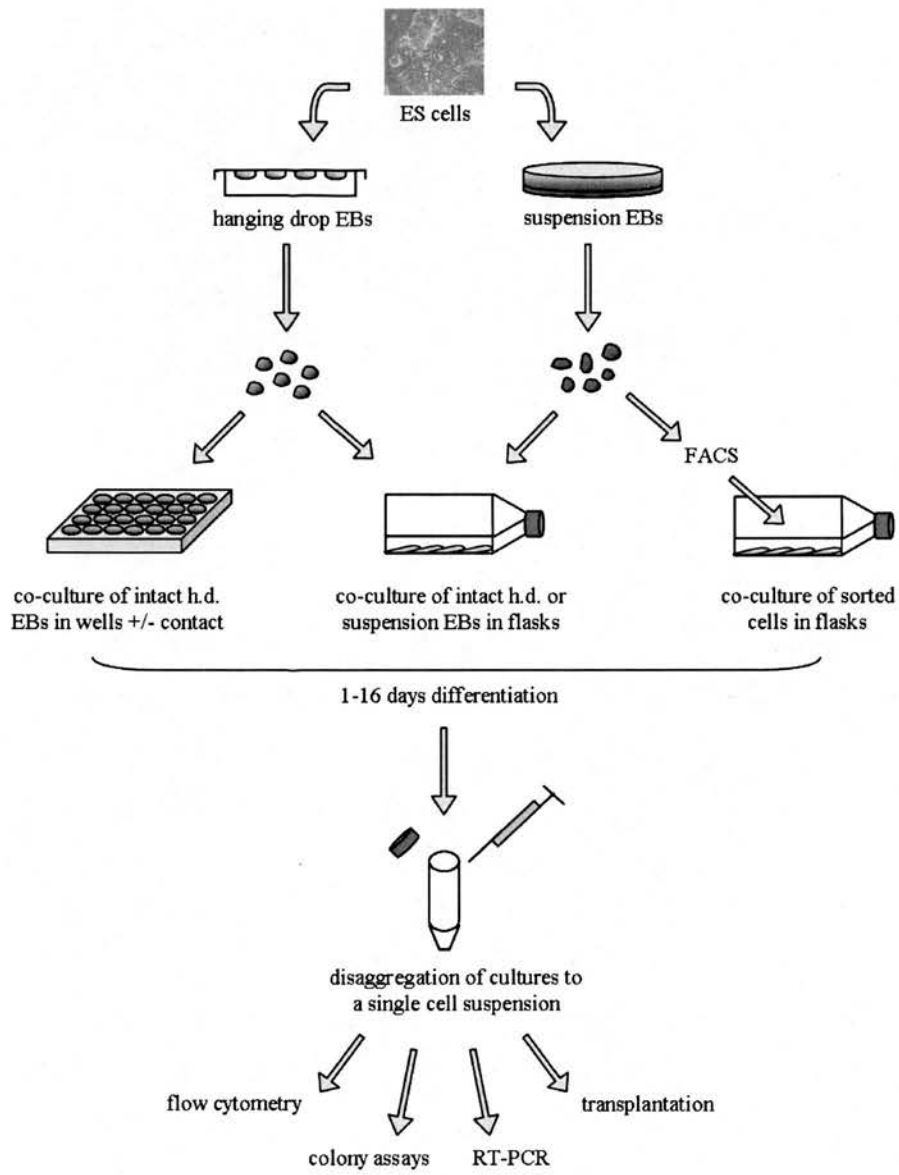


Figure 2.3 Summary of the EB/ stromal co-culture strategies.

EBs were co-cultured in the following ways:

a) Co-cultures with hanging drop EBs:

- 50-100 EBs (1 day old) per 25cm<sup>2</sup> tissue culture flask, in direct contact with stromal cells. This strategy was used for *in vitro* analysis and transplantation experiments.
- A single EB (1 day old) co-cultured per well of a 24 well plate (direct cell contact).
- Approximately 5 EBs (1 day old) per well of a 24 well plate (direct cell contact).
- Single EBs or approximately 5 EBs (1 day old) per transwell in a 24 well plate (no cell contact). Transparent Greiner Bio-one 24 well ThinCert-tissue culture inserts were used to inhibit direct contact between the EBs and stromal cells, while allowing exchange of components through the porous membrane (PET membrane pore size of 0.4µm and pore density of 2x10<sup>6</sup> cm<sup>-2</sup>).

b) Co-cultures with suspension EBs or defined populations of cells isolated from EBs:

Fluorescence activated cell sorting (FACS) was used to isolate defined cell populations from EBs. EBs were generated by the suspension method to maximise the numbers of cells available for FACS and subsequent co-culture on irradiated stromal cells (in 25cm<sup>2</sup> flasks).

c) Co-cultures with extracellular matrix extracted from irradiated stroma:

50 to 100 hanging drop EBs (1 day old) were plated per 25cm<sup>2</sup> tissue culture flask, in contact with extracellular matrices isolated from irradiated stromal cells (procedure described in section 2.2.8).

d) Co-cultures in medium conditioned by the irradiated stromal layers:

ES medium (containing no LIF) was conditioned on irradiated stromal cells for a duration of 1, 2 or 4 days. Equal measures of 1, 2 and 4 day conditioned medium were 0.2µm filter-sterilised and mixed. Hanging drop EBs were differentiated in suspension with 50% conditioned medium and 50% fresh ES medium (-LIF), in

bacteriological grade dishes. The EBs were transferred to fresh petri dishes every 2 days and analysed for haematopoietic activity at defined time points.

#### ***2.2.4 Harvesting co-cultured cells for in vitro analysis or in vivo transplantation***

##### **a) Harvesting cells co-cultured in 25cm<sup>2</sup> flasks**

Co-cultured cells were harvested at defined time points to assess their haematopoietic activity. The medium was aspirated (or kept for analysis) and the adherent cells of the co-cultures were then washed briefly with PBS. Single cell suspensions were obtained by enzymatic digestion with dispase solution (PBS containing 1.2U/ml dispaseII, Roche and 70µg/ml DNaseI, deoxyribonucleaseI, Sigma) for 45 minutes at 37°C. Approximately 2ml dispase solution was used per 25cm<sup>2</sup> flask. Dispase is a neutral protease obtained from *Bacillus polymyxa*, it was used here to disaggregate the cells in co-culture, as it does not damage cell membranes, is stable during incubation and is free of mycoplasma. These properties made it more suitable for this application than proteolytic enzymes such as trypsin or collagenase. DNaseI is an endonuclease that digests double and single stranded DNA into oligo- and mono-nucleotides. This was added to prevent cell clumping due to release of DNA from any sheared cells.

After incubation with dispase solution the flasks were tapped sharply to dislodge the cells, which were transferred to universal tubes containing 8ml ES medium (-LIF) to dilute the dispase and quench its activity. This was followed by passing the cells through a 23-gauge needle, to obtain a single cell suspension by mechanical disruption. Cells were centrifuged at 100xg for 5 minutes and pellets resuspended in ES medium (usually 5ml). Cell suspensions were counted using a haemocytometer. Appropriate numbers of cells were then (i) seeded into colony assays to determine the cell types present, (ii) analysed by flow cytometry to detect expression of different surface markers and (iii) the remaining cells were pelleted by centrifugation, snap frozen on dry ice and stored at -80°C for molecular analysis.

Samples of co-cultured cells were always analysed by flow cytometry to determine the percentage of ES-derived cells in the suspensions. The 7a-GFP ES cells

constitutively expressed GFP and when unlabelled ES cells were used, the stromal layers were dyed prior to irradiation and co-culture. In this way, data could be normalised according to the number of ES-derived cells seeded into assays (described in section 2.2.5).

b) Harvesting EBs co-cultured in 24 well plates

To test whether the effects of co-culture were contact-dependent, 5 EBs were co-cultured per well of a 24 well plate, with or without direct contact with stromal cells. In these experiments, cells were harvested by picking the EBs from the stromal layer or transwell insert with a Gilson pipette with yellow tip. The EBs were then placed into 2ml dispase solution in universal tubes and incubated for 45 minutes at 37°C. One universal tube was used per co-culture condition (stromal line); therefore, a pool of EBs was analysed for each co-culture. The EBs were disaggregated by mechanical disruption, centrifuged, resuspended and counted as described, before assessing haematopoietic activity. The remainder of the cell suspensions were analysed by flow cytometry to normalise the data.

When EBs were co-cultured at 1 EB per well in 24 well plates, individual EBs were picked from the stromal layer or transwell insert using a Gilson pipette with yellow tip. Individual EBs were transferred to separate wells of a 96 well plate with 100µl/well dispase solution and incubated in dispase solution for 45 minutes at 37°C. Subsequently, each EB was gently disaggregated to a single cell suspension by pipetting. Single EB cell suspensions were then transferred to individual wells of 24 well plates containing 100µl of fresh ES medium (-LIF) to neutralise the dispase solution. Each EB cell suspension was individually seeded into its own assay dish to assess haematopoietic activity. Results were expressed as the frequency of colonies obtained per EB, so normalisation of data was not necessary.



### ***2.2.5 Normalisation of data to exclude irradiated stromal cells present in cell suspensions that were seeded into assays***

It was determined that irradiated stromal cells could not give rise to colonies in the assays used. However, in cases where ES cell suspensions containing irradiated stromal cells were seeded into assays, it was necessary to normalise the data according to the actual number of ES-derived cells seeded. This allowed data to be expressed in terms of the frequency of colonies obtained for a defined number of input ES-derived cells. Co-cultured cells were harvested as described above. After removing cells for assays, samples of the cell suspensions were washed and resuspended in FACS PBS (containing 0.1% sodium azide plus 0.1% BSA). A FACSCaliber flow cytometer equipped with 488nm and 633nm lasers (Becton Dickinson) was used to collect data for  $1-5 \times 10^4$  live events. When 7a-GFP cells were used, they constitutively expressed eGFP. Therefore, GFP-positive ES cells (7a-GFP) were used to set the electronic gate on fluorescence channel 1 (green), which was used to exclude GFP-negative stromal cells. In cases where unmarked or reporter ES cells were used, stromal cells were labelled prior to co-culture so that these could be excluded from analysis using electronic gates. In this way, the proportion of ES-derived cells in each cell suspension was determined. This proportion was used to adjust the colony numbers obtained in the assays to take into account the actual number of ES-derived cells seeded. Samples were run by myself and Kay Samuel (SNBTS) helped set the gates on flow cytometric plots. The following formula was used to normalise colony numbers:

$$\text{Normalised number of EB-derived colonies} = \frac{\text{number of colonies scored}}{\text{proportion of ES cells in cell suspension}}$$

### ***2.2.6 Labelling of stromal cells***

When the ES cells carrying a reporter construct or unlabelled wild type E14 or CGR8 ES cells were co-cultured, it was necessary to label the stromal cells to allow the proportion of ES-derived cells to be determined by flow cytometry in order to normalise data appropriately. The Vybrant DiD labelling system from MP-Invitrogen was used to stain the stromal cells. This is an intracellular membrane carbocyanine dye that has low cytotoxicity and is highly resistant to intercellular

transfer. DiD labelling solution could be added directly to normal culture medium to label attached cells uniformly. DiD has an absorption maximum of 644nm and a fluorescence emission spectrum (maximum) of 665nm. Using a conventional flow cytometer, this dye can be detected in the FL3 detection channel. Stromal cells were grown to confluence in flasks or wells and prior to staining they were washed briefly with PBS, which was aspirated before addition of a 1:250 solution of DiD in PBS, at approximately 120 $\mu$ l solution per cm<sup>2</sup> area of adherent stromal cells. Typically, 2 $\mu$ l DiD in 500 $\mu$ l PBS was used per well of a 6 well plate and 12 $\mu$ l DiD in 3ml PBS was used per 25cm<sup>2</sup> flask. The cells were incubated with the dye for 20 minutes at 37°C. The dye solution was then aspirated and the cells washed at least 3 times by repeated addition and aspiration of PBS. Finally, the PBS was aspirated and replaced with ES medium (-LIF). The labelled cells were then ready to be irradiated before a co-culture experiment.

### ***2.2.7 Co-culture of ES cells with labelled stroma***

ES cells carrying a *Brachyury*-eGFP reporter gene (Bry-201 ES cells) were co-cultured on confluent adherent stromal lines that were dyed with Vybrant DiD prior to irradiation in the following ways:

- Approximately 50-100 hanging drop EBs (1 day old) per 25cm<sup>2</sup> flask.
- Approximately 20 hanging drop EBs (1 day old) per well of a 6 well plate (for *Brachyury* kinetics experiments).
- Approximately 50-100 suspension EBs (4 day old) per 25cm<sup>2</sup> flask.
- 4 day old suspension EBs were disaggregated and cells were then sorted using FACS according to *Brachyury*-GFP expression. 1x10<sup>5</sup> sorted *Brachyury*-GFP positive or GFP negative ES-derived cells were co-cultured per 25cm<sup>2</sup> flask of dyed irradiated stromal cells for a further 6 days differentiation.

Bry-201/stromal co-cultures were harvested for analysis as described in section 2.2.4.



### ***2.2.8 Isolation of extracellular matrix (ECM) from irradiated stromal cell layers***

The protocol for the isolation of extracellular matrix (ECM) from cell layers was obtained from Hedman et al., (1979). The isolated ECM is believed to be identical to that of intact cell layers. In these experiments, stromal cells were grown to dense layers and then irradiated as usual, the stromal medium removed, the cells were washed with PBS and placed in fresh ES medium (-LIF) overnight in a 37°C (5% CO<sub>2</sub> humidified atmosphere) incubator. The day after irradiation, the medium was aspirated gently and the cells briefly rinsed three times with PBS at room temperature. The PBS was gently aspirated and the cells were treated with a combination of 0.5% sodium deoxycholate (DOC, cell lysis detergent from Sigma) and 1mM phenylmethyl-sulfonylfluoride (PMSF, stock solution 0.4M in ethanol, Sigma) in 10mM Tris-Cl buffered saline (pH8.0). 6ml solution was used per 25cm<sup>2</sup>, the cells were left in this for 10 minutes on a four-way slow moving shaker at 4°C. The solution was then gently removed by aspiration, being careful to keep the flasks horizontal so that ECM did not peel off during washing. This cell lysis step was repeated three times in total. Following this, the ECMs were treated with a low ionic strength buffer (2mM Tris-Cl pH8.0 containing 1mM PMSF) for 5min at 4°C with gentle shaking. This wash step was also repeated 3 times. The buffer was gently removed and 10ml ES medium (-LIF) placed in each flask and the isolated ECM was ready for use in co-culture.

### ***2.2.9 Serum free culture conditions***

#### ***2.2.9.1 Weaning ES cells***

In order to assess the extent to which the co-culture system relied on the presence of serum in the medium, 7a-GFP ES cells were transferred to serum-free media. ESGRO complete clonal medium (Chemicon International) was used for serum-free maintenance of undifferentiated ES cells. Cells were weaned in serum-free conditions and passaged according to the manufacturer's instructions. Briefly, 1x10<sup>6</sup> ES cells were seeded into 25cm<sup>2</sup> gelatin-coated flasks with 10ml of clonal medium, which contains BMP4 and LIF. These were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. 2-3 days later, the cells were passaged by removing the medium, washing with PBS and treated with 1ml accutase (Chemicon International) per 25cm<sup>2</sup>

flask for 3 minutes at 37°C to detach the cells. The cells were transferred to a universal containing 8ml basal medium (Chemicon International), which does not contain LIF or serum. Due to the absence of serum, the accutase activity is not quenched and must therefore be diluted quickly. Therefore, the cells were washed twice in 8ml basal medium by centrifugation at 100xg for 3 minutes. Finally, the cells were resuspended in clonal medium, counted using a haemocytometer and  $1 \times 10^6$  cells were seeded into fresh gelatin-coated flasks with 10ml clonal medium. The growth rates of the ES cells were assessed for at least three weeks prior to use in co-culture experiments to ensure they were comparable to those grown in serum-containing media.

#### *2.2.9.2 ES cell differentiation in serum-free conditions*

Serum-free ES cells were prepared in hanging drops in the same way as for cells in the presence of serum. Cells in clonal medium, which contained LIF, were prepared in 10µl hanging droplets containing approximately 300 cells per drop. Day 0 EBs were harvested after 2 days incubation at 37°C (in humidified 5% CO<sub>2</sub> atmosphere). In order for differentiation to proceed, the clonal medium containing LIF was withdrawn and EBs were placed in bacterial grade petri dishes in serum-free differentiation medium which was modified for this experiment from a protocol described by Gouon-Evans and colleagues (Gouon-Evans et al., 2006). The medium consisted of 75% IMDM (Gibco), 25% Ham's F12 medium (Gibco), 0.5x N2 (Gibco), 0.5x B27 (Gibco), 2,000 units penicillin (Sigma), 2mg streptomycin (Sigma), 0.05% BSA (Gibco), 2mM glutamine, 0.5mM ascorbic acid,  $4.5 \times 10^{-4}$ M monothioglycerol (MTG) (Sigma). This serum-free differentiation medium was supplemented by adding SCF and BMP4 directly to the wells or flasks.

#### *2.2.9.3 Co-culture in serum-free conditions*

Stromal cells were prepared in 25cm<sup>2</sup> flasks for co-culture as described above in the presence of serum; however, after irradiation and thorough washing of stromal cell layers with PBS, 10ml of serum-free differentiation medium was added per flask.

Co-cultures were setup as follows:

- Approximately 50-100 serum-free 1 day old hanging drop EBs were co-cultured on the stromal layers for up to 6 days of differentiation in the modified Gouon-Evans medium, supplemented with SCF (Stem Cell Technologies) and BMP4 (R&D Systems) at final concentrations of 30ng/ $\mu$ l.

In these experiments, 50% of the medium was replaced every second day. At defined time points, the co-cultured cells were harvested and treated in as described for cells in serum-containing conditions (described above). Subsequent to harvesting, samples of cell suspensions were seeded into assays and analysed by flow cytometry to determine the proportion of GFP positive ES-derived cells for normalisation of data.

### **2.3 ES cell self-renewal assays**

To determine the number of undifferentiated self-renewing ES cells present in a cell suspension, self-renewal assays were setup. Cells were seeded at low density ( $1 \times 10^3$  or  $0.5 \times 10^3$ ), in duplicate wells of a gelatin-coated 6 well plate, with ES medium containing concentrations of LIF between 0U and 100U/ml. The cells were incubated for 5 days at 37°C (in a humidified 5% CO<sub>2</sub> atmosphere) and subsequently stained for alkaline phosphatase (AP) activity with the Leukocyte AP staining kit (Sigma Diagnostics) as directed by the manufacturer. Undifferentiated ES cells are AP+. The following procedure was carried out at room temperature: colonies were fixed with citrate-acetone-formaldehyde fixture [65ml acetone, 8ml 37% formaldehyde and 25ml citrate solution. Citrate solution consisted of 18mM citric acid, 9mM sodium citrate, 12mM sodium chloride with surfactant at pH3.6]. This was applied for approximately 30 seconds and was washed off carefully with water for 45 seconds. Subsequently, colonies were stained with 1.5ml/well alkaline dye solution for 15 minutes in a dry dark place at room temperature. To prepare the dye mixture using the kit: 1ml 0.1M sodium nitrite solution was added to 1ml FRV-Alkaline solution, this was placed into 45ml deionised water containing 1ml Naphthol AS-B1 Alkaline solution. After staining, colonies were washed in water, air-dried and colonies were analysed and categorised according to AP staining by light microscopy (Figure 2.4).

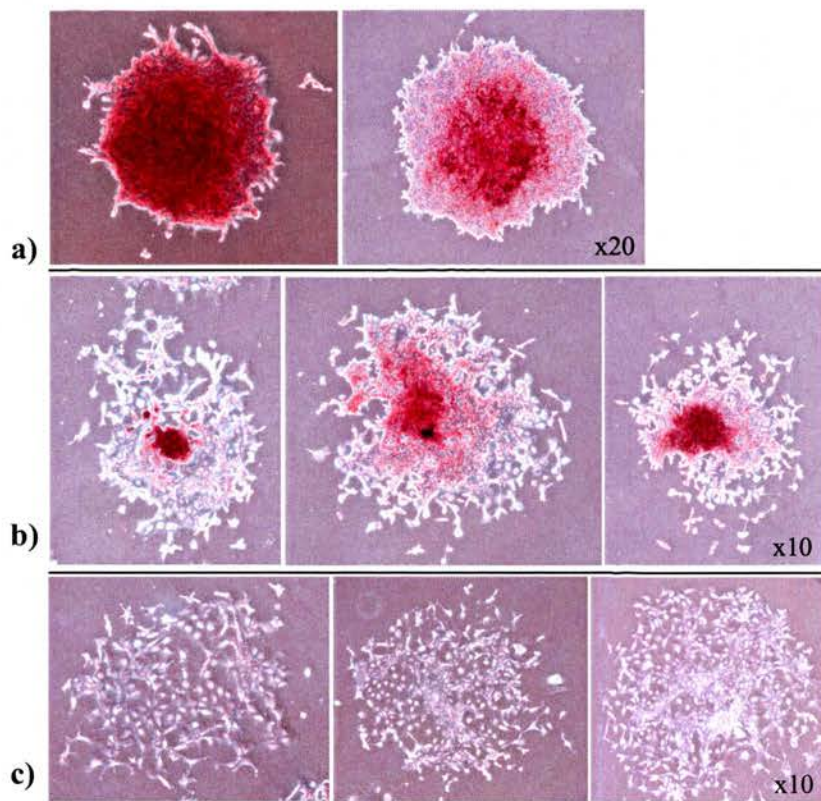


Figure 2.4 Colonies formed in the ES cell self-renewal assay. Colonies were considered to be clonal and were stained for alkaline phosphatase activity, which marked undifferentiated self-renewing ES cells. (a) Undifferentiated ES cell colonies, (b) mixed colonies containing stained undifferentiated and unstained differentiated cells and (c) differentiated colonies containing no stained cells.

## 2.4 Haematopoietic progenitor colony assays

Colony assays involve seeding single cell suspensions into semi-solid medium supplemented with cytokines. Progenitor cells respond to cytokines and generate clonal colonies which can be categorised by their morphology and enumerated under a light microscope. Colony assays allowed retrospective determination of the frequency of haematopoietic colony forming units (CFU) or cells present in a starting sample population.

### 2.4.1 Agarose-based haematopoietic colony assays

In the CFU-A (colony forming unit- A) assay: a bottom feeder layer of 0.6% agarose in specialised Eagle's medium (25%  $\alpha$ -MEM, 20% horse serum, 0.25% sodium bicarbonate and 4mM L-glutamine) that contained 10% conditioned medium from both the L929 and the AF1-19T cell lines (sources of M-CSF and GM-CSF, respectively) was aliquoted (1ml) into each 30mm tissue culture grade dish and allowed to set. Test cells ( $3 \times 10^4$  cells/ml) were seeded into warm 0.3% agarose in modified Eagle's medium (with no conditioned medium) and poured onto the bottom agarose layers (in triplicate). After incubation at 37°C in a 5% O<sub>2</sub> and 10% CO<sub>2</sub> humidified atmosphere for 10 days, the colonies (>2mm in diameter, primarily comprising myeloid cells) were counted.

### 2.4.2 Methylcellulose-based haematopoietic colony assays

Methylcellulose-based assays allowed CFU-Mix (granulocyte/macrophage and red cells), CFU-GM (granulocyte/macrophage), CFU-Mac (macrophage/monocyte) and erythroid/macrophage colony forming cells to be detected. For these assays,  $1 \times 10^5$  test cells were plated into 35mm dishes containing 1.5ml 1% methylcellulose in Iscove's Modified Dulbeccos Medium (IMDM) (Stem Cell Technologies (SCT)), supplemented with 10% fetal bovine serum (SCT); 340 $\mu$ M monothioglycerol; 3 Units/ml mouse erythropoietin (Epo) (Roche); 10 $\mu$ g/ml recombinant human Insulin (Sigma); 10ng/ml murine IL3 (SCT); 10ng/ml recombinant human IL6 (SCT); 50ng/ml recombinant mouse Stem Cell Factor (SCT), 2mM L-Glutamine and 1,000 units penicillin/1mg streptomycin (Sigma). The dishes were placed in a larger culture dish (with lid) containing an open sterilin dish with 5ml UHP water to ensure



the conditions were humid enough to prevent the methylcellulose from drying out. These were incubated at 37°C (in a humidified 5% CO<sub>2</sub> atmosphere). The colonies were identified morphologically by light microscopy and counted after 4 and 10 days.

The haematopoietic readout was scored as follows: Clonal colonies containing red cells and at least two different types of white cell were scored as CFU-Mix, thought to represent multipotent progenitors. CFU-GM were heterogenous white cell colonies with at least two different types of white cell but containing no red cells. CFU-Mac represented macrophage colonies and Erythroid/Macrophage had a similar morphology to CFU-Mac but also contained red cells. Examples of these colonies are provided in Chapter 4, Figure 4.1. Colonies that did not fall into the aforementioned categories were termed “other”; these colonies represented unipotent progenitor cells such as definitive erythroid cells, primitive erythroid cells or mast cells. In this way, all the haematopoietic colonies in the assay dishes were accounted for, to ensure that the total haematopoietic activity could be determined.

When differentiating ES cells were seeded into the methylcellulose-based assay, it was found that secondary EBs formed. These secondary EBs were thought to represent undifferentiated ES cells present in the samples. These undifferentiated cells within the secondary EBs differentiated in response to the exogenous cytokines in the assay, resulting in haematopoietic cells emanating from the secondary EBs, these were called “burst” secondary EBs. These burst secondary EBs did not represent haematopoietic progenitor cells (CFU) which were present in the test population at the initiation of the assay. Therefore, secondary EBs and burst secondary EBs that formed in the methylcellulose assay were not considered as part of the haematopoietic CFU readout. Examples of burst and non-burst secondary EBs are provided in Chapter 3, Figure 3.4.

## 2.5 Cytospins and staining

Colonies picked from methylcellulose assays were prepared as cytopins and stained to determine the cell types present. Individual colonies (or up to 5 pooled colonies) were picked after 10 days in assay using a Gilson pipette (yellow tip) set to 100 $\mu$ l. Colonies ( $1 \times 10^4$  to  $0.5 \times 10^6$  cells) were placed into a universal containing 2 to 6ml warmed PBS (37°C) to dissolve the methylcellulose. The cell suspensions were centrifuged for 5 minutes at 100xg. PBS was aspirated and the cell pellet resuspended in 100 $\mu$ l PBS plus 10% FCS.

Poly-L-lysine coated microscope slides (25x75x1.0mm, BDH Laboratories, pre-treated for superior cellular adhesion) were labelled and a specialised filter card (Shandon Inc.) was placed between the slide and a cytospin funnel, ensuring that the sample chamber and the filter hole lined up. The labelled slide, filter and funnel were then carefully placed into a cytofuge (Cytospin4, Shandon) and held in place by a metal sprung pin, ensuring the holes lined up with the viewer. If fewer than  $5 \times 10^4$  cells were present, 50 $\mu$ l of FCS was loaded into the funnel and centrifuged for a few seconds to wet the filter and to help the cells adhere to the slide. Subsequently, the cell suspension was loaded into the funnel (100-250 $\mu$ l maximum) and centrifuged for 5 minutes at 120xg. The slide/filter/funnel was then carefully removed from the cytofuge and the filter and funnel carefully peeled away from the slide, without smudging the cytospin. Slides were air-dried on a paper towel. Dry slides were carefully placed into coplin jars containing 100% methanol for up to 15 minutes to fix the cytopins. The slides were then air dried in a cool dust-free environment. The cytopins were stained with Quick-DIFF (from Reagen), a form of May Grunwald Giemsa which stains nuclei blue and cytoplasm pink/rose. Fixed and air-dried cytospin slides were dipped into the Quick-DIFF dye I (red eosin) for 10 seconds, followed by dipping into Quick-DIFF dye II (blue hematoxylin) for 10 seconds and then dipped into dH<sub>2</sub>O to wash. The slides were air-dried overnight before analysis by light microscopy.



## 2.6 Molecular analysis of co-cultured cells

### 2.6.1 RNA isolation and reverse transcription

Co-cultures were harvested as described above and, after seeding samples of cells into haematopoietic colony assays or removing cells for analysis by flow cytometry, the remainder of the cell suspensions were briefly centrifuged in eppendorfs and cell pellets were snap frozen on dry ice and stored at  $-80^{\circ}\text{C}$ . Total RNA was extracted using the RNA aqueous-4PCR kit from Ambion or the Qiagen Rneasy extraction kit; in both cases, the RNA was treated with DNaseI to remove residual genomic DNA. cDNA was prepared using the Taqman reverse transcription reagents supplied by Applied Biosystems, or the reverse transcription Superscript III kit from Invitrogen. When using the Applied Biosystems reagents, 400ng RNA was reversed transcribed with random hexamers and oligo(dT) as follows: 1x TaqMan RT buffer, 5.5mM  $\text{MgCl}_2$ , 500 $\mu\text{M}$  each dNTP, 2.5 $\mu\text{M}$  oligo(dT), 2.5 $\mu\text{M}$  random hexamers, 0.4 units/ $\mu\text{l}$  Rnase inhibitor, 1.25 units/ $\mu\text{l}$  Multiscribe RT and 400ng RNA, made up to 20 $\mu\text{l}$  with nuclease-free water. The reaction was incubated at  $25^{\circ}\text{C}$  for 10 minutes, followed by 30 minutes at  $48^{\circ}\text{C}$  and then inactivated at  $95^{\circ}\text{C}$  for 5 minutes. cDNA was kept at  $4^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$  for long term storage. The Superscript III kit from Invitrogen consisted of a 2xRT reaction mix and a 10xRT enzyme mix. These contained 2.5 $\mu\text{M}$  oligo(dT), 2.5ng/ $\mu\text{l}$  random hexamers, 10mM  $\text{MgCl}_2$ , 500 $\mu\text{M}$  each dNTP, Superscript III RT enzyme and RNaseOUT. The reaction was setup with 400ng RNA and was made up to 20 $\mu\text{l}$  with nuclease-free water and incubated at  $25^{\circ}\text{C}$  for 10 minutes, followed by 30 minutes at  $50^{\circ}\text{C}$  and then inactivated at  $85^{\circ}\text{C}$  for 5 minutes. Again, cDNA was kept at  $4^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$  for long term storage.

### 2.6.2 Quantitative real time reverse transcriptase (RT) polymerase chain reaction (PCR)

Quantitative real-time reverse transcription (RT)-PCR was performed on an ABI 7500 FAST qPCR machine (Applied Biosystems) using 10ng cDNA per reaction. Taqman chemistry was used and validated Taqman gene expression assays were purchased from Applied Biosystems (Table 2.2).

<b>Gene</b>	<b>Description</b>	<b>Supplier</b>
Beta-actin	Endogenous control	MWG
HPRT	Endogenous control	MWG
Lmo2	Expressed by definitive haematopoietic cells, required for establishment of primitive haematopoiesis	ABI assay Mm00493153_m1
Runx1	Expressed by definitive haematopoietic cells	ABI assay Mm00486762_m1
Sry	Y chromosome specific marker	ABI assay Mm00441712_s1

Table 2.2 Taqman gene expression kits used for quantitative PCR analysis. Purchased from Applied Biosystems (ABI) or MWG.

The housekeeping gene HPRT was used as the endogenous control to enable relative quantitation of gene expression according to the amount of cDNA loaded. Reactions were set up in triplicate in 96 well plates, using the recommended Taqman program for universal conditions: 55°C for 2 minutes, 95°C for 10 minutes to activate the AmpliTaq Gold, followed by 40 cycles of 95°C for 15 seconds to denature cDNA and 60°C for 1 minute to allow annealing/extension. Relative quantitation was calculated using the delta delta CT method using SDS v1.4 software from Applied Biosystems. Using this software, the gene expression in each reaction was first normalized according to the amount of cDNA loaded (HPRT endogenous control). The data were then shown as fold change in gene expression compared to an internal calibrator sample within each experiment. The gene expression assays supplied by Applied Biosystems were already validated to ensure efficiency of the reactions. In the case of HPRT, the primer set and probe were designed and validated by Dr Melany Jackson in the John Hughes Bennett Laboratory.

## **2.7 *In vivo* experiments**

*In vivo* experiments were carried out with help from Kay Samuel (SNBTS), who also helped set the gates on flow cytometric plots for tissue samples screened (Chapter 6).

### **2.7.1 *Markers of reconstitution***

Prior to carrying out transplantation experiments, it is vital that appropriate markers are in place to allow the investigator to reliably distinguish between donor and host cells. This allows assessment of chimaerism of the recipient (i.e. to determine if donor cells have engrafted host tissues), according to the unique properties of the recipient and donor cells. When 7a-GFP ES cells were used, ES-derived donor cells constitutively expressed an eGFP reporter gene, allowing them to be distinguished by flow cytometry. When unmarked ES cells were transplanted, these were stained with the Vybrant DiD label (Invitrogen) prior to differentiation and injection. When the donor cells originated from primary bone marrow, Rosa26-LacZ (male) mice were used as the donors so that the donor cells could be detected by assessing FDG activity (procedure described in section 2.9.2.2).

As an additional marker, donor cells were male and were transplanted into female recipients so that Y chromosome PCR could be used to detect donor cell contribution to host tissues.

### ***2.7.2 Preparation of cells for in vivo transplantation***

#### *2.7.2.1 Co-cultured ES cells*

7a-GFP ES cells were co-cultured with stromal cells and harvested at defined time points as described and disaggregated to single cell suspensions on the day of transplantation. Samples of cell suspensions were analysed by flow cytometry to determine the proportion of ES cells present. The equivalent of  $1 \times 10^6$  ES-derived cells were transplanted per recipient.

Co-culture cell suspensions could be sorted by FACS prior to transplantation according to expression of surface markers or sorted to separate GFP positive 7a-GFP ES-derived cells from the stromal layers. These sorted cells were injected intravenously (i.v.) or intra-splenically (i.s.) at approximately  $1 \times 10^6$  ES cells (in no more than 200 $\mu$ l or 100 $\mu$ l PBS, respectively) per recipient.

#### *2.7.2.2 Bone marrow or spleen cells*

Bone marrow or spleen cells were harvested from animals as described in section 2.7.4. To accurately enumerate leukocytes in these cell suspensions, red blood cells were excluded using white cell counting fluid. A 10 $\mu$ l aliquot of cell suspension was diluted in 40 $\mu$ l white cell counting fluid (3% acetic acid in distilled water plus 10 $\mu$ g of crystal violet or Gentian). 10 $\mu$ l of this was immediately mounted on a haemocytometer for counting, as prolonged exposure to white cell counting fluid can destroy white cells too. Alternatively, cell counts were obtained using a Beckman-Coulter counter. For transplantation, the cells were counted, pelleted and resuspended in an appropriate volume (maximum 200 $\mu$ l cells in PBS per recipient,  $5 \times 10^7$  cells per ml). For staining and flow cytometry analysis, the remaining cell suspensions were resuspended at  $1 \times 10^7$  cells/ml. .

### 2.7.2.3 Preparation of labelled ES and BM cells for short-term in vivo experiments

Experiments were carried out to test the ability of ES cells to home to different tissues and to assess whether 7a-GFP ES cells continued to express GFP upon transplantation. Disaggregated 4d 7a-GFP EB cells were labelled with Vybrant DiD labelling solution (Invitrogen) as adherent monolayers prior to EB formation, as described in section 2.2.6. Undifferentiated ES cells and male C57 bone marrow cells were dyed in suspension on the day of transplantation. Briefly,  $1 \times 10^6$  cells/ml PBS were incubated with  $5 \mu\text{l/ml}$  DiD cell-labelling solution for 20-30 minutes at  $37^\circ\text{C}$ . Cell suspensions were centrifuged at  $120 \times g$  for 3 minutes, the supernatant removed and pellet resuspended in warm ES medium (no LIF). This wash step was repeated 2 more times. At least 10 minutes recovery time was allowed before injection or flow cytometry of dyed cells. Labelled ES or EB cells and bone marrow-derived cells were injected i.v. at  $1 \times 10^7$  cells per recipient. Recipients were killed 5 minutes, 1 hour or 24 hours later and tissues analysed for donor cells.

### 2.7.3 Preparation of transplant recipients

All animal work was carried out under the provisions of the Animals (Scientific Procedures) Act (UK) 1986. All procedures on live animals were permitted under a Home Office Project Licence (PPL 60/3360) and were carried out by Kay Samuel (Scottish National Blood Transfusion Service, New Royal Infirmary Edinburgh) who holds a Personal Licence also permitting the procedures. The animals were bred and maintained at the Biomedical Research Facility (BRF) of the University of Edinburgh and the daily care of animals was carried out by staff at the BRF. All transplant recipients were euthanased by means of cervical dislocation (Schedule 1) or administration of Euthatal.

Non-obese diabetic severe combined immuno-deficient (NOD/SCID) mice were used as recipients (Jackson Laboratories). These mice are homozygous for the spontaneous SCID mutation  $Prkdc^{scid}$  and are diabetes free. They lack functional B and T cells, have defective myeloid development and have low natural killer cell activity. NOG/SCID or NOD-scidIL2R $\gamma^{\text{null}}$  mice carry the  $Prkdc^{scid}$  and lack the interleukin-2 receptor gamma c. Therefore, they lack T and B lymphoid cells

without leakiness, have extremely low natural killer cell activity and they are resistant to lymphoma formation even after sublethal irradiation, leading to a longer lifespan.

The water supply of recipient mice was supplemented with antibiotics (Baytril) 7 days prior to the transplantation. Prior to transplant, recipient mice were sub-lethally irradiated (250rad) using the GammaCell40 irradiator at the Biomedical Research Facility (BRF). Prior to administration of cells by intra-venous injection, mice were warmed in a 37°C “hot box” for 10-15 minutes to dilate the tail vein. Mice were held in a restraint for injection of cells using a 1ml syringe and 25G needle. On the day of transplantation, a single cell suspension of the donor sample was prepared; for instance, for a 6-8 week old recipient, no more than 200µl cell suspension in PBS was injected per recipient.

For intra-splenic injections, mice were sub-lethally irradiated, warmed and anaesthetised using vetlar (ketamine anaesthetic and domitor analgesic) injected intra-peritoneally. Once unconscious, the recipient was placed on its back on a heated pad and swabbed with 70% ethanol to sterilise the site of incision. A small area to the left of the midline, just below the sternum, was shaved and a small incision made in the skin to reveal the peritoneum. Once the spleen had been visually located, a small incision was made in the peritoneum and the spleen was gently grasped near one end and carefully externalised to lie on the peritoneum. A maximum of 100µl of cell suspension was carefully injected directly into the spleen using an insulin syringe and 29G needle. The spleen was then tucked back into the peritoneal cavity and the peritoneal membrane sutured closed. The skin was closed with surgical stainless steel clips. Anaesthesia was reversed using the reversal agent atipamezole injected sub-cutaneously and their recovery monitored closely. Antibiotic supplementation of recipients’ drinking water was withdrawn 28 days after transplantation. After 56 days (8 weeks), reconstitution should be complete, but to detect long-term reconstituting cells, recipients had to be repopulated for up to 6 months.

### *2.7.3.1 Primary, secondary and tertiary transplants*

8-12 weeks post transplantation, peripheral blood samples were obtained from recipients which were to be kept alive for a further 12 weeks. Alternatively, 8 weeks post-primary transplant, recipients were killed. Single cell suspensions of bone marrow and spleen of each primary recipient were counted, pelleted and resuspended in an appropriate volume (200 $\mu$ l cells in PBS per recipient). The BM and spleen cells were transplanted separately into two sub-lethally irradiated female secondary recipients and assessed after 8 weeks for reconstitution. Samples of bone marrow and spleen cells from each primary recipient were also analysed by flow cytometry or stored at -80°C for molecular analysis at a later date. Tertiary transplants were carried out in the same way 8 weeks post-secondary transplant.

### *2.7.4 Recovery and preparation of tissues from mice for transplantation or analysis*

Mice were killed at appropriate time points and tissues analysed to determine if reconstitution had taken place, to determine the level of chimaerism (donor cell engraftment) and to carry out secondary or tertiary transplantation.

#### *2.7.4.1 Peripheral blood*

For sampling of peripheral blood (PBL) during reconstitution, mice were anaesthetised using halothane and allowed to recover. While unconscious, a few droplets of PBL (from the retro-orbital sinus) were collected into an eppendorf using a tube containing 101U heparin to prevent clotting. Samples were pelleted and stored at -80°C for later analysis of genomic DNA by Y chromosome PCR. In addition, a few droplets were collected into 15ml conical tubes containing 10ml PBS supplemented with 101U heparin. Following removal of red blood cells by hypotonic shock, isolated white cells were subjected to analysis by flow cytometry.

Briefly, to carry out hypotonic shock PBL samples in PBS were centrifuged for 5 minutes at 160xg. The supernatant (containing plasma) was removed by aspiration (as pellets are loose). The pellet was loosened by flicking the tube base. 9ml dH<sub>2</sub>O was added and the sample mixed by inversion for 12 seconds. 1ml 10x PBS was added and mixed by inversion, followed by another centrifuge for 5 minutes at



160xg. The supernatant was removed by aspiration to preserve the loosely packed pellet. This process of hypotonic shock lysed the red blood cells, though the pellet needed to be washed to remove residual red cell “ghosts”. In order to do this, the supernatant was removed by aspiration, the pellet loosened by flicking and 10ml of PBS was added. The suspension was centrifuged again and the supernatant tipped off. The pellet was then loosened and resuspended in 100-500 $\mu$ l. After hypotonic shock, the samples should contain mainly white blood cells. However, some red blood cells would remain and, in order to count the cells, these needed to be removed. Therefore, the cells were counted using white blood cell counting fluid and a haemocytometer, or using a Beckman-Coulter counter.

#### 2.7.4.2 Bone Marrow

Bone marrow was harvested from both femurs. All the instruments used for dissection were sterilised, the freshly killed mouse was placed on its back, swabbed with 70% ethanol and both femurs dissected. The muscle was trimmed away from the femurs and they were immediately placed in a petri dish of PBS. To harvest the bone marrow, the bones were flushed with 500 $\mu$ l to 1ml PBS per femur. The bone was held with forceps and the ends carefully removed with scissors. A 1 or 2ml syringe was filled with PBS and a 25G needle attached, which was inserted into one end of the bone and the PBS was flushed through into an eppendorf (1.5ml) to collect the marrow. A single cell suspension was prepared by gently passing the sample through a series of needles of increasing gauge. For secondary or tertiary transplantation, half the sample was kept for analysis and counting and the other half transplanted.

#### 2.7.4.3 Peritoneal exudate cells (PEC)

To harvest PECs, the mouse was laid on its back and the skin pulled back to expose the peritoneal membrane. A 5ml syringe was filled with PBS and a 21G needle attached, which was inserted into the peritoneal cavity low on the left hand side. The PBS was injected into the cavity, the needle removed and the mouse was gently shaken holding its hind feet. The same syringe and needle was used to recover the PBS from the peritoneal cavity. This wash step was repeated three times in total to

maximise PEC recovery. The cell suspension was transferred to a universal tube and made up to 25ml with PBS. The samples were centrifuged at 160xg for 5 minutes to pellet the cells. The supernatant was discarded and the pellet resuspended in 1ml PBS. The cells were counted with a haemocytometer, no white blood cell counting fluid was necessary unless bleeding had occurred. The cells were either stored as a pellet at -80°C for later molecular analysis or analysed by flow cytometry immediately.

#### *2.7.4.4 Spleen*

Spleens were usually harvested after removal of the femurs to harvest bone marrow. The spleens were removed and homogenised using sterile technique. Using forceps, the spleens were individually transferred to a 5ml loose fitting glass homogeniser. 1ml PBS was added and the glass rod inserted. A gentle force was exerted and the rod turned 3 times to release the pulp from the spleen capsule. The cell suspension was transferred to an eppendorf using a Pasteur pipette. This was left to stand for a few minutes to allow the debris to settle. The supernatant was then decanted to a fresh tube with a Pasteur pipette. The suspension was divided between two eppendorfs, one for analysis and one for secondary transplantation.

## **2.8 Molecular analysis of tissue samples from transplant recipients**

### *2.8.1 Genomic DNA extraction and quantitative real time Y chromosome PCR*

Genomic DNA was prepared from tissues of recipient mice to assess donor cell contribution by Y chromosome PCR. The QIAGEN blood and tissue DNA extraction kit was used to extract genomic DNA, according to manufacturer's instructions. The optical densities of the DNA preparations were determined using an eppendorf BIO photometer or a Nanodrop spectrophotometer to ascertain the DNA concentrations. To detect the Y chromosome present in male donor cells, quantitative real time PCR was performed on an ABI 7500 FAST qPCR machine (Applied Biosystems) using the recommended program for universal conditions.

The following Y chromosome qPCR assay was established and validated for this project (with technical advice from Dr Melany Jackson, JHBL) to facilitate the quantification of male donor cells in female recipient tissues.

The SRY gene is the sex-determining region of Y chromosome and as SRY is a single exon gene, primers against it will detect genomic DNA and transcript. Therefore, the SRY TaqMan® Gene Expression Assay (Assay ID: Mm00441712\_s1) from Applied Biosystems was used to detect the Y chromosome. The PCR reactions were carried out in triplicate using 40ng genomic DNA per reaction and the validated SRY 20xGEX mastermix (containing 900nM forward primer, 900nM reverse primer and 250nM FAM probe).

Beta-actin was chosen as an internal (endogenous) control to detect the amount of DNA loaded per well. Primers for Beta-actin were obtained from MWG and were designed in primer express by Wang and colleagues (Wang et al., 2002, Liver Transplantation): BACTINreverse 5'-CAA GAA GGA AGG CTG GAA AAG A-3' and BACTINforward 5'-ACG GCC AGG TCA TCA CTA TTG-3'. A VIC labelled probe was purchased from Applied Biosystems: BACTIN probe-VIC CAACGAGCGGTTCCGATGCCCT, MGBNFQ (minor groove binder non-fluorescent quencher). The primers and probe were used at concentrations of 900nM forward primer, 900nM reverse primer and 250nM VIC probe. Beta-actin was used as the endogenous control to detect and quantify the DNA loaded in each reaction.

Prior to use with experimental samples, the Y chromosome quantitative PCR strategy was validated with control tissue samples consisting of known ratios of male and female cells. In these control experiments, spleen and bone marrow were harvested from male and female C57/Bl6 control mice, cell suspensions made and counted as described above. A dilution series of male cells in a background of female cells was prepared (1:10, 1:30, 1:1x10<sup>2</sup>, 1:3x10<sup>2</sup>, 1:1x10<sup>3</sup>, 1:3x10<sup>3</sup>, 1:1x10<sup>4</sup>, 1:3x10<sup>4</sup>, 1:1x10<sup>5</sup>, 1:3x10<sup>5</sup> and 1:1x10<sup>6</sup>), each preparation containing a total of 1x10<sup>6</sup> cells. Genomic DNA was extracted from this series of cell preparations using the QIAGEN kit and assessed using the SRY and Beta-actin primers. Using the standard curve method, it

was determined that 1 in  $10^3$  male cells in a female background could be detected. This would represent 0.001% reconstitution of a recipient, making this a highly sensitive method of detecting host-donor chimaerism.

## **2.9 Flow cytometry and fluorescence activated cell sorting (FACS)**

The flow cytometry described in this thesis was carried out with the guidance of Kay Samuel at the Scottish National Blood Transfusion Service, New Royal Infirmary Edinburgh; or using the flow cytometry facilities at the Centre for Inflammation Research, Queen's Medical Research Institute, Edinburgh. A BD FACSCalibur flow cytometer (with 488nm and 633nm lasers, Becton Dickinson) and CellQuest software (Becton Dickinson) were used. Fluorescence activated cell sorting (FACS) was carried out by Dr Martin Waterfall at the Roslin Institute or with Shonna Johnson at the Queen's Medical Research Institute. Cells were sorted with a FACS Vantage SE cell sorter (Beckton Dickinson & co., Mountain View, CA) equipped with a dual output 351nm/488nm laser and a 633nm laser using FACSDiva software (Beckton Dickinson).

### ***2.9.1 Flow cytometry analysis and FACS of ES-derived cells***

Flow cytometry was used to determine the proportion of ES-derived cells in co-cultures and to detect expression of various lineage-specific surface markers. Co-cultured cells were harvested as described above and washed in FACS PBS (highly toxic, PBS containing 0.1% BSA and 0.1% sodium azide). When analysing cells by flow cytometry, approximately  $2 \times 10^5$  cells per FACS tube were incubated for 20 minutes at 4°C with optimal concentrations (titrated) of monoclonal antibodies. Table 2.3 summarises the antibodies used. Samples were quenched in FACS PBS and centrifuged at 160xg for 5 minutes. This wash removed unbound primary antibody. A secondary avidin-phycoerythrin (PE) conjugate was used to detect biotinylated primary antibodies. This was added after the first wash, incubated for 20 minutes at 4°C, washed and centrifuged again. Samples were analysed using a BD FACSCalibur flow cytometer and CellQuest software (Becton Dickinson).

Surface Marker	Conjugate	Cell populations marked	Supplier
cKit	Biotin	Haematopoietic stem cells	Caltag
Sca-1	PE	Haematopoietic stem cells	BD
CD45	APC	Pan-haematopoietic marker	Caltag
Gr-1	APC	Granulocytes (myeloid)	Caltag
CD11b	PE	Macrophages/monocytes (myeloid)	Caltag
CD49d	PE	Alpha-4-integrin, marks haematopoietic and endothelial cells	BD
Ter119	PE	Erythroid cells	BD
B220	PE or APC	B-lymphoid cells	Caltag
CD106	Biotin	VCAM-1, marking endothelial cells	Caltag
CD34	PE or APC	Cord blood HSC, mobilised adult BM derived HSC	Caltag
CD31	PE	PECAM-1 (platelet endothelial cell adhesion molecule). Platelets, endothelial cells.	Caltag
Flk-1	PE	Endothelial cells, haemangioblast	BD
CD54	PE	Intracellular adhesion molecule-1. Resting and activated lymphocytes and monocytes.	BD
CD41	purified	Megakaryocytes, embryonic HSCs	BD
CD150	APC	SLAM receptor. LTR-HSCs	Biolegend
CD48	PE	SLAM receptor. STR-HSCs	BD
CD244	Biotin	SLAM receptor. Haematopoietic progenitor cells.	BD
Avidin	APC	Secondary antibody	Caltag

\*APC = allophycocyanin, PE = phycoerythrin

Table 2.3 Monoclonal antibodies used for flow cytometry analysis or fluorescence activated cell sorting (FACS).

For co-cultured cells, data for  $1 \times 10^5$  live cells were acquired. Control samples included unstained cell samples and cells stained with the second step (avidin-PE) only. For cell sorting experiments, approximately 5 to  $10 \times 10^6$  cells were resuspended in PBS containing 2% FCS. Sorted cells were collected into PBS containing 10% FCS and were used in an experiment immediately.

### ***2.9.2 Flow cytometry analysis of recipient tissue samples – detection of reconstitution***

To assess reconstitution, samples of peripheral blood, bone marrow and spleen were analysed by flow cytometry. Tissue samples were obtained from recipients and single cell suspensions made as described above. Cell suspensions were centrifuged for 5 minutes at 160xg, the supernatant discarded and the pellet loosened by flicking the tube base. The pellet was resuspended in 2ml PBS and counted. For flow cytometry, the cells were resuspended at  $1 \times 10^7$ /ml FACS PBS.

#### ***2.9.2.1 Detection of ES donor cells***

Donor 7a-GFP ES cells would be detected in the FL1 channel. If GFP positive cells were present in haematopoietic tissues, repopulation of the different blood compartments could be assessed by flow cytometry of cells stained with fluorescently labelled monoclonal antibodies against CD45 (a pan-haematopoietic marker), B220 (marking B-lymphocytes), CD4 and CD8 (T-lymphocytes), Gr1 and CD11b (granulocytes, monocytes and macrophages).

#### ***2.9.2.2 Detection of Rosa26 bone marrow donor cells***

Rosa26 mice (and cells) have LacZ (which encodes the enzyme Beta-galactosidase) under the control of the Rosa26 promoter, which is expressed ubiquitously. Beta-galactosidase activity can be measured in Rosa26-derived tissues or in cells transfected with Beta-galactosidase reporter construct. This is done by exposing the cells to fluorescein di-D-galactopyranoside (FDG, Sigma), which is a non-fluorescent substrate that is converted by beta-galactosidase to give a fluorescent product that can be readily detected by flow cytometry.



Stock solutions of 100mM FDG (100mM per  $\mu\text{l}$ ) in DMSO were kept in 5 $\mu\text{l}$  aliquots at  $-20^{\circ}\text{C}$ . In order to obtain a working solution of 2mM FDG, 245 $\mu\text{l}$  distilled water was added to 5 $\mu\text{l}$  of 100mM stock solution (1 in 50 dilution). To assess FDG conversion, cells were pelleted in eppendorfs by centrifugation for 4 minutes at 160xg. Supernatant was poured off and the pellet was resuspended in residual PBS (150 $\mu\text{l}$ ). 40 $\mu\text{l}$  of this cell suspension was aliquotted into two FACS tubes (one for staining and one as an unstained control). These cells were warmed to  $37^{\circ}\text{C}$  in a water bath. 40 $\mu\text{l}$  of water (unstained controls) or 40 $\mu\text{l}$  FDG working solution (2mM in distilled water) was added to each cell suspension. These were incubated for 2 minutes at  $37^{\circ}\text{C}$  in a water bath. Following this, 2ml of ice cold PBS was added to each tube and left on ice for 30 minutes. Cell suspensions were then centrifuged for 5 minutes at 160xg and a Pasteur pipette was used to remove supernatant, leaving 500 $\mu\text{l}$  supernatant in which to resuspend the cells. These suspensions were then analysed by flow cytometry to assess fluorescence present in the FDG treated samples compared to the water controls.

It should be noted that Beta-galactosidase mediates its effects in membranes of cells, so cells with more membranes (e.g. granulocytes have more internal membrane than lymphocytes) would be brighter due to more fluorescent product being released in the same length of time.

## **2.10 Microscopy and Photography**

Cells were routinely inspected and counted using a Leitz Labovert light microscope. A Zeiss Axiovert 25 fluorescence microscope (with a UV light source) was used to analyse and count haematopoietic colonies and to visualise eGFP expression. eGFP excites at 488nm and emits at 509nm and was therefore visualised using the Zeiss 09II filter set (excitation 450-490nm, emission 520nm). Images were captured with an AxioCam digital camera and AxisVision 3.1 software. The white balance of some images was edited using Adobe Photoshop 7.0; in these cases, the adjustments were applied to the entire image.



## **2.11 Statistical analysis**

GraphPad Prism4 software was used to determine whether or not there was a significant difference between data sets. Mann-Whitney U tests and Wilcoxon Signed Rank (paired) tests were used to analyse non-parametric data which did not have a normal distribution, whereas paired or unpaired student's t tests were used to analyse parametric data with normal distribution.

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Chapter Three  
**Effects of stromal cell lines on maintenance of ES  
cells**

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### 3.1 Aims

To determine the effects of stromal cell lines derived from the haematopoietic tissues of mid-gestational mouse embryos on the maintenance and growth rates of ES cells.

### 3.2 Introduction

A novel haematopoietic differentiation system has been established whereby embryoid bodies (EBs) are co-cultured in contact with stromal cell lines derived from mid-gestational mouse haematopoietic niches (see Chapter 2, Figure 2.1 and Table 2.1). The stromal cell lines were derived by Elaine Dzierzak and colleagues from transgenic mice carrying the temperature sensitive SV40 large T antigen (Tag) immortalising transgene (*tsA58*), which was expressed under the control of Beta-actin or PGK promoters. A large panel (~100) of clonal stromal cell lines was derived from foetal livers and AGM subregions of embryos (E10-11). Oostendorp and others screened the stroma for their capacity to maintain/expand mouse bone marrow LTR-HSCs and CFUs (Oostendorp et al., 2002a, 2002b, 2005). Briefly, the AM20.1B4 and AM20.1A4 stromal cell lines were derived from the aorta and surrounding mesenchyme (AM subregion) of AGMs from E10 transgenic embryos. AM14.1C4 was derived from E11 AM subregions. UG26.1B6 and UG26.2D3 were derived from urogenital ridge (UG) subregions of E11 embryos. EL08.1D2 was derived from E11 foetal liver. In the experiments described in this thesis, mouse EBs were co-cultured with these stromal cell lines to determine whether they can promote haematopoietic differentiation (Chapter 4). Oostendorp and colleagues (2002b) reported that one of the stromal lines used here (UG26.1B6) expresses low levels of LIF transcript; therefore, it was possible that stromal co-culture might inhibit differentiation of ES cells. In light of this observation, it was considered necessary to first determine the effects of the stromal cell lines on basic aspects of ES cell biology; such as self-renewal and growth rates.

### 3.3 Experimental approach

- Determine whether the growth rates of the differentiating ES cells under the different co-culture conditions were statistically comparable.
- Determine whether ES cells were maintained in their undifferentiated state in co-cultures by carrying out self-renewal assays and by assessing secondary EB formation in colony assays.

### 3.4 Results

#### *3.4.1 There were no significant differences in the growth rates of ES cells in co-culture with different stromal cell lines*

7a-GFP ES cells were prepared in hanging drops in the presence of LIF to form EBs. These were harvested 2 days later and placed into suspension cultures in petri dishes, at which time LIF was withdrawn to initiate differentiation. The EBs were termed day 0, marking the time after LIF withdrawal. After 1 day of differentiation in suspension, EBs were co-cultured on irradiated stromal cell layers in 25cm<sup>2</sup> flasks in the absence of exogenous LIF. At defined time points (2, 4, 6 and 10 days of differentiation) whole co-cultures were harvested and counted. The 7a-GFP ES cells used here constitutively expressed eGFP (Gilchrist et al., 2003); therefore, the proportion of ES cells present in each sample was determined by flow cytometry, as shown in Figure 3.1. The rate of growth of the ES-derived cells was determined by normalising the cell counts according to the proportion of ES cells present in co-cultures at each time point (Figure 3.2). Using this strategy, it was found that there was no significant difference between the growth rates of ES cells in the AM20.1B4, UG26.1B6 and EL08.1D2 co-cultures as compared to the control, where EBs were cultured on gelatin alone (-LIF) ( $p=0.75$ ).

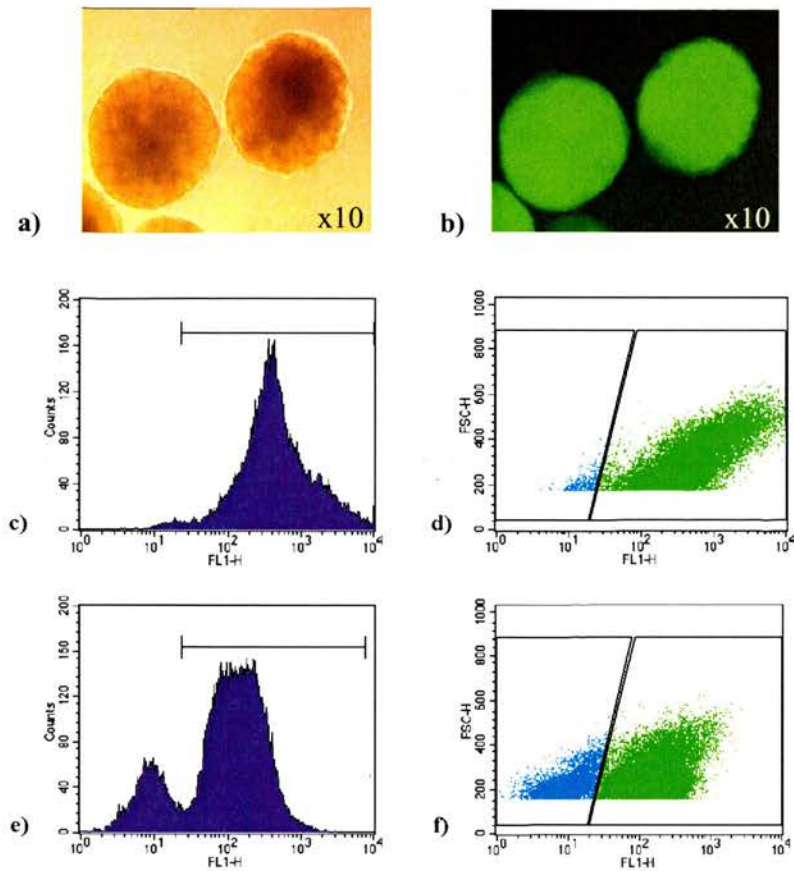


Figure 3.1 Co-culture of 7a-GFP hanging drop EBs on stromal layers. (a) Light and (b) fluorescence microscopy of 4 day 7a-GFP EBs in suspension. (c, d) Flow cytometric analysis of 6 day 7a-GFP EBs cultured on gelatin. (e, f) 6 day 7a-GFP EBs on unstained AM20.1B4 stromal cells. The proportion of GFP+ ES cells present in the co-cultures could be readily determined.

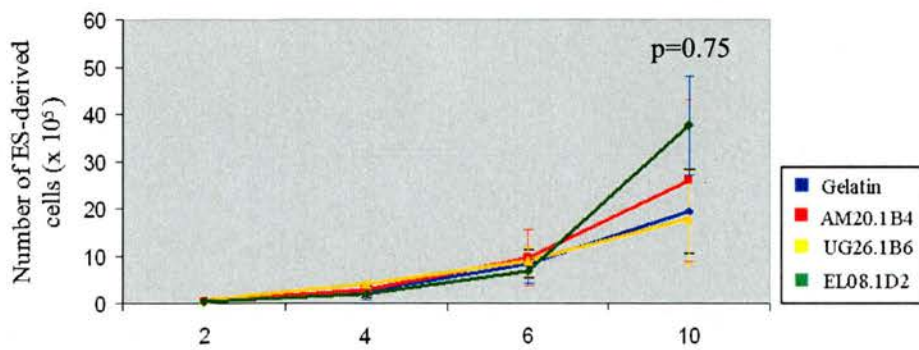


Figure 3.2 Growth rates of ES cells differentiated in co-culture. There were no significant differences in the growth rates of 7a-GFP EB cells co-cultured on AM20.1B4, UG26.1B6, EL08.1D2 stromal cells or on gelatin (4 independent experiments,  $p=0.75$ . Data analysed using a paired non-parametric one way ANOVA or Friedman test).

### ***3.4.2 Stromal cells do not maintain ES cells in their undifferentiated state during co-culture***

Oostendorp and colleagues (2002b) reported that the UG26.1B6 stromal cell line expresses low levels of LIF transcript. In their study, AM14.1C4, UG26.2D3 and EL08.1D2 did not express LIF transcripts, but the data are unavailable for the AM20.1B4 and AM20.1A4 stromal cell lines. Since the presence of LIF could inhibit ES cell differentiation, it was determined whether the stromal lines had the capacity to maintain ES cells in their undifferentiated state.

#### Self-renewal assays:

In order to quantify the numbers of undifferentiated ES cells present in co-cultures, the cells were subjected to self-renewal assays. One day old hanging drop 7a-GFP EBs were co-cultured on irradiated stromal cell layers for up to 6 days of differentiation in the absence of exogenous LIF. At defined time points during differentiation, co-cultures were disaggregated to single cell suspensions and samples of cells seeded into self-renewal assays (Figure 3.3). Cells were plated at low densities onto gelatin in the presence of optimal concentrations of LIF (100U). After 5 days incubation, resultant colonies were fixed and stained for alkaline phosphatase activity. In the presence of optimal concentrations of LIF, undifferentiated ES cells respond by forming tight ES colonies, which are positive for alkaline phosphatase activity. By contrast, when differentiated cells are seeded into the self-renewal assay, they form colonies that are alkaline phosphatase negative (images in Chapter 2, Figure 2.4)(Berstine et al., 1973; Wobus et al., 1984). Self-renewal assays were carried out for EBs co-cultured on AM20.1B4, UG26.1B6, EL08.1D2 and gelatin for 2, 4 and 6 days differentiation (Figure 3.4). Assays carried out at 8 and 10 days gave results comparable to those at 6 days (data not shown). The numbers of undifferentiated ES cells, represented by mixed and stem cell colonies, were greatly reduced by 4 days of differentiation and this was maintained for up to 10 days (Figures 3.4 b and c, respectively). Therefore, AM20.1B4, UG26.1B6 and EL08.1D2 stromal cell lines did not maintain ES cells in their undifferentiated state during co-culture, suggesting that LIF was not expressed at a level which could inhibit differentiation.



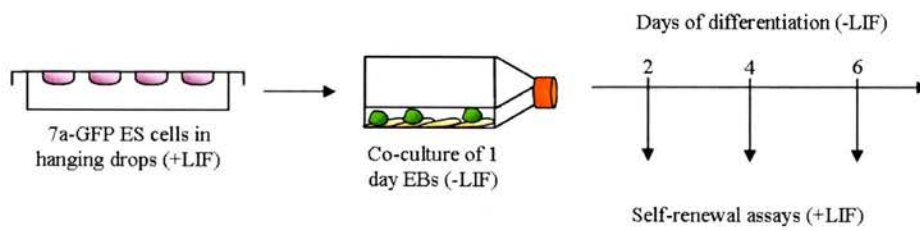


Figure 3.3 Testing whether ES cells are maintained in their undifferentiated state in co-cultures.

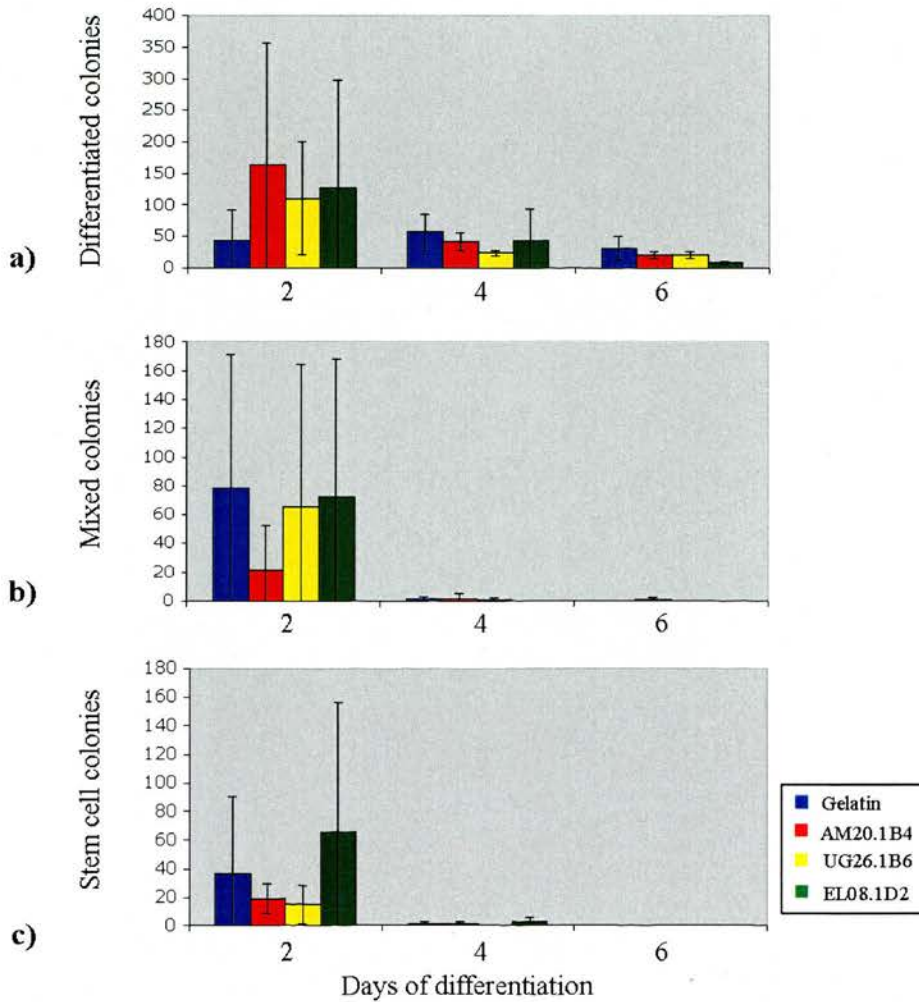


Figure 3.4 Self-renewal assays setup with co-cultured cells after 2, 4 and 6 days of differentiation. The numbers of (a) differentiated, (b) mixed and (c) stem cell colonies obtained per  $1 \times 10^3$  input ES-derived cells, in the presence of 100U LIF. Colony numbers were normalised according to the proportion of 7a-GFP ES cells seeded. Data represent 3 independent co-culture experiments. Friedman tests showed there were no significant differences in the colony numbers obtained from the gelatin, AM20.1B4, UG26.1B6 and EL08.1D2 co-culture conditions at any of the time points analysed ( $p > 0.05$ ).

The decrease in stem cell and mixed colonies was concomitant with the generation of a relatively constant number of differentiated colonies (Figure 3.4 a). There was no significant difference between the number of differentiated colonies under the various co-culture conditions at any of the time points tested ( $p>0.05$ ). Between days 4 and 10 of differentiation, a range of 25 to 100 differentiated colonies was obtained from all the co-cultures; indicating that the stromal lines tested here (and gelatin) permitted survival of differentiated cells that are detectable in self-renewal assays (in the presence of 100U LIF), but that there was no increase in the numbers of these differentiated cell types.

#### Methylcellulose-based colony assays:

In the absence of LIF, undifferentiated ES cells seeded directly into semi-solid medium (such as methylcellulose supplemented with cytokines) form aggregates or secondary EBs, which then undergo differentiation (Keller et al., 1993; Dang et al., 2002). The methylcellulose colony assays used here were designed to retrospectively measure the frequency of haematopoietic progenitor cells (HPCs) or colony forming units (CFU) present in a test cell population. Though, seeding increasing numbers of undifferentiated ES cells in the assay was found to correlate with an increase in the number of secondary EBs (data not shown). When EBs which were co-cultured with stromal layers to 6 days of differentiation were subjected to the haematopoietic colony assays, colonies representing HPCs as well as some secondary EBs were observed. As shown in Figure 3.5, the secondary EBs were morphologically distinct from HPC-derived colonies; though, some of the secondary EBs did appear to be undergoing haematopoietic differentiation, seen as a halo of myeloid or erythroid cells emanating from the EB. It was possible that the secondary EBs represented undifferentiated ES cells present in the co-cultures at the time of assay setup, even though the ES cells had undergone 6 days differentiation in the absence of exogenous LIF and the colony assay did not contain LIF. In order to test this, secondary EBs were picked directly from the assay dishes and analysed by flow cytometry for expression of SSEA1 and SSEA4 surface antigens. SSEA1 (stage-specific embryonic antigen 1) is a carbohydrate antigen which is strongly expressed by cells of the murine inner cell mass (ICM) (Solter and Knowles, 1978).

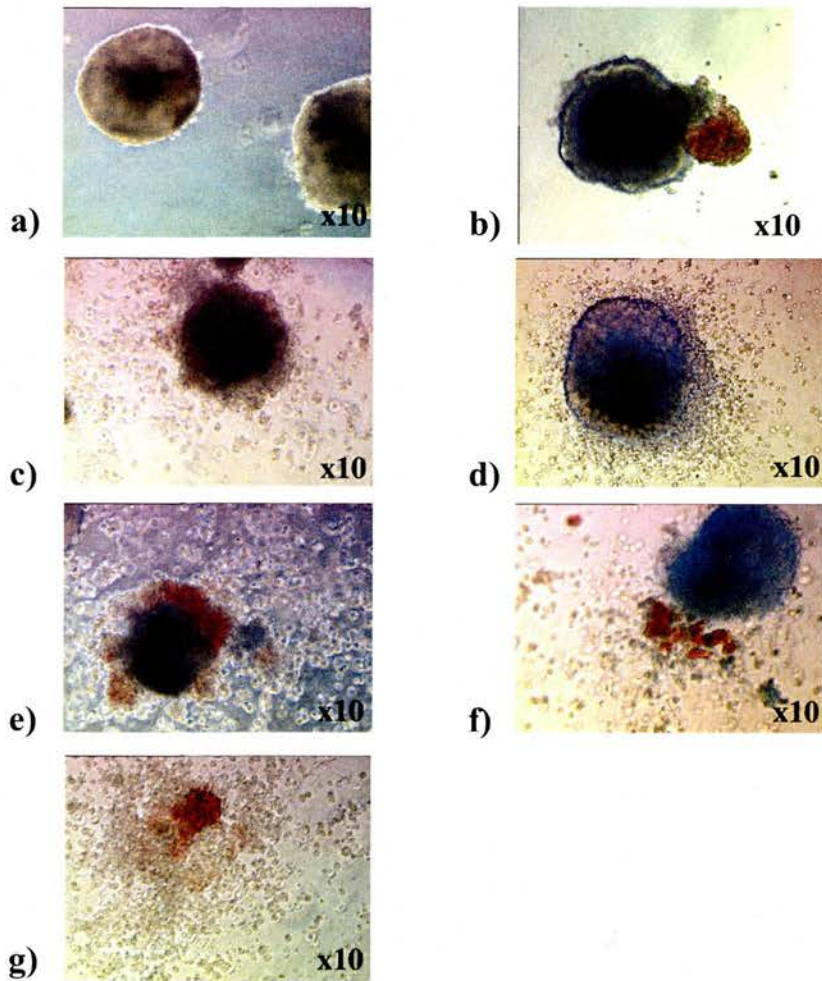


Figure 3.5 Undifferentiated ES cells formed aggregates (secondary EBs) in the colony assays. These ES cells appeared to respond to cytokines in the assay by undergoing haematopoietic differentiation, resulting in generation of erythrocytes and a halo of myeloid cells emanating from the secondary EB. (a) Secondary EBs, (b) secondary EBs containing erythrocytes, (c and d) secondary EBs with a myeloid burst, (e and f) secondary EBs containing erythroid and myeloid cells, (g) a non-secondary EB CFU-Mix colony for comparison.



By contrast, SSEA4 is expressed by cells of early cleavage mouse embryos, but is not expressed in the ICM (Kannagi et al., 1983). In culture, SSEA1 is known to be expressed on the surface of undifferentiated mouse ES cells and differentiation is characterised by loss of SSEA1 expression and may be accompanied by the appearance of SSEA4 (Henderson et al., 2002; Solter and Knowles, 1978). It was found that 28.4% of cells within secondary EBs expressed the undifferentiated mouse ES cell marker SSEA1 and 9% of cells expressed the differentiated cell marker SSEA4 (Figure 3.6), confirming that secondary EBs generated in the haematopoietic colony assays indeed consisted of undifferentiated ES cells.

In light of these results, the frequency of secondary EBs in the colony assays was used to measure the numbers of undifferentiated ES cells present in co-cultures. Colony assays were setup with EBs co-cultured with stroma for 6 days of differentiation (Figure 3.7). UG26.2D3 co-cultures had reduced numbers of secondary EBs compared to gelatin ( $p=0.02$ ), but there was no significant difference in numbers of secondary EBs formed in assays setup with cells from the other co-cultures ( $p$ -values ranged from 0.2 to 1.0). These data demonstrate that none of the stromal cell lines used in this project, namely AM20.1B4, AM20.1A4, AM14.1C4, UG26.1B6, UG26.2D3, EL08.1D2 and OP9, maintain ES cells in their undifferentiated state.

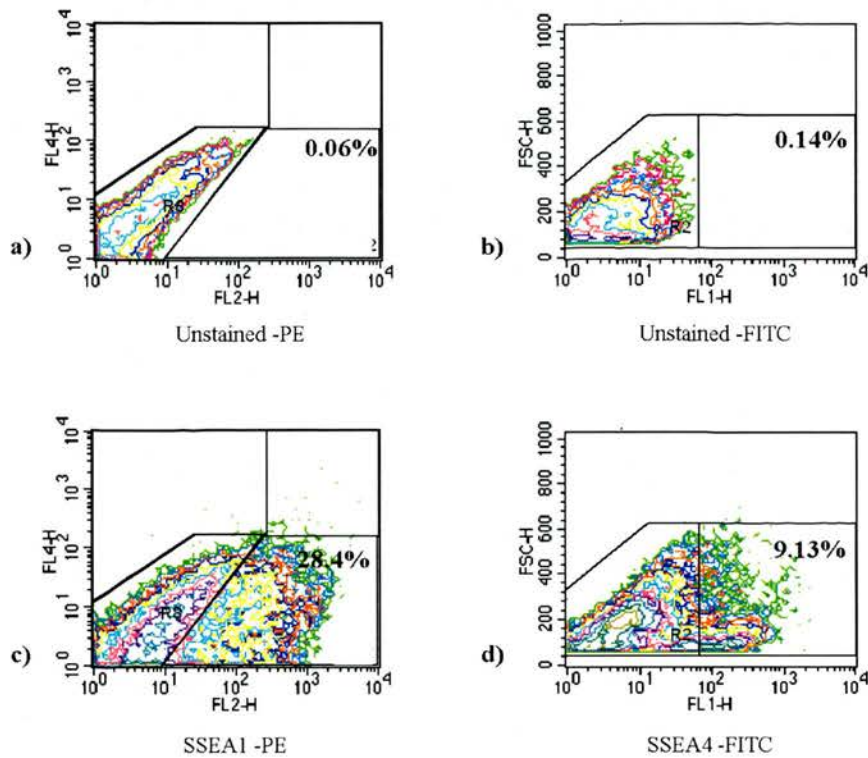


Figure 3.6 Flow cytometry showing SSEA1 and SSEA4 expression in secondary EBs that were picked from colony assays. Unstained cell controls for (a) PE and (b) FITC channels are shown. (c) SSEA1 marking undifferentiated mouse ES cells. (d) SSEA4, a marker of differentiated cells. Colony assays were initially set up with disaggregated 6 day old EBs that had been differentiated in co-culture and secondary EBs were picked 10 days after assay setup.

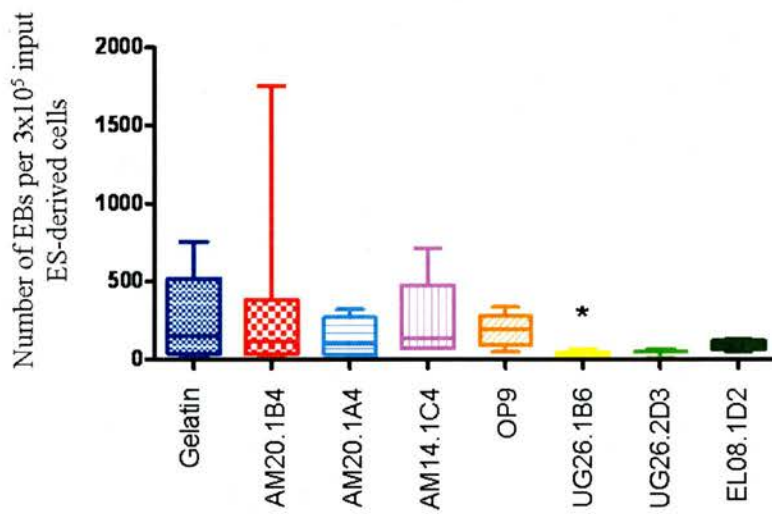


Figure 3.7 Frequency of secondary EBs generated in the haematopoietic colony assays. 1 day 7a-GFP EBs were co-cultured on gelatin or stromal cell layers to 6 days differentiation and disaggregated for analysis. Data represent 5 to 11 independent experiments. p-values ranged from 0.2 to 1.0 when stromal co-cultures were compared to gelatin controls. Except for UG26.1B6, which had reduced numbers of secondary EBs compared to gelatin (p=0.02)(Mann Whitney U tests).



### 3.5 Discussion

The aim of these experiments was to determine the effects of stromal co-culture on basic aspects of ES cell biology, such as ES cell growth rates and self-renewal. The UG26.1B6 stromal cell line has been reported to express low levels of LIF transcript (Oostendorp et al., 2002b). However, self-renewal assays demonstrated that in the absence of exogenous LIF, ES cells co-cultured on this stromal line were not maintained in their undifferentiated state (Figure 3.4). This suggested that the levels of LIF expressed are too low to sustain self-renewal of undifferentiated ES cells. The results of self-renewal assays were confirmed by measuring the frequency of secondary EBs generated in the colony assays setup with 6 day co-cultured cells (Figure 3.7). None of the co-cultures were found to maintain ES cells in their undifferentiated state; therefore, it is unlikely that any of the stromal cell lines used in this study express high levels of LIF.

The haematopoietic colony assays used here were designed to measure the frequency of ES cell -derived HPCs generated in co-culture. When considering haematopoietic colony assay data, it is important to bear in mind that undifferentiated ES cells may respond to cytokines in the colony assays by undergoing haematopoietic differentiation and therefore could contribute to the haematopoietic readout. Thus, even though undifferentiated ES cells were not maintained by stromal culture (Figures 3.4), subtle differences in the numbers of undifferentiated ES cells persisting in co-cultures could skew the colony assay readout. It was found that undifferentiated ES cells contributed to haematopoietic colony assays in the form of secondary EBs. These contained a high proportion (28.4%) of cells expressing SSEA1, a marker of undifferentiated mouse ES cells. These secondary EBs were morphologically distinct from colonies originating from HPCs, and could be readily distinguished by light microscopy, even when they appeared to be undergoing haematopoietic differentiation. This feature made it possible to use the numbers of secondary EBs to measure undifferentiated ES cells present in the co-cultures; and importantly, this also enabled the secondary EBs to be excluded from the colony readout when assessing the HPC-derived haematopoietic activity in co-cultures. This

precluded the possibility of undifferentiated ES cells skewing the haematopoietic colony readout.

It was determined that none of the stromal lines enhanced the growth rates of the ES-derived cells over that of gelatin control cultures (Figure 3.2). Thus, there was no difference in differentiated ES cell output from the different co-cultures. Importantly, these data also serve to validate the interpretation of subsequent haematopoietic colony assay data. To illustrate, when co-cultured cells were analysed by colony assay, the cell suspensions consisted of ES-derived cells as well as irradiated stromal cells. Even though equal numbers of co-cultured cells were seeded into each assay dish, it was possible that a difference in differentiated ES cell output from the co-cultures (i.e. growth rates) could lead to a difference in the number of ES-derived cells seeded into the colony assays. This could in turn bias a particular co-culture towards a higher CFU readout. However, the growth rate data demonstrate that co-cultures with different stromal lines generated comparable numbers of ES-derived cells and therefore it is unlikely that there was any bias towards enhanced colony readout dependent upon the stromal cell line. Even so, a labelling strategy was employed, whereby fluorescently labelled ES cells were used to allow normalisation of colony data (in retrospect) according to the proportion of ES-derived cells seeded into each assay. In this way, the colony readout of the different co-cultures could be directly compared by expressing the data in terms of the frequency of CFU obtained from a defined number of input ES-derived cells.

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Chapter Four  
**Effects of stromal cell lines on haematopoietic  
differentiation of ES cells**

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## 4.1 Aim

To investigate whether haematopoietic differentiation of mouse ES cells can be enhanced by co-culture with AGM-derived clonal stromal cell lines.

## 4.2 Introduction

Previous work in the John Hughes Bennett Laboratory (JHBL) demonstrated that co-culture of EBs with primary E10.5 AGM explants significantly increased ES cell derived haematopoietic activity (Krassowska, Gordon-Keylock et al., 2006). After 6 days of differentiation, a 20-fold increase in CFU-A and a 50-fold increase in HPP-CFC frequencies were observed, as compared with that seen in the absence of AGM explants. The experiments presented in this chapter aimed to determine whether clonal stromal cell lines derived from AM or UG subregions of the E10-11 AGM region retained the enhancing capacity of primary AGM explants. It was hypothesised that this might provide insight into which subregion(s) of the AGM mediated the enhancement of haematopoiesis. Oostendorp and colleagues found that UG26.1B6, as well as an E11 foetal liver derived line, EL08.1D2, provided potent support to adult mouse bone marrow LTR-HSCs (Oostendorp et al., 2002a, 2002b, 2005). Therefore, it was of particular interest to test the effects of these two stromal lines on differentiating ES cells. The stromal co-culture system also offered a number of advantages over the use of AGM explants; for instance, the stromal cell lines would be readily available and amenable to large-scale culture. Furthermore, a simplified co-culture system would be more likely to yield reproducible results.

In order to determine whether the efficiency of the novel stromal co-culture system compared favourably with other culture methods, the OP9 stromal cell line was included in the study as a positive control. The only way to unequivocally demonstrate that definitive LTR-HSCs are present in a cell population is by transplanting the cells into immuno-compromised adult recipients and demonstrating that donor cells can achieve long-term reconstitution of the recipient haematopoietic system (>6 months). However, prohibitive numbers of recipients would have been

required to assess all the co-cultures by *in vivo* repopulating assays. Therefore, the stromal cell lines were screened in the first instance for haematopoietic enhancing activity by a number of *in vitro* methods; namely, haematopoietic colony assay, flow cytometry and reverse transcriptase (RT) quantitative PCR. Haematopoietic colony assays, such as CFU-A, CFU-Mix, CFU-GM, CFU-M and Ery/Mac, should reflect the ability of cultures to induce and/or maintain multipotent HPCs. Flow cytometry and RT-PCR were used to determine whether cells differentiated in co-culture display the surface phenotype and molecular characteristics of definitive haematopoietic cell types.

### 4.3 Experimental approach

- Validate the use of colony assays as an *in vitro* measure of ES cell-derived haematopoietic progenitors. To confirm that colonies consisted of haematopoietic cells types, individual colonies were picked and the presence of haematopoietic lineage cells confirmed by their morphology and surface phenotype analysed by microscopy and flow cytometry, respectively.
- 1 day old hanging drop 7a-GFP EBs were co-cultured on confluent irradiated stromal cell layers and assessed at various time points for haematopoietic activity using haematopoietic colony assays.
- The enhancing effects of co-culture were confirmed by flow cytometric analysis to detect expression of markers associated with terminally differentiated haematopoietic cells of myeloid, lymphoid and erythroid lineages.
- Flow cytometry was used to detect ES-derived cells expressing combinations of surface markers which are known to be associated with adult bone marrow HSCs or embryo-derived HSCs.
- Co-cultured ES cells were analysed by quantitative RT-PCR for expression of gene transcripts associated with definitive haematopoietic cells.

## 4.4 Results

### 4.4.1 Colonies in the assays consist of haematopoietic cells

Haematopoietic colony assays used semi-solid medium (methylcellulose), supplemented with a number of haematopoietic cytokines (including IL3, IL6, SCF and Epo). These were seeded with single cell suspensions of recovered EB/stromal co-cultured cells. Haematopoietic progenitor cells (HPCs) respond to the assay conditions by forming morphologically identifiable colonies such as CFU-Mix, CFU-GM and CFU-M, which can be readily distinguished by light microscopy. Examples of ES-derived HPC colonies and their categorisation are provided in Figure 4.1. Each colony is believed to originate from a single progenitor (i.e. colony forming unit, CFU) present in the test cell suspension; thus, the frequency of ES-derived HPCs in co-cultures could be determined in retrospect. To confirm that these colonies comprised of haematopoietic cells, cytopins of individual colonies picked directly from the assay dishes were prepared, fixed and subsequently stained for H and E (using a quick-DIFF kit) to allow visualisation of intracellular components. The morphology and staining of the cells was consistent with haematopoietic cell types. Figure 4.2 shows a single CFU-Mix colony which consisted of cells with granulocytic, erythroid, macrophage and megakaryocytic morphologies.

Colonies picked from the assay dishes (CFU-Mix, CFU-GM, CFU-M and Ery/mac) were pooled for flow cytometric analysis to detect expression of surface markers associated with undifferentiated ES cells and haematopoietic cell types. It was found that these cells did not express SSEA1, a surface marker of undifferentiated mouse ES cells (Figure 4.3) (Solter and Knowles, 1978; Henderson et al., 2002). Therefore, it is unlikely that the colonies originated from undifferentiated ES cells which may have persisted during co-culture and would therefore have also been taken forward into the colony assay. Only a small proportion (1.42%) of the cells within the colonies expressed SSEA4, a marker of differentiated mouse ES cells (Figure 4.3). This was probably due to the length of time in co-culture (6 days) and colony assay (10 days), as SSEA4 can be down-regulated as cells terminally differentiate.



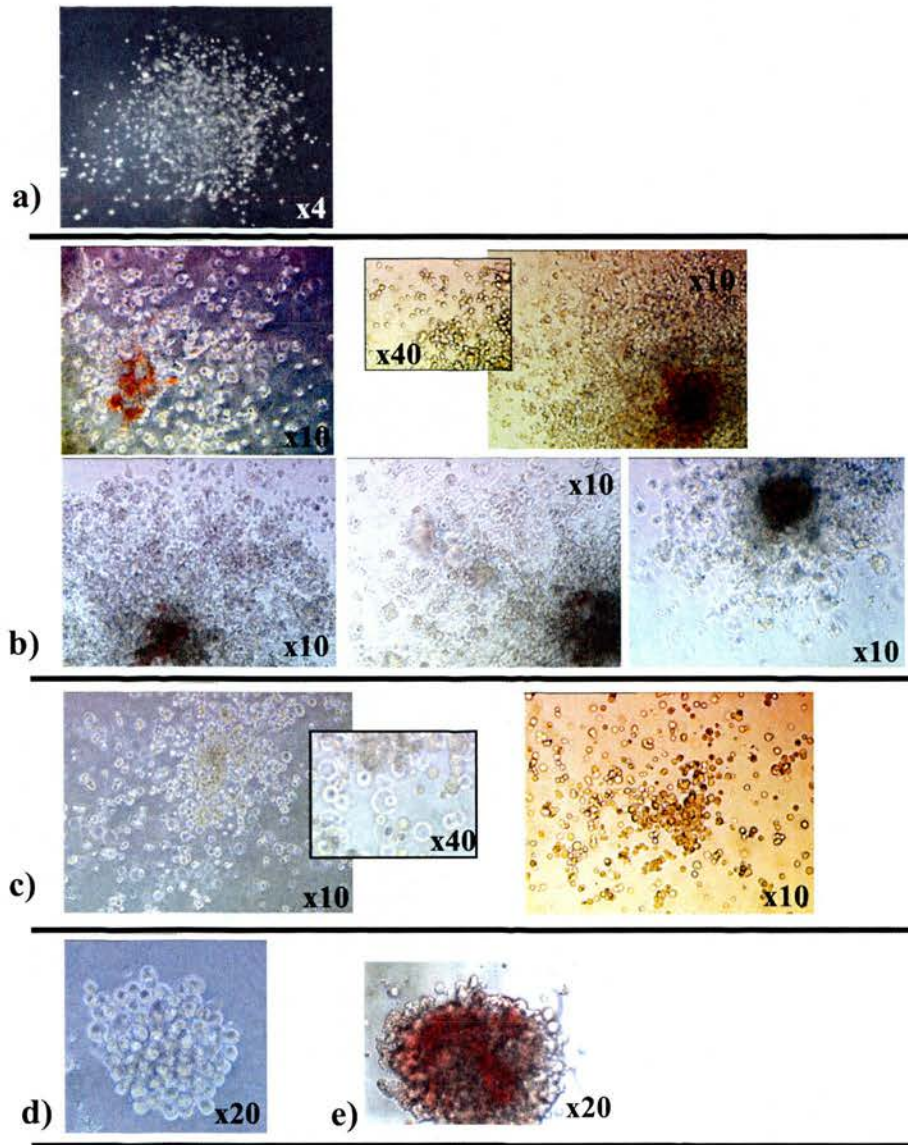


Figure 4.1 Examples of colonies observed in the haematopoietic colony assays and their categorisation. Colony assays were initially set up with disaggregated 6 day old EBs that had been differentiated in co-culture. (a) CFU-A colony containing myeloid cells, (b) CFU-Mix colonies contained erythrocytes and at least two types of white cell, (c) CFU-GM contained at least two types of white cell and no erythrocytes, (d) CFU-M consisted of macrophages and (e) erythroid/macrophage colony.



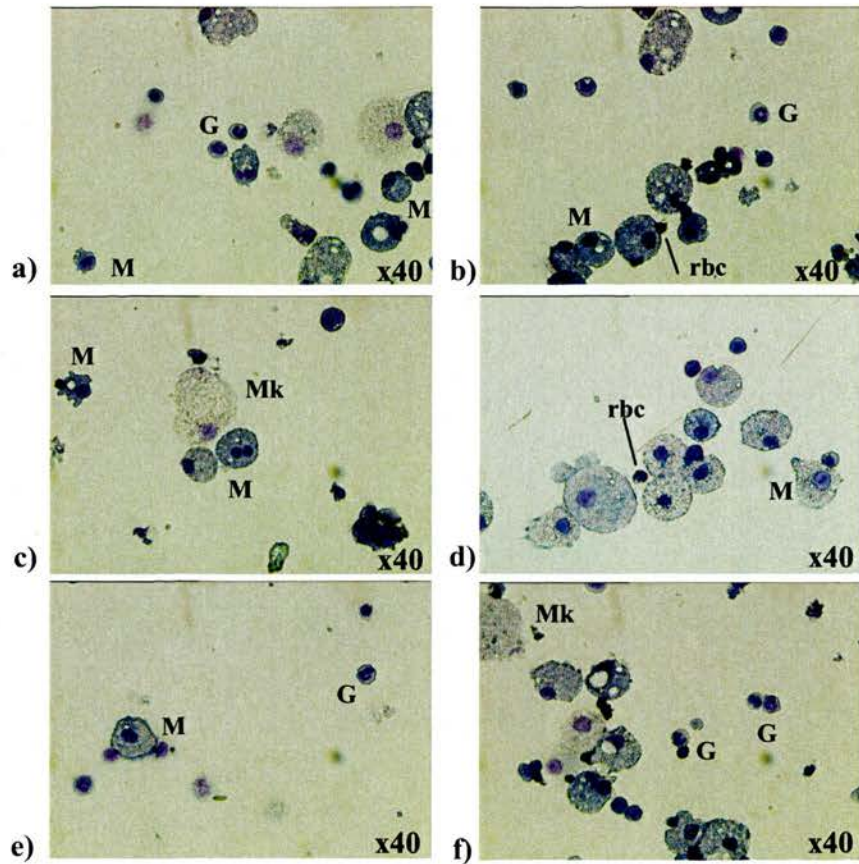


Figure 4.2 Cytospin analysis of colonies picked from methylcellulose-based assays. (a) to (f) represent cells from a single CFU-Mix colony, which was cytocentrifuged and stained with H&E to visualise intracellular components. CFU-Mix colonies were found to contain cells with macrophage morphology (M), as well as granulocytes (G), megakaryocytes (Mk) and erythrocytes (rbc).

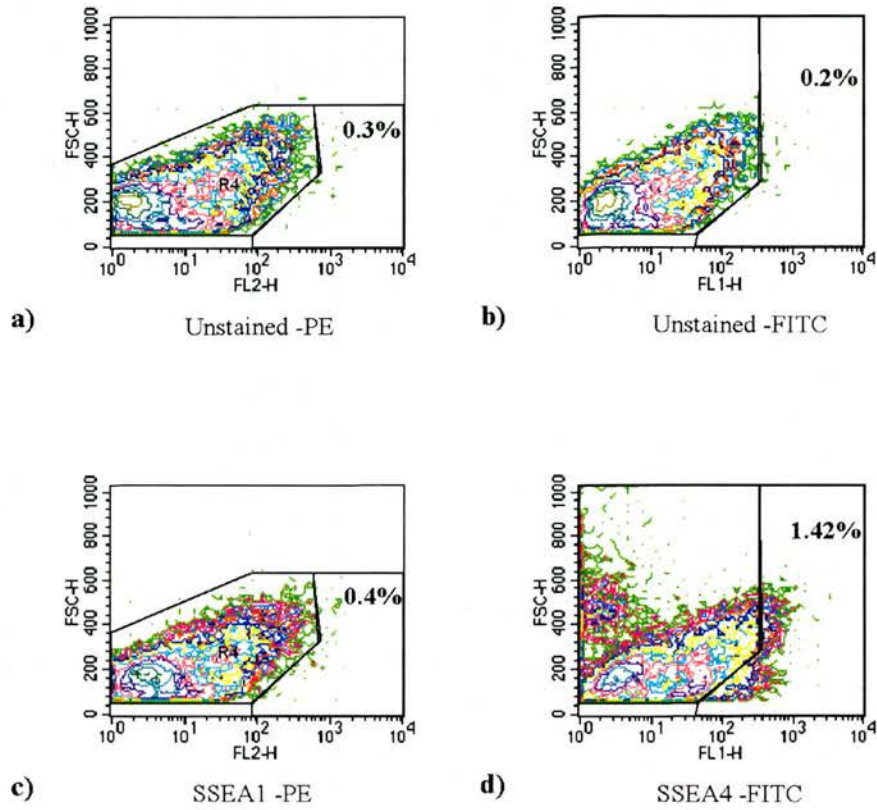


Figure 4.3 Flow cytometry analysis of haematopoietic colonies that were picked from methylcellulose-based assays. CFU-Mix, CFU-GM, CFU-M and Ery/Mac colonies were pooled for analysis. (a) Unstained cells in PE channel, (b) unstained control for FITC channel. (c) SSEA1-PE stained cells, (d) SSEA4-FITC stained cells.

Further flow cytometry analysis confirmed that haematopoietic surface markers of myeloid, erythroid and lymphoid lineages were expressed by cells within the colonies (Figure 4.4). The plots in Figure 4.4 show that CD11b (Mac-1) marked 34% of cells. This antigen is expressed by cells of the macrophage/monocytic lineage (Springer et al., 1979). 7.2% of cells expressed Gr1, which is a granulocytic marker. These data demonstrate that myeloid cells were represented in the colonies. Erythroid cells were also present in the colonies, as Ter119 was expressed on 5.4% of the cells. Ter119 antibody marks mouse erythroid cells from early pro-erythroblast to mature erythrocyte stages, but is not expressed by cells with BFU-E or CFU-E activities (Kina et al., 2000). A small proportion (2%) of B220 positive cells was also detected in the picked colonies. B220 is expressed on resting as well as activated B cells and pro-B cells (Hardy et al., 1991) and is used as a lineage marker for B lymphocytes. It has been noted that these markers only represent 48.6% of cells within the colonies. It is possible that making use of other haematopoietic markers, such as cKit, Sca-1 or CD45, could account for more haematopoietic cell types present in the colonies.

Taken together, these data confirmed that the colonies scored according to their gross morphology indeed consisted of haematopoietic cells and that they were likely to represent HPCs which were present in the co-culture cell suspension at the time of assay setup. In other experiments, it was found that primary adult bone marrow cells formed colonies in this methylcellulose-based assay (data not shown), confirming that this assay is capable of detecting definitive adult haematopoietic cell types.

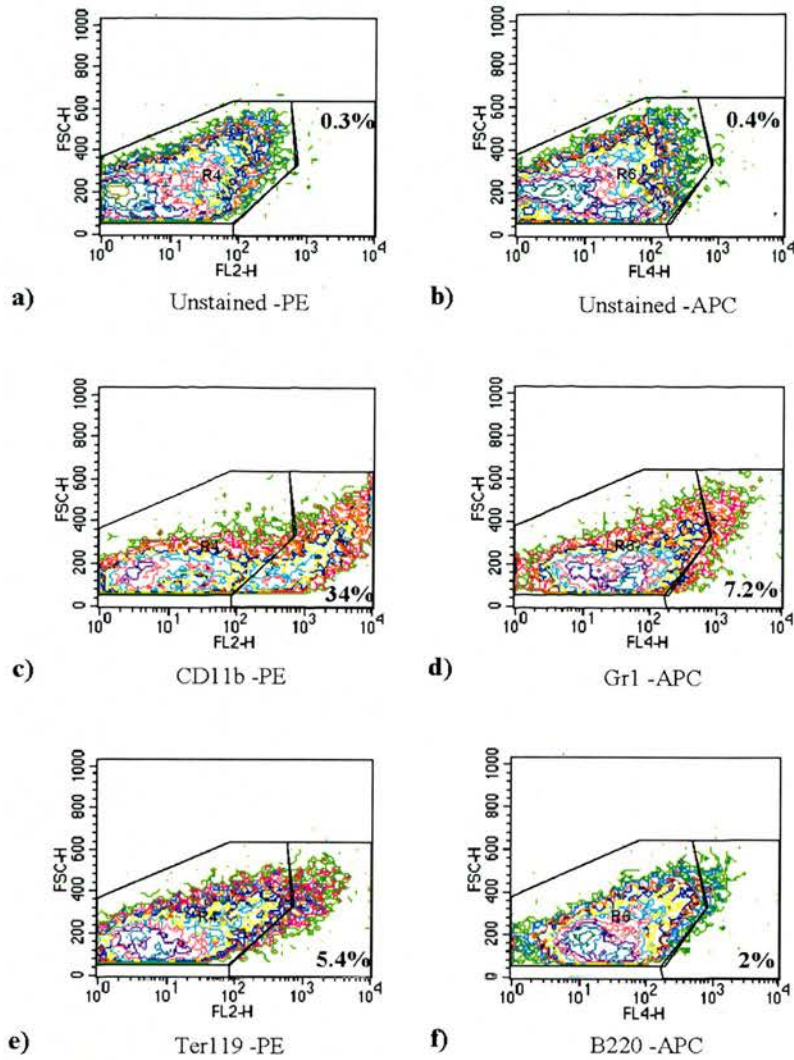


Figure 4.4 Flow cytometry analysis showing expression of haematopoietic surface markers in haematopoietic colonies that were picked from methylcellulose-based assays. Colonies were pooled for analysis. The unstained controls for the PE and APC channels are shown in (a) and (b), respectively. Haematopoietic surface markers such as (c) CD11b, (d) Gr1, (e) Ter119 and (f) B220 were expressed on cells in the colonies.



***4.4.2 The haematopoietic enhancing activity of primary AGM co-culture is partially retained by a stromal cell line derived from the AM subregion of the AGM***

One day old 7a-GFP EBs that were prepared in hanging drops were co-cultured on confluent irradiated stromal cell layers in 25cm<sup>2</sup> flasks. AM20.1B4, derived from the AM subregion of the AGM was used and this was compared with UG26.1B6 and EL08.1D2, which are reported to be highly supportive to adult mouse bone marrow LTR-HSCs (Oostendorp et al., 2002b). Co-cultured cells were harvested at defined time points, assessed by colony assays and haematopoietic CFU were categorised as shown in Figure 4.1. Even though the growth rates of ES cells in the co-cultures were statistically comparable (Chapter 3, Figure 3.2), it was possible that small differences in ES-derived cell numbers could bias the colony readout. In order to circumvent this, 7a-GFP ES cells were used to enable colony numbers to be normalised in retrospect according to the proportion of ES-derived (GFP<sup>+</sup>) cells present in the cell suspensions seeded into each assay. This strategy facilitated the direct comparison of different co-cultures, as the colony data could be expressed in terms of the frequency of CFU observed for a defined number of input ES-derived cells.

Co-cultures were assessed by CFU-A colony assay after 4, 6 and 10 days differentiation (Figures 4.5). EB/AM20.1B4 co-culture resulted in significantly enhanced CFU-A activity compared with EBs differentiated on gelatin (p=0.001 at 4 days and p=0.0009 at 6 days). By 10 days of differentiation, CFU-A activity in AM20.1B4 co-cultures was reduced such that they were not significantly different from 10 day gelatin controls (p=0.17). Krassowska et al (2006) reported that co-culture of EBs with primary E10.5 AGM explants resulted in an average of 148 CFU-A per 3x10<sup>4</sup> input ES-derived cells at 6 days differentiation, representing a 20 fold increase in CFU-A progenitors compared to control EBs, which were cultured alone at the air-surface interface. By contrast, at 6 days in EB/AM20.1B4 co-cultures there was only an average of 3 fold increase in CFU-A progenitors compared to EBs differentiated on gelatin (Figure 4.5).

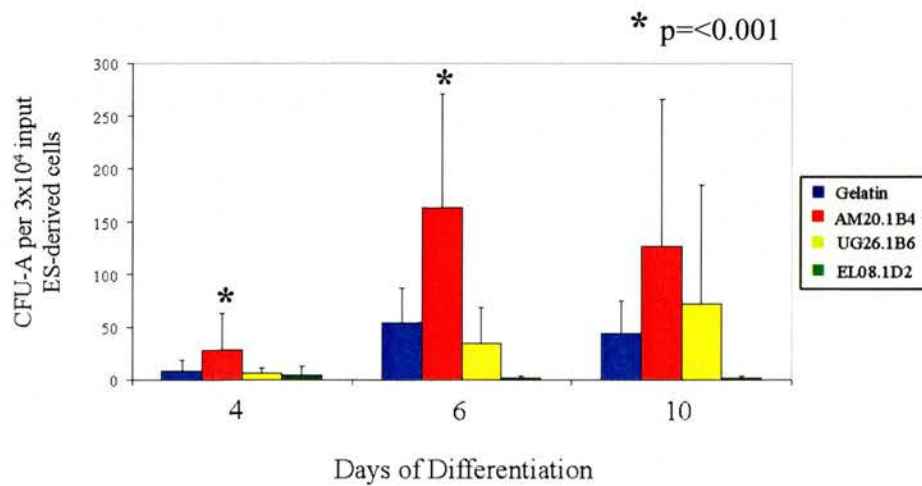


Figure 4.5 CFU-A colony assays of AM20.1B4, UG26.1B6, EL08.1D2 and gelatin/ EB co-cultures at 4, 6 and 10 days differentiation. Data represent 5 independent co-culture experiments, which were normalised according to the proportion of ES cells seeded into the assays. Paired Mann Whitney U tests were used to calculate p-values comparing stromal co-cultures to gelatin controls.



The enhancing capacity of the primary AGM region appeared to be only partially retained by the AM20.1B4 stromal cell line. However, in EB/AM20.1B4 co-cultures  $163 \pm 108$  CFU-A were generated per  $3 \times 10^4$  ES cells, demonstrating that the frequency of CFU-A progenitors was broadly comparable with that of primary AGM explant co-cultures. These data probably reflect the differences between the two co-culture systems. For example, the primary AGM/EB co-cultures were carried out at the air-surface interface and the AGM tissue was not irradiated. While, in the stromal cell co-culture system EBs were differentiated on confluent irradiated stromal layers in  $25\text{cm}^2$  flasks and were fully submerged in medium. Furthermore, primary AGMs comprise of a number of cell types, which are likely to interact or have additive effects in their provision of a haematopoietic microenvironment. Irradiation of the stromal cell layers prevents the cells from proliferating; though they do continue to secrete factors, but the exact effects of irradiation are unknown.

By contrast to AM20.1B4, CFU-A activity in EB/UG26.1B6 co-culture was not significantly different from gelatin controls at 4 or 10 days of differentiation and was significantly reduced at 6 days ( $p=0.002$ ). CFU-A activity following co-culture on EL08.1D2 stromal cells was significantly reduced compared to gelatin controls at 4, 6 and 10 days differentiation ( $p=0.03$ ,  $p=0.001$  and  $p=0.0005$ , respectively).

Primary AGM/EB co-cultures were only assessed by CFU-A and HPP-CFC assay; however, in order to further characterise the haematopoietic cell types generated in AM20.1B4 co-cultures, methylcellulose-based colony assays were carried out at 4, 6 and 10 days (Figure 4.6). At all the time points tested, it was found that EB-derived CFU-Mix, CFU-GM, Ery/Mac and CFU-M frequencies were significantly increased in AM20.1B4 co-cultures as compared to gelatin controls ( $p<0.04$ ). Haematopoietic activity in AM20.1B4 co-cultures peaked at 6 days and was reduced by 10 days differentiation. At 16 days, haematopoietic colony readout from the co-cultures was even further reduced (data not shown). Again, UG26.1B4 and EL08.1D2 were not found to enhance haematopoietic differentiation of ES cells.

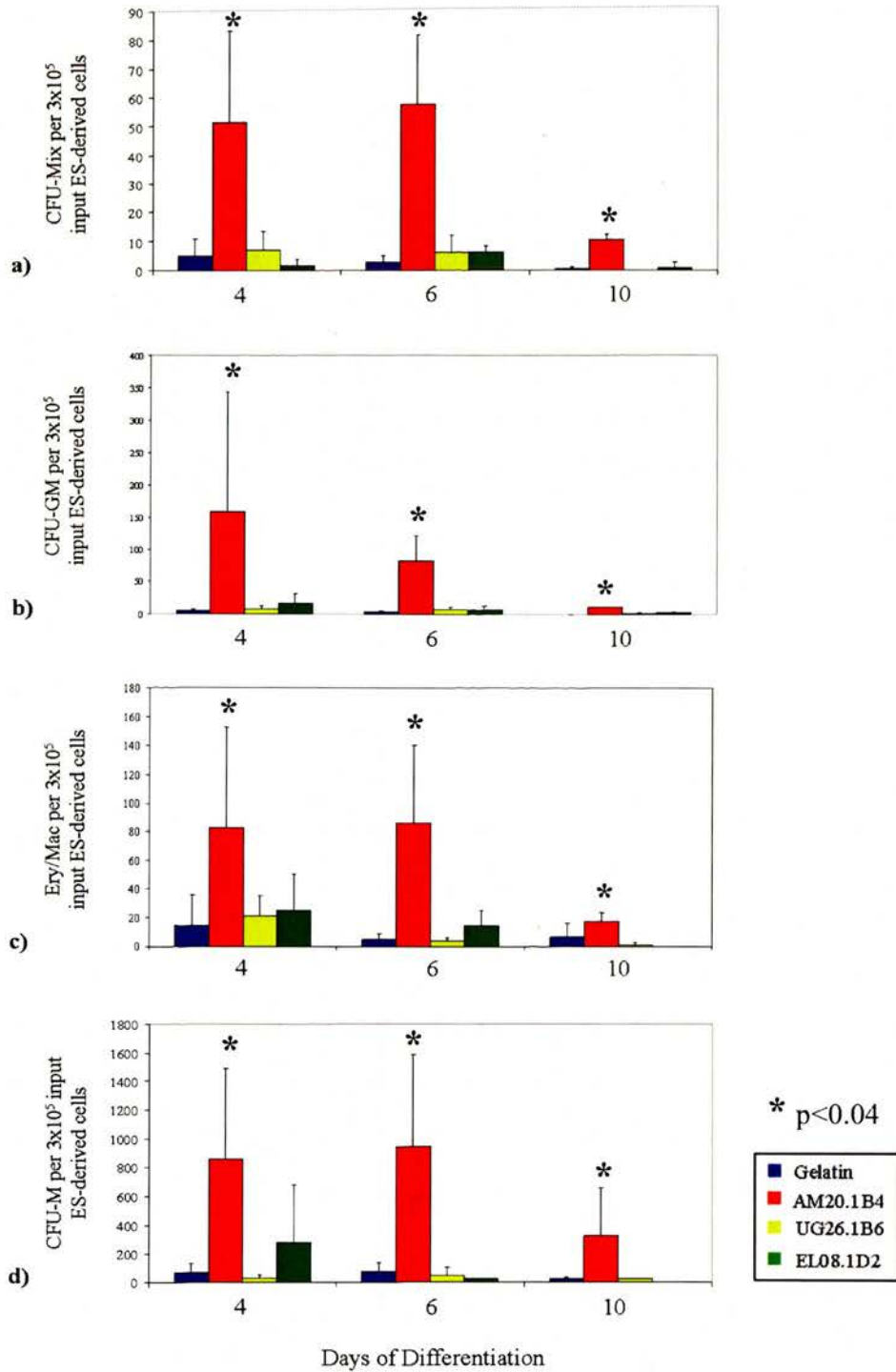


Figure 4.6 Methylcellulose-based haematopoietic colony assays of AM20.1B4, UG26.1B6, EL08.1D2 and gelatin/ EB co-cultures at 4, 6 and 10 days differentiation. Shown are the frequencies of (a) CFU-Mix, (b) CFU-GM, (c) Ery/Mac and (d) CFU-M colonies per  $3 \times 10^5$  input ES-derived cells. Data represent 3 independent co-culture experiments and p-values were calculated by paired Mann Whitney U tests.

These data demonstrate that a stromal line from the AM subregion of the AGM partially retains the enhancing effects of primary AGM co-culture, while a UG-derived line does not. It is interesting that AM20.1B4 had a potent haematopoietic enhancing effect on EBs (Figure 4.5), yet it was non-supportive of adult BM derived HSCs (Oostendorp et al., 2002a; 2002b). Conversely, UG26.1B6 and EL08.1D2 were reported to provide powerful long term support to adult HSCs, yet these lines did not enhance haematopoietic differentiation of ES cells. These data could highlight that the signals required for the support of adult LTR-HSCs and those required to promote haematopoietic differentiation of ES cells differ.

#### ***4.4.3 The enhancing effects of three AM-derived stromal lines are comparable with those of the OP9 stromal cell line***

In order to confirm that cells in the AM subregion of the AGM mediated the enhancing effects of primary AGM culture, further AM and UG derived stromal cell lines were screened for enhancing activity. In addition, OP9 stromal cells were included as a positive control to facilitate comparison of colony data with other published work. It was found that only the AM20.1B4 stromal cell line enhanced CFU-A activity at 6 days differentiation (Figure 4.7), corroborating previous data for this cell line (Figure 4.6). AM20.1A4, AM14.1C4, UG26.2D3 stroma and OP9 did not enhance CFU-A activity as compared to gelatin controls. Though, this does not rule out the possibility that the timing of CFU-A emergence in these stromal co-cultures differs from that of AM20.1B4. Therefore, it would be interesting to test CFU-A activity over a time course of differentiation in these co-cultures.

Methylcellulose-based colony assays revealed that after 6 days of differentiation, all three stromal cell lines derived from the AM subregion of the AGM significantly enhanced the overall haematopoietic CFU readout over that of gelatin controls,  $p < 0.0035$  (Figure 4.8). Statistically, the total haematopoietic activity in AM20.1B4 and AM20.1A4 co-cultures was comparable with OP9 co-cultures ( $p > 0.3$ ). While, AM14.1C4 co-culture resulted in significantly higher haematopoietic activity than OP9 co-culture ( $p = 0.038$ ).

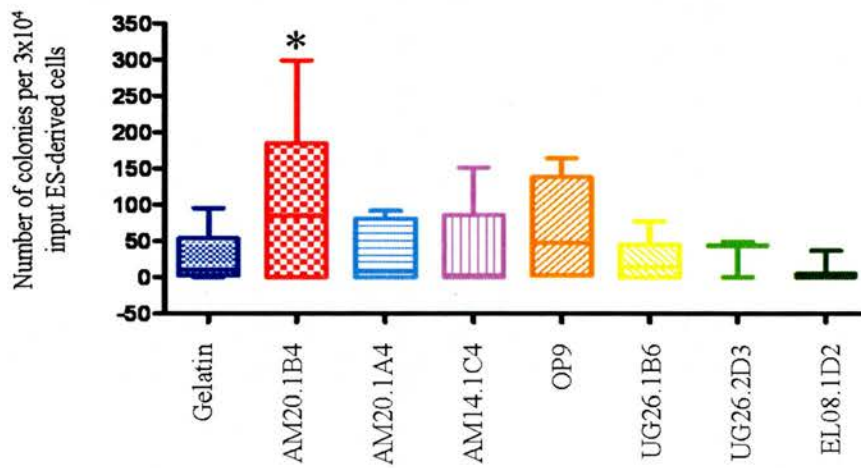


Figure 4.7 Resultant CFU-A activity when 7a-EBs were co-cultured to 6 days of differentiation. Only AM20.1B4 co-culture led to a significant increase in ES-derived CFU-A activity compared gelatin controls (\* p=0.02, according to Mann Whitney U test). These data represent 3 to 11 independent co-culture experiments.



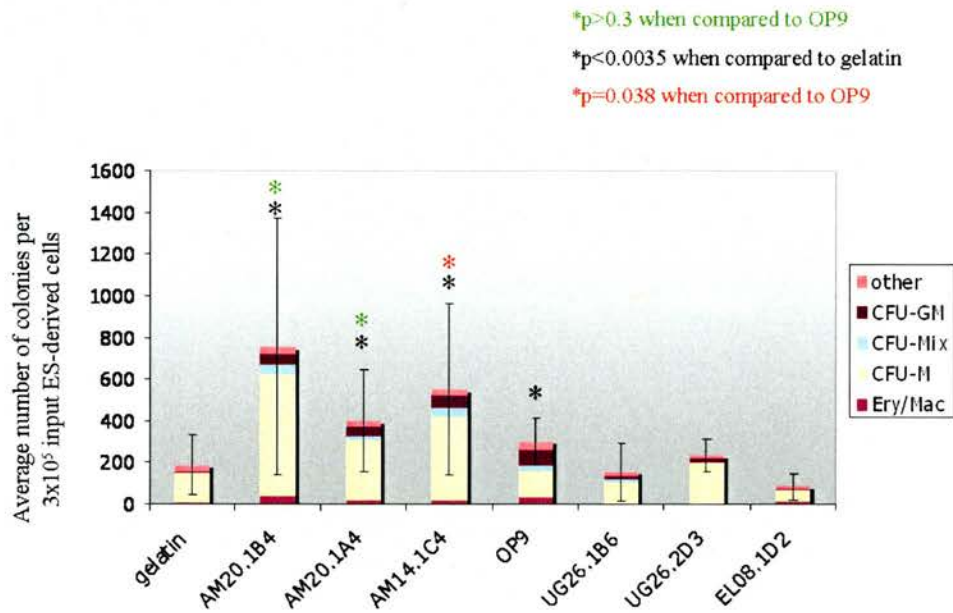


Figure 4.8 Total haematopoietic readout when 7a-GFP EBs were co-cultured to 6 days differentiation. The frequency of CFU per  $3 \times 10^5$  ES-derived cells in seeded into methylcellulose assays is shown. Data represent between 3 and 9 independent co-culture experiments. Paired Mann Whitney U tests were used to compare data sets.

The haematopoietic readouts of AM and OP9 co-cultures were all significantly better than that of gelatin controls, where EBs were differentiated in the absence of a stromal layer ( $p < 0.0035$ ). In contrast, the two UG-derived lines (UG26.1B6 and UG26.2D3) and the foetal liver stromal line (EL08.1D2) were not significantly different from gelatin controls ( $p > 0.05$ ). These data indicate that it was not merely the presence of a stromal layer which enhanced haematopoietic differentiation, but that the effects were specific to the AM subregion derived cell lines and OP9.

When individual CFU colony types were assessed, there were no significant differences in the frequencies of CFU-Mix present in AM20.1B4, AM20.1A4 and AM14.1C4 co-cultures compared to OP9 cultures,  $p > 0.05$  (Figure 4.9 a). The average number of colonies and standard deviations between experiments are provided in Table 4.1. From  $3 \times 10^5$  input ES-derived cells,  $43 \pm 23$  CFU-Mix colonies were generated by AM20.1B4 co-culture,  $15 \pm 17$  CFU-Mix were obtained from AM20.1A4 and  $37 \pm 44$  from AM14.1C4. These frequencies were statistically comparable with OP9 cultures ( $25 \pm 19$  CFU-Mix per  $3 \times 10^5$  ES-derived cells). When these data were expressed in terms of fold change relative to gelatin controls; on average in AM20.1B4, AM20.1A4, AM14.1C4 and OP9 co-cultures, CFU-Mix frequencies were 23x, 8x, 19x and 13x (fold) higher than gelatin controls, respectively. Therefore, co-culture of ES cells with AM-derived stromal lines significantly enhanced numbers of multipotent progenitors with CFU-Mix potential *in vitro*.

There is a considerable standard deviation between experiments. This is likely to be a consequence of pooling data from 3 to 9 independent co-culture experiments; which were performed with different batches of foetal calf serum. When considering each AM co-culture experiment separately, however, there was a consistent increase in haematopoietic activity over gelatin controls (Table 4.2). Furthermore, the AM co-cultures consistently compared favourably with OP9 positive controls.



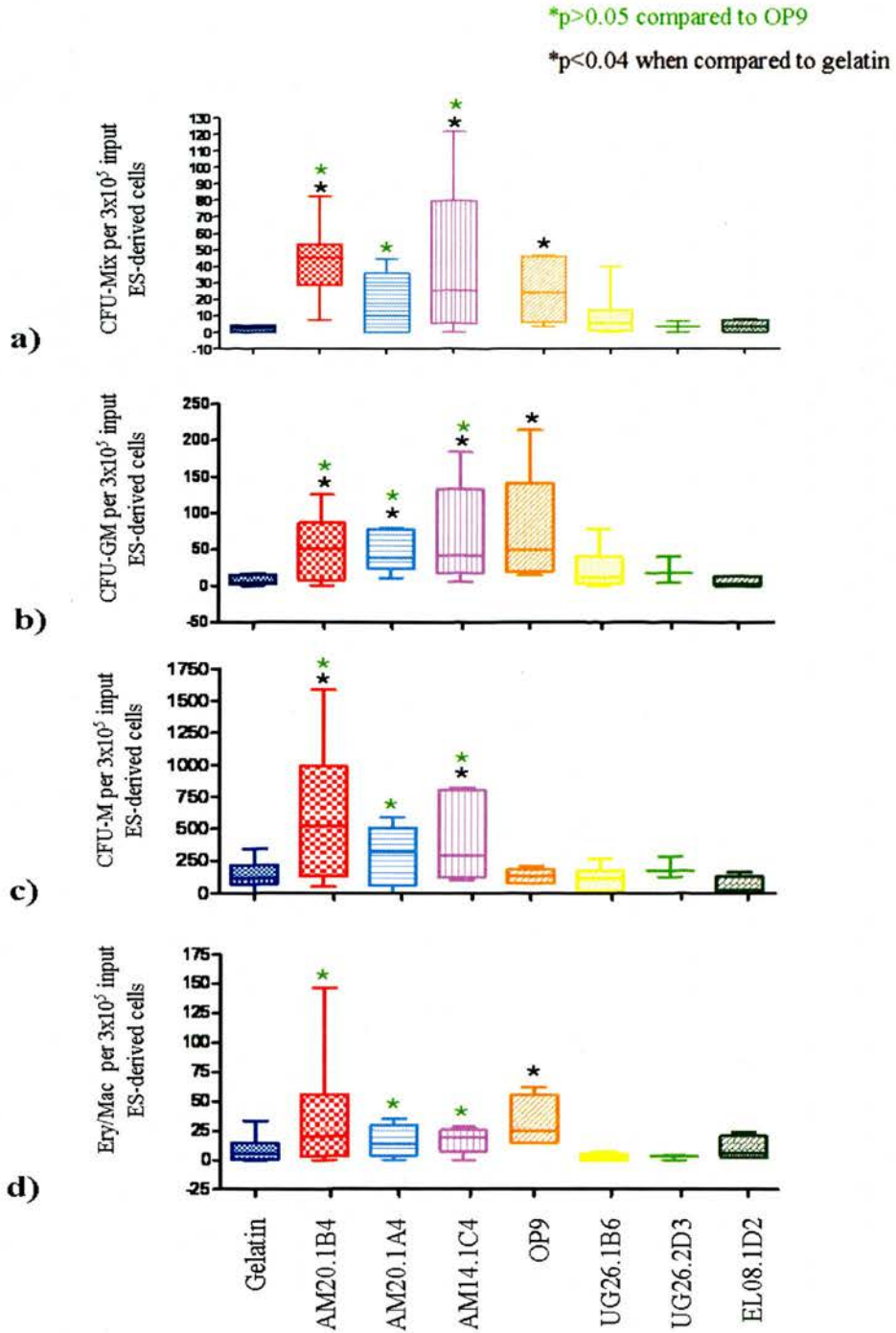


Figure 4.9 Haematopoietic activity in 7a-GFP embryoid bodies co-cultured to 6 days differentiation. (a) CFU-Mix, (b) CFU-GM, (c) CFU-M, (d) Ery/Mac. Data represent between 3 and 9 independent co-culture experiments. Mann Whitney U tests showed that haematopoietic activity in co-cultures with AM-derived lines was statistically comparable with OP9 co-cultures (\*).

Co-culture	Exp	CFU-Mix	CFU-GM	Ery/Mac	CFU-M
<b>Gelatin</b>	n=9	1.9 ± 1.8	7.1 ± 6.5	9.0 ± 11.1	137.4 ± 113.4
<b>AM20.1B4</b>	n=9	42.9 ± 22.5	48.9 ± 44.5	35.4 ± 47.2	589.7 ± 514.7
<b>AM20.1A4</b>	n=6	15.2 ± 17.4	46.3 ± 26.3	15.6 ± 12.4	292.4 ± 214.8
<b>AM14.1C4</b>	n=6	36.8 ± 43.7	64.1 ± 63.6	17.2 ± 9.7	403.9 ± 327.6
<b>OP9</b>	n=6	25.3 ± 18.8	69.3 ± 73.2	31.5 ± 19.3	129.7 ± 48.3
<b>UG26.1B6</b>	n=9	9.8 ± 12.4	21.4 ± 28.6	2.8 ± 2.6	102.7 ± 90.1
<b>UG26.2D3</b>	n=3	3.4 ± 4.8	20.5 ± 18.5	2.4 ± 2.1	192.2 ± 80.3
<b>EL08.1D2</b>	n=6	3.5 ± 3.3	5.2 ± 6.4	9.7 ± 8.7	57.2 ± 57.6

Table 4.1 The frequencies of CFU-Mix, -GM, -M and Ery/Mac colonies in stromal co-cultures and gelatin controls at 6 days differentiation. Average frequencies (per  $3 \times 10^5$  input ES-derived cells) and standard deviations of between 3 and 9 co-culture experiments are shown.

Co-culture	Fold increase in CFU-Mix over gelatin control	Number of experiments in which CFU-Mix frequencies in AM co-culture were higher, comparable or reduced relative to OP9 system		
		Higher	Comparable	Reduced
AM20.1B4	22.6 ± 20.6	2 of 5	2 of 5	1 of 5
AM20.1A4	7.6 ± 6.5	2 of 6	3 of 6	1 of 6
AM14.1C4	19.4 ± 16.7	3 of 6	2 of 6	1 of 6
OP9	13.3 ± 5.7	N/A	N/A	N/A

Table 4.2 Summary of the fold change in frequency of CFU-Mix observed in co-cultures relative to gelatin. Shown are the colony numbers per  $3 \times 10^5$  input ES-derived cells and the number of experiments in which CFU-Mix frequencies in AM co-cultures were comparable with or better than OP9 culture.

It was found that the frequency of CFU-Mix in AM14.1C4 co-cultures was greater than or comparable to OP9 culture in 5 out of 6 independent experiments. AM20.1B4 was comparable to OP9 in 4 of 5 experiments and AM20.1A4 was comparable in 5 of 6 experiments (Table 4.2). This indicates that, while there was variability in the base level of haematopoietic activity between experiments, the AM co-cultures were relatively consistent in their ability to enhance haematopoietic differentiation as compared to OP9.

The frequency of CFU-GM, CFU-M and Erythroid/Macrophage colonies in AM co-cultures were also statistically comparable to that in OP9 cultures and enhanced compared to gelatin (Figure 4.9 b, c, d). It should be noted that OP9 stromal cells do not express functional M-CSF and this is thought to allow lymphoid differentiation to take place as ES cells do not preferentially undergo differentiation into macrophage/monocyte lineages. In accordance with this, there appears to be a minimal CFU-M readout from OP9 co-cultures and the frequency was not significantly different from gelatin controls ( $p>0.05$ ) (Figure 4.9 c).

When co-cultures were harvested and analysed, irradiated stromal cells were still present in the cell suspensions which were seeded into colony assays. Control experiments demonstrated that irradiated stromal cells seeded into the colony assays alone were unable to generate colonies and therefore would not affect the ES-derived colony readout (Appendix 1 and 2). This verified that colonies represented ES cell-derived HPCs which were present in the co-cultures at the time of assay setup.

Other ES cell lines have also been tested in this co-culture system; including wild-type E14 and CGR8 ES cells (data not shown). In these co-cultures, the AM-derived stromal cell lines consistently had significant haematopoietic enhancing effects which were comparable to or better than OP9 cultures. This demonstrates that the enhancing activity was not specific to 7a-GFP ES cells and that the novel stromal co-culture system described here can be used to differentiate other ES cell lines in a reproducible and efficient manner.

#### ***4.4.4 Enhanced haematopoietic activity in AM-derived stromal co-cultures demonstrated by flow cytometry analysis at 10 days differentiation***

To further characterise the cell types generated in co-culture, flow cytometry analysis was carried out at defined time points during differentiation. Monoclonal antibodies against a variety of surface markers were used and for each co-culture, no less than  $1 \times 10^5$  cells were collected for analysis (Chapter 2, Table 2.2). In Tables 4.3, 4.4 and 4.5 to follow, gating on the flow cytometry plots was carried out by Kay Samuel.

Flow cytometry analysis demonstrated that at 4 and 6 days differentiation, there were no differences in the proportions of ES-derived cells expressing haematopoietic surface antigens in gelatin or AM20.1B4, UG26.1B6 and EL08.1D2 co-cultures (Appendix 3). Further analysis revealed that by 10 days of differentiation there was an increase in the proportion of cells expressing haematopoietic surface markers in AM20.1B4 stromal co-culture compared to gelatin. Figure 4.10 shows an increase in cells expressing adult HSC markers, Sca-1 and cKit, as well as CD49d, which is reported to be expressed on E11 AGM-derived LTR-HSCs (Gribi et al., 2006). Increases in cells expressing myeloid markers Gr-1 and CD11b, lymphoid lineage marker B220 and the erythroid marker Ter119, were also observed.

To further the investigation, the three AM-derived stromal co-cultures were compared to OP9 co-cultures and gelatin controls at 10 days of differentiation. These data are summarised in Table 4.3 and markers that were comparable to OP9 culture are highlighted. All three AM stromal lines enhanced the proportions of EB cells expressing haematopoietic surface markers characteristic of myeloid (CD11b, Gr1), lymphoid (B220) and erythroid (Ter119) lineages. This was coupled with an increase in the proportion of cells expressing the pan-haematopoietic cell marker CD45, with an average of 7 to 11% of ES-derived cells expressing CD45 in enhancing co-cultures compared to only 1.6% in gelatin cultures.

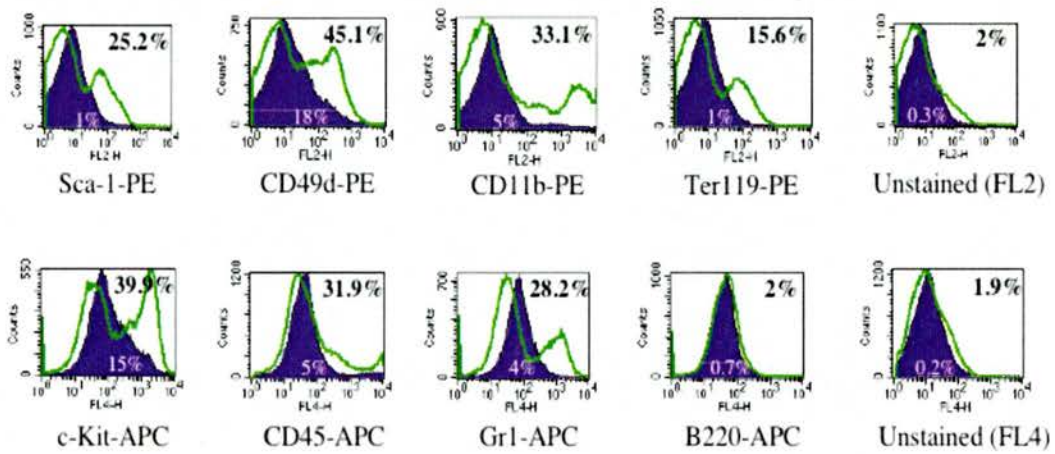
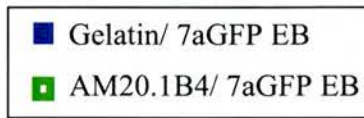


Figure 4.10 Flow cytometry analysis of 7a-GFP EBs co-cultured to 10 days of differentiation. AM20.1B4 co-cultures contained higher proportions of EB-derived cells expressing haematopoietic surface markers compared to EBs differentiated on gelatin. Data represent one co-culture experiment, histograms were prepared by Kay Samuel.



Surface Marker	% Positive ES-derived cells				
	Gelatin	AM20.1B4	AM20.1A4	AM14.1C4	OP9
CD45	1.6 ± 1.6	7.2 ± 10.2	9.6 ± 4.8	7.0 ± 0.3	11.0 ± 9.3
Gr1	0.8 ± 1.2	6.1 ± 9.5	2.2 ± 1.1	1.4 ± 0.1	4.4 ± 3.2
CD11b	1.5 ± 1.5	7.5 ± 11.0	15.1 ± 14.0	2.3 ± 1.3	5.1 ± 2.7
B220	2.9 ± 6.0	3.6 ± 3.0	17.0 ± 12.1	4.4 ± 0.7	4.8 ± 4.7
Ter119	0.5 ± 0.6	2.9 ± 5.0	2.4 ± 1.3	2.1 ± 2.3	1.6 ± 2.2
Gr1+ CD11b+	0.8 ± 1.2	6.0 ± 9.6	1.9 ± 1.0	0.8 ± 0.4	3.5 ± 2.8

Table 4.3 Flow cytometry analysis of 7a-GFP EBs co-cultured on irradiated stromal cells to 10 days differentiation. The proportions (%) of ES-derived cells expressing haematopoietic surface markers in co-cultures with AM-derived stromal lines were broadly comparable to that of OP9 co-cultures. Data represent up to 9 independent co-culture experiments and in each condition, no less than  $1 \times 10^5$  cells were collected for analysis.

In the literature, ES cells are plated directly onto OP9 stromal layers which are not irradiated and cells are replated onto fresh OP9 layers after 5 and 10 days differentiation. This differs from the differentiation strategy employed here, whereby EBs were co-cultured on irradiated stroma for 6 days differentiation with no replating step. Nonetheless, when ES cell/OP9 stromal co-culture was first described by Nakano and colleagues (1994), they reported that 15% of cells were cKit<sup>+</sup>, 25% Ter119<sup>+</sup>, 5% CD11b<sup>+</sup> and 7% B220<sup>+</sup>. These data broadly correlate with the expression pattern observed reported here (Table 4.3 and 4.4); other than for Ter119 expression, which was only expressed on an average of 1.6% of OP9 co-cultured EB cells.

It is interesting that 10 days of differentiation was required to detect enhanced frequencies of cells expressing these haematopoietic surface markers, whereas CFU activity peaked at 6 days of differentiation and was reduced by 10 days differentiation (Figures 4.5 and 4.6). One explanation may be that CFU progenitors present at 6 days terminally differentiate to give rise to cells which express myeloid, lymphoid and erythroid markers by 10 days, but can no longer generate colonies in the assays. This may also explain the variability in flow cytometry data between co-culture experiments, as the ability of the stromal microenvironment to promote production of haematopoietic progenitors may be more consistent than the ability of the microenvironment to support continual self-renewal and expansion of these progenitors, as opposed to their differentiation. In attempt to improve the self-renewing support of the co-cultures, a selection of cytokines (including IL3, IL6 and SCF) was added to test whether haematopoietic activity could be prolonged or enhanced further. It was found that addition of these cytokines during co-culture did not appear to make a difference to colony readout (data not shown), but these preliminary findings do not preclude that the addition of other cytokines as a means to improving the co-cultures further. Nevertheless, flow cytometry analysis of markers associated with terminally differentiated haematopoietic cells has confirmed that AM-derived stromal co-cultures enhance haematopoietic differentiation of ES cells such that they are comparable to OP9 positive control cultures.

#### 4.4.5 Enhanced proportions of cells expressing combinations of surface markers associated with adult BM-derived HSCs

The haematopoietic colony assays indicated that numbers of multipotent HPCs (CFU-Mix) were enhanced in the AM stromal co-cultures and that these progenitors were functional *in vitro*, in that they could respond to cytokines and undergo multi-lineage differentiation to form mixed colonies. Since the surface phenotypes of adult and embryo LTR-HSCs have been well documented, it was of interest to analyse co-cultured cells for expression of combinations of surface markers associated with these progenitors. It was found that expression of cKit and Sca-1 were greatly enhanced in AM co-cultures compared to gelatin controls and that they were comparable to or better than OP9 (Table 4.4). On average, there were 15 to 18% cKit<sup>+</sup> cells in AM co-cultures and 10% in OP9 cultures compared to only 4% in gelatin. An average of 7 to 22% Sca-1<sup>+</sup> cells were detected in AM cultures compared to 5% in OP9 and only 1.5% in gelatin cultures. Notably, the average proportions of cKit<sup>+</sup>Sca-1<sup>+</sup> co-expressing cells in AM20.1B4 (2.6±2.3%), AM20.1A4 (5.8%) and AM14.1C4 (2.7%) co-cultures were comparable to OP9 (2.9±4.8%) and these populations were enhanced compared to gelatin controls (0.05±0.07%). A combination of cKit and Sca-1 expression is characteristic of definitive adult BM-HSCs and it is possible that these cells represent a small population of definitive HSCs present in the co-cultures. Though, it is yet to be shown that these double positive cells are negative for lineage markers.

Kiel and colleagues (2005) reported that differential expression of SLAM family receptors can be used to distinguish between adult BM-derived LTR-HSCs, HPCs and more restricted progenitors; these populations were reported to express CD150<sup>+</sup>CD48<sup>-</sup>CD244<sup>-</sup>, CD150<sup>-</sup>CD48<sup>-</sup>CD244<sup>+</sup> and CD150<sup>-</sup>CD48<sup>+</sup>CD244<sup>+</sup>, respectively. In attempt to identify ES-derived HSCs within the stromal co-cultures, expression of the SLAM receptors was assessed. As shown in Table 4.4, CD150<sup>+</sup> cell populations were enhanced in all the AM and OP9 co-cultures as compared to gelatin controls. In particular, AM20.1A4 cultures contained 14.8±20% CD150<sup>+</sup> cells and OP9 contained 12.8±3.5% compared to only 0.9±0.2% in gelatin controls.

Surface Marker	% Positive ES-derived cells				
	Gelatin	AM20.1B4	AM20.1A4	AM14.1C4	OP9
<b>cKit+</b>	3.95 ± 5.0	14.9 ± 16.7	18.4	16.9	9.8 ± 8.0
<b>Sca1+</b>	1.5 ± 3.1	6.7 ± 8.6	21.6	8.2	5.0 ± 8.5
<b>cKit+ Sca1+</b>	0.05 ± 0.1	2.6 ± 2.3	5.8	2.7	2.9 ± 4.8
<b>CD150+</b>	0.9 ± 0.2	2.0 ± 1.4	14.8 ± 20	3.7 ± 5.0	12.8 ± 3.5
<b>CD150+ CD48+</b>	0.1 ± 0.06	0.9 ± 0.8	0.8 ± 0.6	0.3 ± 0.3	1.6 ± 1.0

Table 4.4 Flow cytometry analysis of 7a-GFP EBs co-cultured on irradiated stromal cells to 10 days differentiation. These analyses aimed to detect combinations of surface markers which are expressed on adult BM-HSCs. Highlighted in yellow are the co-cultures where the proportions (%) of ES-derived cells expressing the surface markers were enhanced. Data represent between 1 and 9 independent co-culture experiments.

A significant finding was that these CD150<sup>+</sup> cells did not co-express CD48, which is a pan-haematopoietic cell marker and is not expressed on mouse bone marrow (or foetal liver) LTR-HSCs (Kiel et al., 2005; Kim et al., 2006). In addition, it was found that CD244 was not expressed by cells in any of the co-cultures (data not shown). Therefore, it is possible that the CD150<sup>+</sup>CD48<sup>-</sup> cells present in AM and OP9 co-cultures may represent definitive HSCs.

There is evidence that ES-derived HSCs may not display a complete surface phenotype consistent with that of adult BM-HSCs. This is reflected by the apparent inefficiency in ES-derived HSC homing to appropriate niches upon intra-venous transplantation (Burt et al., 2004). Alternatively, ES-HSCs present in the co-cultures may have a surface phenotype that is similar to that of AGM-derived HSCs. Therefore, it was determined whether co-cultured cells expressed surface markers which are characteristic of AGM-derived HSCs and markers that were previously reported to be expressed on definitive ES-derived HPCs (Table 4.5). CD34 is expressed on cord blood HSCs, mobilised BM-HSCs and endothelial cells (Sato et al., 1999). In the mouse embryo, E11 AGM HSCs co-express cKit and CD34 and the repopulating fraction of cells all express CD49d (alpha4 integrin) (Sanchez et al., 1996; Taoudi et al., 2005; Gribi et al., 2006). Analysis for these markers revealed that AM20.1B4 and OP9 co-cultures did not contain enhanced numbers of CD34<sup>+</sup> or cKit<sup>+</sup>CD34<sup>+</sup> co-expressing cells. However, AM20.1B4 cultures did contain a high proportion of CD49d<sup>+</sup> cells (41.7±4.7%) compared to gelatin controls (17.9%). The expression of this marker was not assessed in AM20.1A4, AM14.1C4 or OP9 co-cultures.

Mikkola and colleagues (2003) found that CD41 was expressed by CD34<sup>+</sup>cKit<sup>+</sup> cells in the E9 YS, a population which was previously reported to be able to repopulate newborn recipients and is therefore thought to contain pre-HSC cells (Yoder et al., 1997a, 1997b). Mikkola et al (2003) also reported that during EB differentiation *in vitro*, cells co-expressing cKit<sup>+</sup>CD41<sup>+</sup> appeared to represent definitive ES-derived HPCs.

Surface Marker	% Positive ES-derived cells				
	Gelatin	AM20.1B4	AM20.1A4	AM14.1C4	OP9
CD34	0.1 ± 0.07	0.3 ± 0.2	nd	nd	0.4 ± 0.06
cKit+ CD34+	0.1 ± 0.07	0.2 ± 0.2	nd	nd	0.3 ± 0.08
CD49d	17.94	41.7 ± 4.7	nd	nd	nd
CD41	0.06 ± 0.1	0.06 ± 0.1	18.1	1.4	0.2 ± 0.3
cKit+ CD41+	0.02 ± 0.02	0.06 ± 0.1	nd	nd	0.2 ± 0.3

*\*nd = not determined*

Table 4.5 Flow cytometry analysis of 7a-GFP EBs co-cultured on irradiated stromal cells to 10 days differentiation. These analyses aimed to detect combinations of surface markers which are expressed on embryo-derived HSCs. Data represent between 1 and 9 independent co-culture experiments.



The proportions of cKit<sup>+</sup>CD41<sup>+</sup> double positive cells were not enhanced in AM20.1B4 (0.06±0.02%) or OP9 co-cultures (0.2±0.3%) (Table 4.5). However, AM20.1A4 co-cultures contained a high proportion of cells expressing CD41 (18.1%) compared to gelatin controls (0.06±0.09%). It was not determined whether these CD41<sup>+</sup> cells co-expressed cKit. This would be an important control, as CD41 is also a marker of megakaryocytes.

These analyses were all carried out on co-cultures at 10 days differentiation; however, CFU-Mix progenitor numbers were found to peak at 6 days differentiation and were reduced by 10 days. Therefore, it may be useful to carry out flow cytometry analysis for these definitive HSC markers at 6 days differentiation. Taken together, the data so far could suggest that adult-type HSCs may have been generated in the co-cultures; though, these surface markers are not necessarily exclusively expressed on haematopoietic cells and expression of surface markers is not a measure of function or potential of the cells. Repopulation assays need to be carried out to determine the *in vivo* potential of the co-cultured ES cells.

#### ***4.4.6 Co-cultured cells display the molecular characteristics of definitive haematopoietic cells***

It has been suggested that haematopoietic differentiation in EBs recapitulates primitive haematopoietic lineage specification within the yolk sac (Keller et al., 1993; Keller et al., 2005). Therefore, it cannot be assumed that the haematopoietic cells generated in the AM stromal co-culture system arose from definitive ES-derived HSCs which have repopulating ability. According to the flow cytometry data described above, definitive HPCs or HSCs might be present in some co-cultures. In order to reliably detect definitive haematopoietic cell types, quantitative real-time RT-PCR was carried out to detect definitive gene transcripts. One day old hanging drop EBs (Bry-201) were co-cultured to 5 days of differentiation and then isolated from stromal layers (DiD stained) by FACS. When these sorted ES cell samples were assessed by quantitative RT-PCR, it was found that Runx1 and Lmo2 gene transcripts were expressed. As a control, RT-PCR was carried out on adult bone marrow samples (C57/Bl6), which confirmed that definitive haematopoietic cells

express these gene transcripts (Figures 4.11 and 4.12). In these experiments, the target gene expression is expressed as fold increase relative to a calibrator (1 day old EBs), which is assigned a value of 1.

In a further experiment, 1 day old hanging drop 7a-GFP EBs were co-cultured to 10 days differentiation and then analysed by RT-PCR without prior sorting to remove the irradiated stromal cells, thus irradiated stromal cells alone were included as controls (Figure 4.13). It should be noted that the control stromal layers were harvested 5 days post-irradiation, as the RNA content in the stromal cells 10 days after irradiation was considerably lower than at earlier time points. Therefore, these control samples have been used only as an indication of haematopoietic transcript expression, but do not represent the levels of transcript which the stromal layers contribute to the 10 day co-culture samples. As shown in Figure 4.13, Runx1 was expressed in AM20.1B4, AM20.1A4, AM14.1C4 and OP9 co-cultures at 10 days differentiation. The irradiated stromal cell controls appeared to express lower levels of Runx1. Therefore, these cells were unlikely to account for the high levels of Runx1 transcript in 10 day co-cultures. In these experiments the RT- controls, where no reverse transcriptase enzyme was included in the RT reaction, showed no gene expression in any of the samples (data not shown). This confirmed that the PCR amplification arose from cDNA and not genomic DNA. Taken together, these data demonstrate that definitive genes, Runx1 and Lmo2, are expressed by ES cells differentiated in AM20.1B4, AM20.1A4, AM14.1C4 and OP9 co-cultures. Runx1 gene expression was detected at 5 and 10 days differentiation in co-cultures; therefore it is possible that definitive cell types detected at 10 days by flow cytometry could have arisen from definitive cells present at earlier time points.

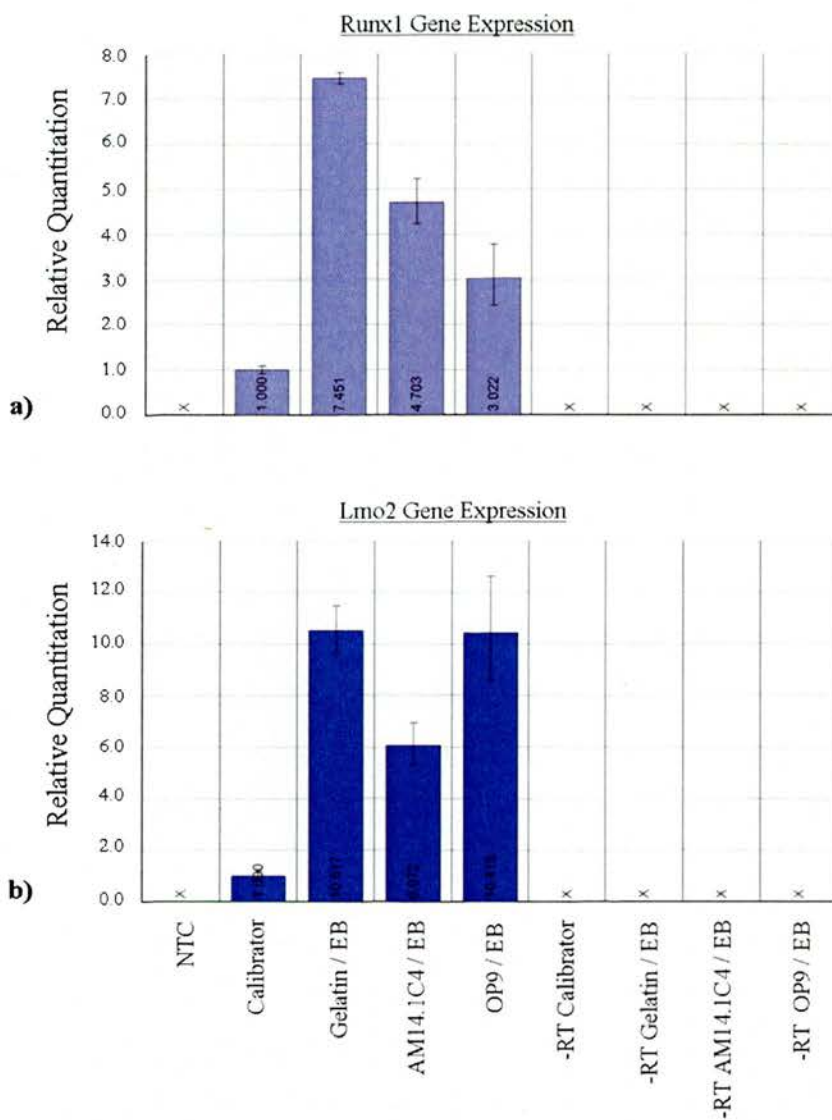


Figure 4.11 Quantitative RT-PCR analysis of co-cultured ES cells. One day hanging drop EBs were co-cultured to 5 days differentiation and sorted from stromal layers by FACS for RT-PCR analysis for (a) Runx1 and (b) Lmo2 gene expression. Relative quantitation was calculated using the delta delta Ct method in ABI FAST7500 software. One day EBs were used as the calibrator and HPRT was used as the endogenous control. NTC, no template control; -RT, no reverse transcriptase enzyme control.

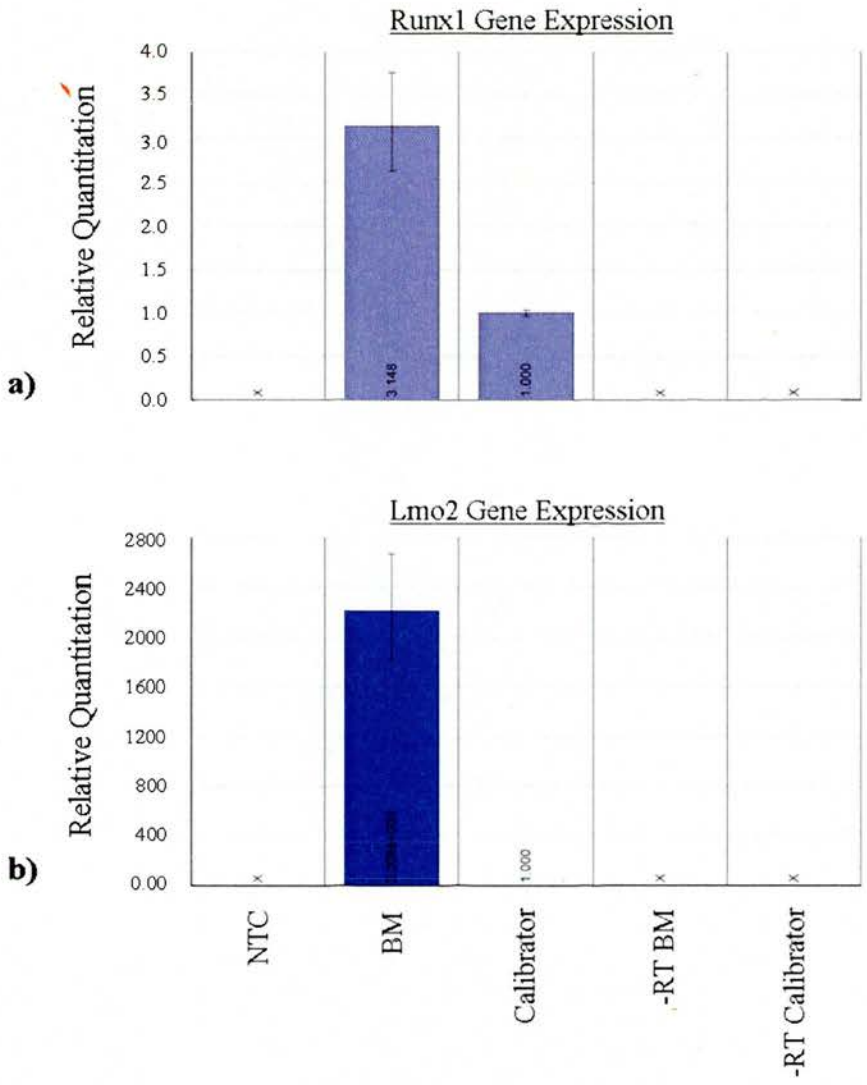


Figure 4.12 Quantitative RT-PCR analysis of bone marrow cells. (a) Runx1 and (b) Lmo2 gene expression. Calibrator: 1 day EBs; endogenous control, HPRT. NTC, no template control; BM, bone marrow; -RT, no reverse transcriptase enzyme control.

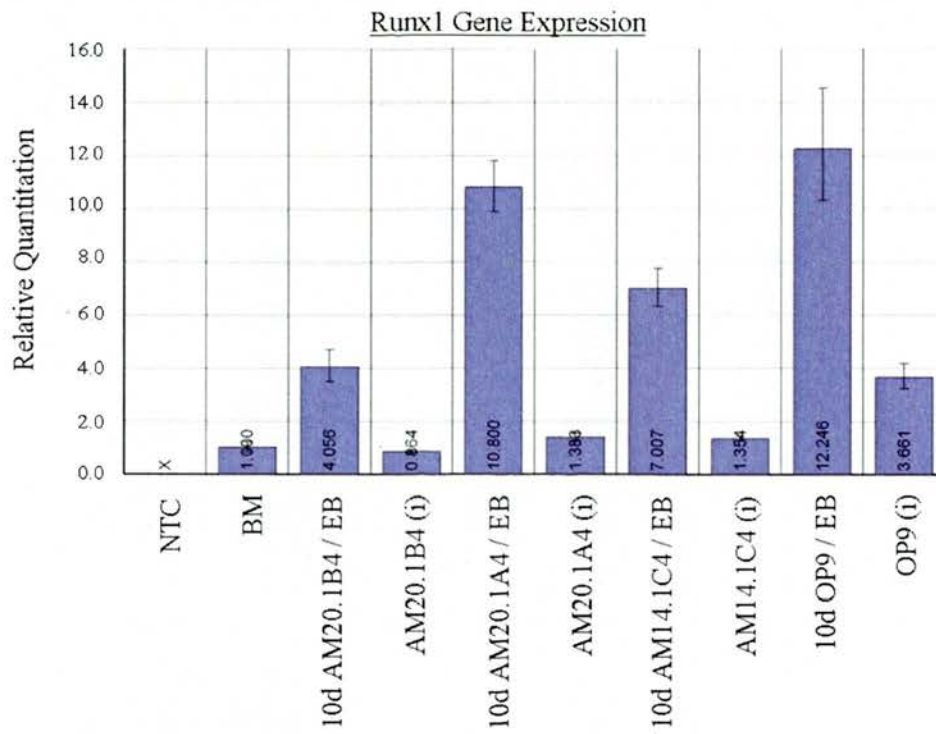


Figure 4.13 Quantitative RT-PCR analysis of 7a-GFP EBs co-cultured to 10 days of differentiation. Irradiated (i) stromal cells alone were also analysed. Bone marrow (BM) cells were used as the calibrator and HPRT was used as the endogenous control. NTC, no template control.

## 4.5 Discussion

### *4.5.1 A reliable and efficient AM stromal co-culture system has been established which potently promotes haematopoietic differentiation of ES cells*

Krassowska et al (2006) reported that haematopoietic activity was significantly enhanced when EBs were co-cultured with primary E10.5 AGM explants. The experiments reported in this chapter aimed to screen clonal stromal cell lines derived from AM and UG subregions of the midgestational AGM region for their ability to promote haematopoietic differentiation of ES cells. The AM20.1B4 stromal cell line was found to partially retain the CFU-A enhancing activity of primary AGM/EB co-culture (Figures 4.5 and 4.7). It has been demonstrated that all three stromal cell lines derived from AM subregion of the AGM (AM20.1B4, AM20.1A4 and AM14.1C4) significantly enhanced the frequencies of multipotent HPCs. In contrast, urogenital ridge (UG26.1B6, UG26.2D3) and foetal liver (EL08.1D2) stromal lines did not promote haematopoietic differentiation as compared to gelatin (no feeder) controls when assessed by colony assay or by flow cytometry. This indicated that it was not simply the presence of a stromal cell layer which promoted haematopoietic differentiation, but that the haematopoietic enhancing effects were specific to AM-derived stromal cell lines. It is possible that the UG and EL stromal lines promote ES cell differentiation into other lineages, generating cell types which are not detected by haematopoietic colony assays.

The effects of AM stromal co-culture were compared with that of the OP9 cell line, which is a bone marrow derived stromal cell line well-known to promote haematopoietic differentiation of ES cells. In the literature, ES cells are plated directly onto non-irradiated OP9 stromal layers and cells are replated onto fresh OP9 layers after 5 and 10 days differentiation. This differs from the differentiation strategy employed here, whereby ES cells were prepared in hanging drops to form EBs, which were co-cultured on irradiated stroma for 6 days differentiation with no replating step. Nonetheless, using this EB co-culture strategy, OP9 stroma consistently enhanced haematopoietic activity compared to gelatin controls. Furthermore, the enhancing activity of the AM lines was comparable to that of OP9 co-cultures. A range of haematopoietic progenitors, represented by CFU-Mix, CFU-



GM, CFU-M and Ery/Mac colonies, were enhanced in AM co-cultures (Figures 4.8 and 4.9). In addition, at 10 days differentiation AM co-cultures contained increased proportions of ES-derived cells expressing haematopoietic surface markers associated with cells of myeloid, lymphoid and erythroid lineages such as CD45, Gr1, CD11b, B220 and Ter119 (Table 4.3). Importantly, quantitative RT-PCR analysis demonstrated that gene transcripts associated with definitive haematopoiesis, Runx1 and Lmo2, were expressed by co-cultured ES cells at 5 and 10 days differentiation (Figures 4.11 and 4.13). Therefore, it is possible that definitive haematopoiesis had occurred, though this does not rule out the presence of primitive haematopoietic cell types. One way to conclusively demonstrate that definitive cells were present (*in vitro*) would be to pick colonies containing erythrocytes from haematopoietic colony assays (CFU-Mix, Ery/Mac) and to analyse them individually by RT-PCR for expression of embryonic and adult globins (such as  $\beta$ H1 and  $\beta$ major, respectively). Colonies derived from definitive progenitors would only express adult globins, while primitive cells would express either embryonic or a combination of embryonic and adult globins.

It is interesting that the frequencies of CFU-Mix progenitors were significantly enhanced in AM co-cultures (Figure 4.9). Early studies using mouse bone marrow cells reported that CFU-GEMM could be closely related to CFU-S in the haematopoietic hierarchy (Humphries et al., 1981). Nakahata and Ogawa (1982) suggested that the pre-CFU-S (or HSC) is an earlier progenitor to the CFU-GEMM and that CFU-GEMM forming cells overlap with the CFU-S population. In a study by Kerk and colleagues (1985), it was shown that when adherent bone marrow cells were seeded into colony assays, primary CFU-GEMM were likely to be derived from early multilineage haematopoietic progenitors. The primary CFU-GEMM themselves were not able to generate secondary CFU-GEMM upon replating. However, it was found that the adherent HPCs from which the primary colonies formed were able to generate secondary colonies and these progenitors were correlated with cells that are capable of achieving long-term repopulation *in vivo*. Hence, they concluded that the presence of CFU-GEMM *in vitro* could be used as an indicator for the presence of LTR-HSCs in a cell population. It should be noted that

these initial studies of the CFU-GEMM colony were carried out with BM-derived cell populations and the assays were setup with conditioned medium, unlike the system used in this thesis, which used known concentrations of recombinant cytokines and where the cells were ES-derived. Despite this, it is possible that the presence of CFU-Mix cells in the EB/AM co-cultures could be an indication that HSCs had been present prior to the time at which the colony assays were performed.

#### ***4.5.2 Co-cultured cells display a surface phenotype characteristic of adult BM-derived HSCs***

It cannot be assumed that ES-derived HSCs or HPCs will have the same cell surface phenotype as their *in vivo* adult counterparts. To this end, co-cultured ES derived cells were assessed for expression of surface markers characteristic of AGM-derived HSCs or were previously reported to be expressed on definitive ES-derived HPCs. The analysis of cell surface markers associated with AGM-derived LTR-HSCs was inconclusive, since CD49d was expressed on large proportions of cells in AM20.1B4 co-cultures, but cKit<sup>+</sup>CD34<sup>+</sup> cells were not detected at high levels (Table 4.5). In addition, high proportions of CD41<sup>+</sup> cells were present in AM20.1A4 co-cultures, but it was not determined whether these cells co-expressed cKit. However, the data were not determined for all the enhancing co-cultures and further analysis might still reveal these populations.

An important finding was that between 2.6 and 5.8% of cells were cKit<sup>+</sup>Sca1<sup>+</sup> in AM-derived co-cultures compared to 3% on OP9 and only 0.05% on gelatin (Table 4.4). Since adult BM-HSCs express cKit and Sca1, these double positive cells could represent HSCs. Kiel et al (2005) and Kim et al (2006) reported that the differential expression pattern of CD150, CD48 and CD244 SLAM receptors on adult bone marrow and foetal liver mouse LTR-HSCs, STR-HSCs and restricted haematopoietic progenitors correlates with the *in vivo* potential of the cells. CD150 is expressed on T and B cell subsets and on thymocytes, whereas CD48 is expressed on leukocytes and CD244 marks natural killer cells and a T cell subset. In the co-cultures, the frequency of CD150<sup>+</sup> expressing cells was enhanced in all the AM and OP9 co-cultures. In particular, AM20.1A4 co-cultures contained 14.8±20% CD150<sup>+</sup> cells

and OP9 contained  $12.8 \pm 3.5\%$  compared to only  $0.9 \pm 0.2\%$  in gelatin controls. These CD150<sup>+</sup> cells did not co-express CD48 (Table 4.4) and CD244 expression was not detected in any of the co-cultures (data not shown). The presence of this CD150<sup>+</sup>CD48<sup>-</sup> population may suggest that HSCs were generated in co-cultures. Though, surface markers are not a measure of cell potential or function and transplantations of these cells is required to confirm the potential of co-cultured cells *in vivo*.

#### ***4.5.3 Timing of haematopoietic activity***

No differences in expression of haematopoietic surface markers of terminally differentiated cell types were evident at 4 and 6 days differentiation in any of the enhancing or non-enhancing co-cultures. At 10 days differentiation high proportions of cells in AM and OP9 co-cultures expressed these markers compared to gelatin and non-enhancing controls. At first glance, this timing of surface marker expression appears to contradict the colony assay data, which showed that (i) significantly more HPCs were present in AM-derived stromal co-cultures at 6 days differentiation compared to the other co-cultures (Figure 4.8) and (ii) haematopoietic activity peaks at 6 days differentiation and is reduced by 10 days (Figures 4.5 and 4.6). These observations support the hypothesis that HPCs present at 6 days differentiation can respond to assay conditions to form CFUs, but that they do not yet display the surface phenotype of terminally differentiated haematopoietic cells. It may be that the HPCs present in co-cultures at 6 days (which are detectable by CFU assay) expand in numbers and terminally differentiate in culture such that by 10 days, high proportions of cells express these surface markers, but are too differentiated to respond to and form colonies in assays. If this were the case, it could suggest that AM stromal co-culture provided a potent microenvironment for the production and/or expansion of HPC/HSCs from ES cells over the course of 6 days differentiation, but that the stroma did not provide long-term support for the maintenance of these cells in their self-renewing state.

It is interesting that both CD150<sup>+</sup>CD48<sup>-</sup> and Sca-1<sup>+</sup>cKit<sup>+</sup> populations, which could represent adult-type HSC populations, were detected at 10 days differentiation, when

CFU-Mix activity is reduced. It is possible that analysis for these markers at earlier time points could reveal higher proportions of these populations. In support of this hypothesis, co-cultured ES cells were found to express Runx1 and Lmo2 gene transcripts as early as 5 days differentiation, suggesting that definitive haematopoiesis could have taken place at earlier time points. Keller and colleagues (1993; 2005) have reported that EB differentiation appears to recapitulate the yolk sac pattern of primitive haematopoietic lineage specification. Therefore, in the co-cultures, primitive haematopoiesis might take place first, giving rise to primitive cells with limited self-renewal potential, which then exhaust their ability to self-renew and so terminally differentiate. This might be followed by a wave of definitive haematopoiesis, where HSCs arise and expand in numbers if the conditions are appropriate. It is likely that both primitive and definitive haematopoiesis takes place in the co-cultures. Further *in vitro* analysis at different time points and transplantation studies will help to clarify the timing of HSC emergence (if any) in the co-cultures, as well as demonstrating their *in vivo* repopulating potential.

#### ***4.5.4 Why would AM but not UG subregions promote haematopoietic differentiation of ES cells?***

The effects of AM-derived lines on haematopoietic differentiation of ES cells is interesting; since, in the embryo, the AM subregion of the AGM is where LTR-HSCs are believed to first arise *de novo* (de Bruijn et al., 2000a, 2000b, 2002). Definitive HSCs bud from rounded cell clusters present on the ventral floor of the dorsal aorta at E10-10.5. These intra-aortic clusters are thought to represent haemogenic endothelium as they express both haematopoietic and endothelial markers (CD45<sup>+</sup>CD34<sup>+</sup>) (Wood et al., 1997) and Runx1 is expressed in these clusters at E10 (North et al., 1999). The AM stromal cell lines were derived from E10-11 AGM regions; therefore, it is possible that the factors responsible for the initial induction and emergence of HSCs are potently expressed by these lines. This could explain why undifferentiated ES cells, which have not yet committed to a particular lineage, can readily respond by undergoing haematopoietic differentiation; or why ES-derived haematopoietic progenitors which arise spontaneously in EBs can respond.

It is thought that HSCs which emerge from the AM subregion of the AGM subsequently move to urogenital ridges at E11 where they undergo proliferation (de Bruijn et al., 2000a, 2000b, 2002). The urogenital ridges appear to be the only site in the embryo where HSC proliferation occurs without differentiation (Taoudi et al., 2007, 2008). At E12-13, HSCs migrate to the foetal liver where they expand in numbers prior to colonising the bone marrow (Kumaravelu et al., 2002). Therefore, the foetal liver and urogenital ridges are sites where HSC proliferation occurs and it is likely that the UG- and EL-derived stromal lines produce a plethora of proliferative factors, while AM-derived stroma produce HSC inductive factors. In agreement with this, Oostendorp and colleagues (2002b) have reported that the UG26.1B6 and EL08.1D2 lines are potent supporters of transplantable adult BM-derived HSCs. The best supporter, UG26.1B6, was found to maintain adult BM HSCs for up to 4 weeks in culture without the loss of repopulating ability (Oostendorp et al., 2002b). AM14.1C4 stroma could maintain repopulating cells for up to 1 week, but were unable to provide such potent long-term support; AM20.1B4 and AM20.1A4 were not tested. In another study, UG26.1B6 and EL08.1D2 stromal lines were reported to support CFU production from human CD34<sup>+</sup> cord blood HSCs for up to 12 weeks in culture and the AM-derived lines tested (including AM20.1B4 and AM20.1A4) were not found to provide this support (Oostendorp et al., 2002a). UG26.1B6 and EL08.1D2 were also able to support transplantable E11 AGM-derived HSC (CD34<sup>+</sup>cKit<sup>+</sup> sorted cells); however, they did not support CD34<sup>+</sup>cKit<sup>+</sup> pre-HSCs derived from E10 AGM or E10 yolk sac and were unable to induce these cells to become transplantable LTR-HSCs. The AM20.1B4, AM20.1A4 and AM14.1C4 stromal lines were not tested in this way. Collectively, these data show that UG26.1B6 and EL08.1D2 stroma used in this thesis can support adult HSCs and embryo-derived HSCs, but cannot maintain pre-HSCs or induce them to become transplantable.

It could be that AM-derived stromal lines have the ability to induce haematopoietic cells and can support subsequent haematopoietic commitment. While, the UG and EL stromal lines may only act on haematopoietic stem cells once they have reached a certain level of maturation; conversely, haematopoietic cells might only be able to



respond to the stroma after they have reached this level. In support of this, the EL stromal lines were derived from E11 foetal livers and at E11-12, the foetal liver is not yet a highly supportive HSC niche. This may be reflected by the fact that a low number of clones from this tissue were found to be supportive to adult HSCs (Oostendorp et al 2002b). During embryogenesis, HSCs emerge in the AM subregion and migrate to and expand in other haematopoietic sites in a highly reproducible manner, before colonising the bone marrow niche. Therefore, it is possible that during embryogenesis, HSCs need to mature in a highly regulated fashion and that this is mediated by their interaction with specific niches in different sites in the embryo. If this is the case, this might explain why the UG and EL lines tested in the co-culture system were unable to promote haematopoietic induction of ES cells. Given that UG26.1B6 is able to maintain/expand mature BM-HSC, a two step co-culture has been attempted whereby EBs were differentiated on AM20.1B4 for 6 days, followed by culture on UG26.1B6 for 4 further days. In these preliminary experiments, however, the haematopoietic activity was not increased over that of AM20.1B4 co-culture (data not shown).

Taken together, the data presented here suggest that there are biological differences between supporting embryonic cells (AGM or ES-derived cells) and adult BM-derived haematopoietic cells. Since AM20.1B4, AM20.1A4 and AM14.1C4 were able to enhance haematopoietic differentiation of ES cells, it may be interesting to test the ability of these lines to induce and/or maintain pre-HSC ( $CD34^+cKit^+$ ) populations isolated from AGM or yolk sac. This is plausible, as Matsuoka et al (2001) reported that another AGM-derived stromal cell line, AGM-S3, was able to support induction of LTR-HSCs from E8.5 yolk sac and para-aortic splanchnopleural cells.

UG and EL lines were derived from E11 embryos, while AM20.1B4 and AM20.1A4 were derived from E10 embryos. Therefore it may seem as though the differential effects of the lines on ES cells could be due to a timing difference. However, this is unlikely to be the case, as AM14.1C4 was derived from E11 AGM tissue and has also been found to be a potent promoter of ES cell haematopoietic differentiation. It



could also be argued that the effects of the lines could be due to the difference in transgenic embryos used, since AM20.1B4 and AM20.1A4 lines were derived from *tsA58* transgenic embryos and EL08.1D2 was derived from BL1b *Sca-1-LacZ* transgenic embryos. However, AM14.1C4 cells were also derived from BL1b embryos, therefore this does not correlate with the differential effects of the lines. In light of this, the data presented here strongly suggest that the AM subregion of the E10 and E11 AGM is rich in haematopoietic inductive factors which can significantly promote haematopoietic differentiation of ES cells.

It is interesting that, to date, few embryo-derived stroma have been found to be comparable with OP9 bone marrow stroma in their ability to promote haematopoietic differentiation of ES cells. For instance, Weisel and Moore (2006) generated 106 clonal stromal lines from E10.5 AGM regions. Of ten selected stromal lines, only one was reported to be comparable with OP9 stroma. In their study, and in most others, stromal lines have been derived from whole AGM regions (Xu et al., 1998; Ohneda et al., 1998) as opposed to AM or UG subregions. Here, 3 stromal lines have been identified which are comparable to OP9 and are all derived from the same subregion of the AGM. Taken together, these observations could indicate that the differential effects of the AM, UG and EL stromal lines are due to the difference in anatomical sites from which the lines were derived. However, it cannot be assumed that all stromal clones derived from the AM subregion will be potent promoters of haematopoietic differentiation of ES cells, nor can it be assumed that other UG or EL derived lines will not have enhancing capabilities. In accordance with this, Oostendorp and colleagues (2002a; 2002b) found that some AM-derived lines had intermediate supportive effects on adult HSCs and cord blood HPCs. Therefore, they did not conclude that the supportiveness of the stromal lines was wholly dependent on their embryonic site of origin. It would be necessary to test a larger panel of clonal stromal cell lines from the AM and UG subregions in the EB co-culture system to conclusively determine whether the ability to enhance haematopoietic differentiation of ES cells falls mainly in the AM subregion of the E10-11 AGM.

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Chapter Five

**Investigating the cellular interactions involved in the  
haematopoietic enhancing effects of co-culture**

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## 5.1 Aim

To identify cellular interactions involved in the enhancement of haematopoietic differentiation of ES cells mediated by AM-derived stromal cell lines.

## 5.2 Introduction

Three clonal stromal cell lines derived from the AM subregion of the E10-11 AGM have been identified which are capable of significantly enhancing haematopoietic differentiation of mouse ES cells. The experiments described here investigate cellular interactions which may have a role in this enhancing activity. Experiments have been designed to test (i) whether EB/stromal cell-cell contact was important or if haematopoietic enhancing factors were secreted; (ii) whether extracellular matrices isolated from the stromal cell layers retained the enhancing effects of co-culture and (iii) whether stromal lines act by inducing ES cells to haematopoietic fates or by promoting proliferation of ES cell derived haematopoietic progenitors. In addition, ES cells carrying a *Brachyury*-eGFP reporter gene (Bry-201 ES cells), which is a marker of mesoderm, have been used in co-culture to test whether AM stroma mediate their enhancing effects by promoting commitment of ES cells to mesodermal fates.

## 5.3 Experimental approach

- To determine whether the enhancing effects of co-culture were dependent on direct cell-cell contact, EBs were cultured in medium conditioned on stromal layers or co-cultured in transwell inserts above the stroma.
- To determine if extracellular matrices (ECMs) retain the enhancing effects of co-culture, EBs were co-cultured on ECMs that were isolated from confluent irradiated stromal cells.
- To test whether the stromal lines stimulate *de novo* induction of ES cells to haematopoietic fates or whether they act by promoting proliferation of

haematopoietic progenitors, individual EBs were co-cultured on stromal cells, before disaggregation and seeding into colony assays to determine the proportion of single EBs with haematopoietic activity and the number of haematopoietic CFU in each EB.

- To determine if the AM-derived lines mediate their enhancing effects by promoting numbers of ES cells committing to mesodermal fates, Bry-201 EBs were co-cultured. The kinetics of *Brachyury*-eGFP expression during co-culture was determined to reveal if a higher proportion of *Bry*<sup>+</sup> cells were present in AM co-cultures compared to gelatin controls.
- To test whether the *Bry*<sup>+</sup> cells and their progeny are the populations which are responsive to the haematopoietic inductive effects of co-culture, *Bry*-eGFP positive and negative ES cells were sorted by FACS and co-cultured to determine whether haematopoietic CFU activity correlated with cells that had expressed *Brachyury*.

## 5.4 Results

### 5.4.1 *The enhancing effects of the AM-derived lines are dependent on direct cell-cell contact or on short-range secreted factors*

Hanging drop 7a-GFP EBs were differentiated in suspension in the presence of medium which had been conditioned on AM20.1B4, UG26.1B6 or EL08.1D2 irradiated stromal cell layers (-LIF). The resultant CFU-A activity in EBs was measured at 2, 4, 6, 8 and 10 days differentiation (Figure 5.1). CFU-A activity was markedly reduced in EBs differentiated in conditioned medium, as compared to those in unconditioned medium ( $p=0.004$ ). However, there were no significant differences between AM20.1B4 conditioned medium and that of UG26.1B6 ( $p=0.56$ ) or EL08.1D2 ( $p=0.77$ ). Therefore, AM20.1B4 conditioned medium did not retain the CFU-A enhancing effects of co-culture. On the contrary, conditioned media appeared to inhibit CFU-A activity in differentiating EBs.

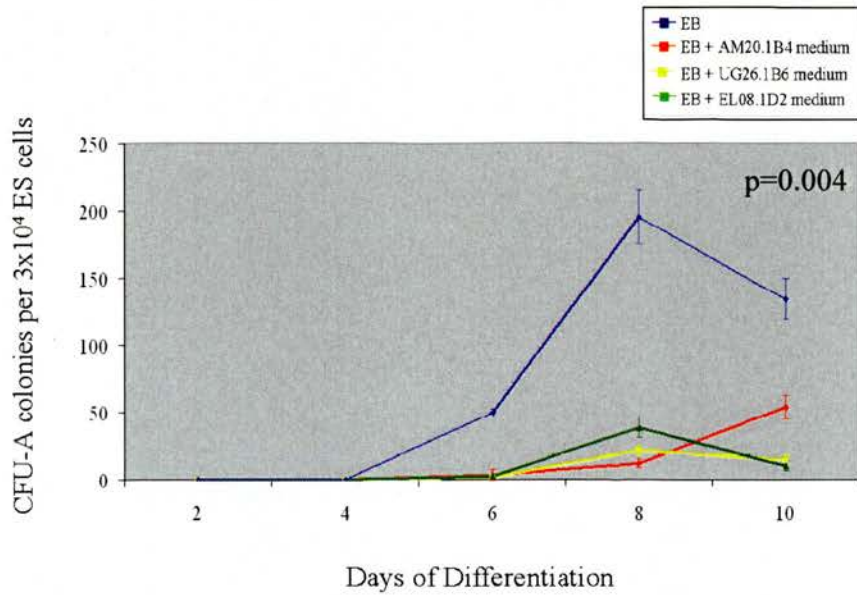


Figure 5.1 CFU-A activity in 7a-GFP EBs differentiated in suspension cultures containing medium conditioned on irradiated stromal layers. CFU-A activity was significantly reduced in EBs differentiated in the presence of medium conditioned on the stromal cell lines,  $p=0.004$ . There were no significant differences between AM20.1B4 conditioned medium and that of UG26.1B6 ( $p=0.56$ ) or EL08.1D2 ( $p=0.77$ ). Shown are triplicate assays from one representative experiment. P-values calculated using paired Mann Whitney U tests.



In the AM stromal/EB co-culture system, EBs were plated down on stromal layers and it is possible that this direct interaction with EBs might affect the factors or concentration of factors secreted by the stroma. In order to mimic this microenvironment when testing the role of cell-cell contact, 1 day 7a-GFP EBs were cultured in transwell inserts above the stroma. The transwells prevented direct cell-cell contact, but allowed secreted factors to pass through a porous membrane (pore size  $0.4\mu\text{m}$ , pore density  $2 \times 10^6 \text{ cm}^{-2}$ ). Thus, direct contact between ES cells and stromal cells was prevented, while the supernatant microenvironment remained the same. After 6 days of differentiation, EBs were harvested from the wells or transwell inserts and analysed by colony assay (Figure 5.2). The frequency of multipotent progenitors (CFU-Mix, CFU-GM and Ery/Mac) in AM20.1B4, AM20.1A4, AM14.1C4 and OP9 cultures was significantly reduced in non-contact cultures compared to the corresponding contact cultures ( $p < 0.03$ , paired Mann Whitney U tests). The frequency of unipotent CFU-M colonies was also reduced when contact was prevented with AM stroma,  $p < 0.05$  (Figure 5.2b). There were no significant differences between contact and non-contact gelatin control cultures, demonstrating that the transwell inserts themselves did not affect haematopoietic output. Taken together, these data demonstrate that AM-derived stroma are likely to mediate their haematopoietic enhancing effects in a contact-dependent manner. As stated previously, OP9 stromal cells do not express functional M-CSF, which promotes macrophage differentiation. In agreement with this, low numbers of CFU-M colonies were generated in OP9 cultures (Figure 5.2b). Interestingly, CFU-M activity in contact and non-contact OP9 cultures was comparable ( $p = 0.13$ ), suggesting that EBs might express M-CSF which allows some CFU-M formation.

The enhancing effects of the stromal cell lines in the wells did not appear as pronounced as previously observed in  $25\text{cm}^2$  flasks (chapter 4). This may indicate that the overall haematopoietic output is affected when the surface area into which the ES cells can expand is limited. Nevertheless, non-contact cultures did not show any enhancing activity in 4 independent experiments. Haematopoietic activity was significantly higher in contact cultures compared to non-contact cultures and conditioned medium caused a reduction in CFU-A activity in EBs.



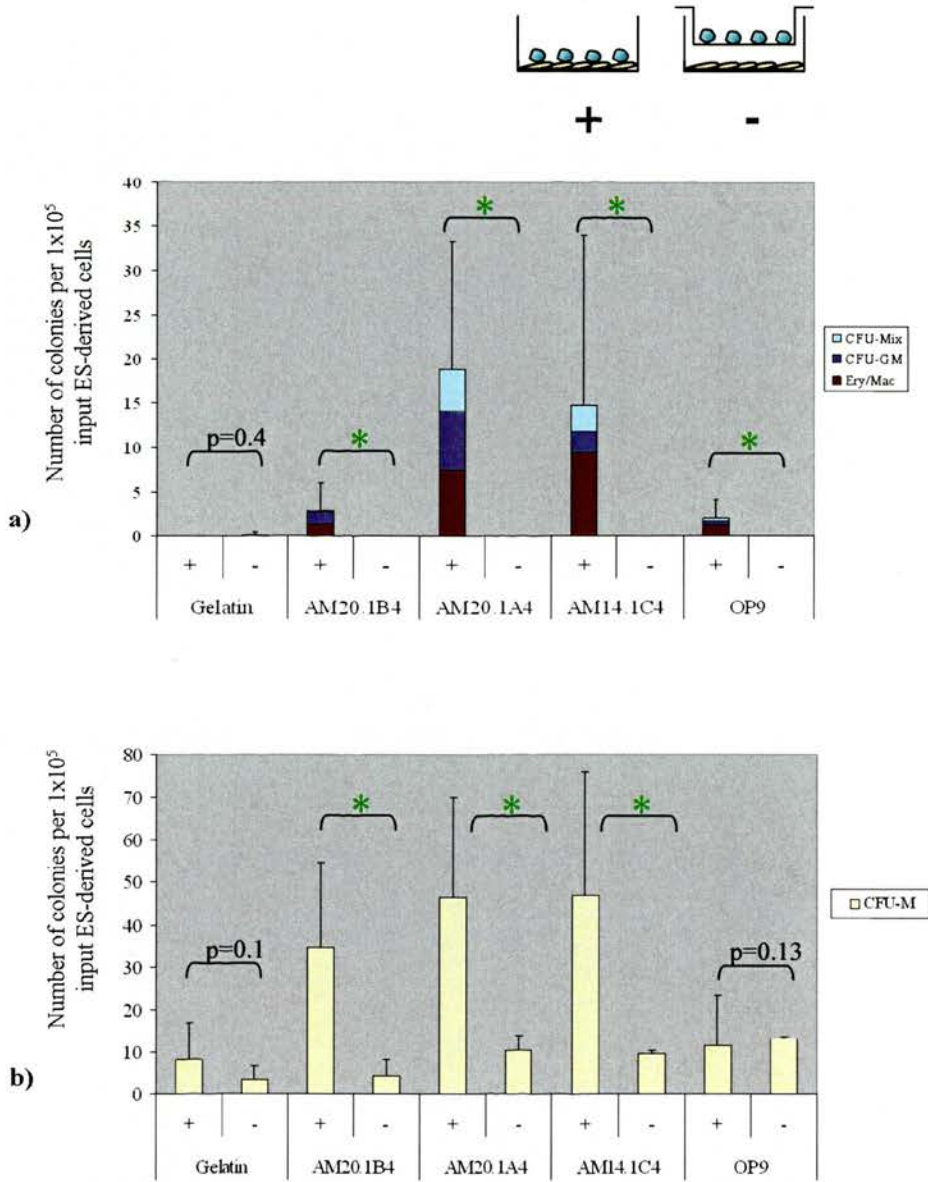


Figure 5.2 Haematopoietic CFU generated when 1 day 7a-GFP EBs were co-cultured to 6 days differentiation in direct contact with stromal cell layers (+) or in transwell inserts (-). Shown are the frequencies of (a) multipotent CFU-Mix, CFU-GM and Ery/Mac ( $*p < 0.03$ ) and (b) CFU-M ( $*p < 0.05$ ). Data represent 4 independent stromal co-culture experiments and 1 gelatin control experiment.

Given these observations, it is possible that the enhancing activity of stromal co-culture relies on a delicate balance between positive regulators and secreted inhibitory factors. The data presented here have highlighted the importance of direct cell-cell contact in mediating the haematopoietic enhancing effects of the AM-derived stromal cell lines. It should be noted, however, that the effects of high concentrations of short-range secreted factors cannot be ruled out using this non-contact transwell strategy.

#### ***5.4.2 Enhancing effects of the stromal lines are not retained by extracellular matrices isolated from AM stromal cell layers***

In light of the above results, it was determined whether extracellular matrices (ECMs) isolated from the stromal lines retained the haematopoietic enhancing activity of co-culture. ECMs were extracted from confluent stromal cell layers 1 day after irradiation using the deoxycholate method described by Hedman et al (1979). One day old 7a-GFP EBs were cultured on intact stromal layers or on ECMs for up to 6 days differentiation before disaggregation and analysis by haematopoietic colony assay (Figure 5.3). Co-culture of EBs with ECMs isolated from AM20.1A4 and AM14.1C4 stroma resulted in a significant reduction in overall CFU activity compared to the corresponding stromal co-cultures ( $p < 0.05$ , paired Mann Whitney U tests). Thus, ECM components did not retain the enhancing effects of co-culture. The reduction in haematopoietic readout in ECM cultures appeared to be due to a decrease in multipotent CFU-Mix, CFU-GM and Ery/Mac, as well as CFU-M. Therefore, the ECMs did not retain enhancing activities specific to particular haematopoietic lineages.

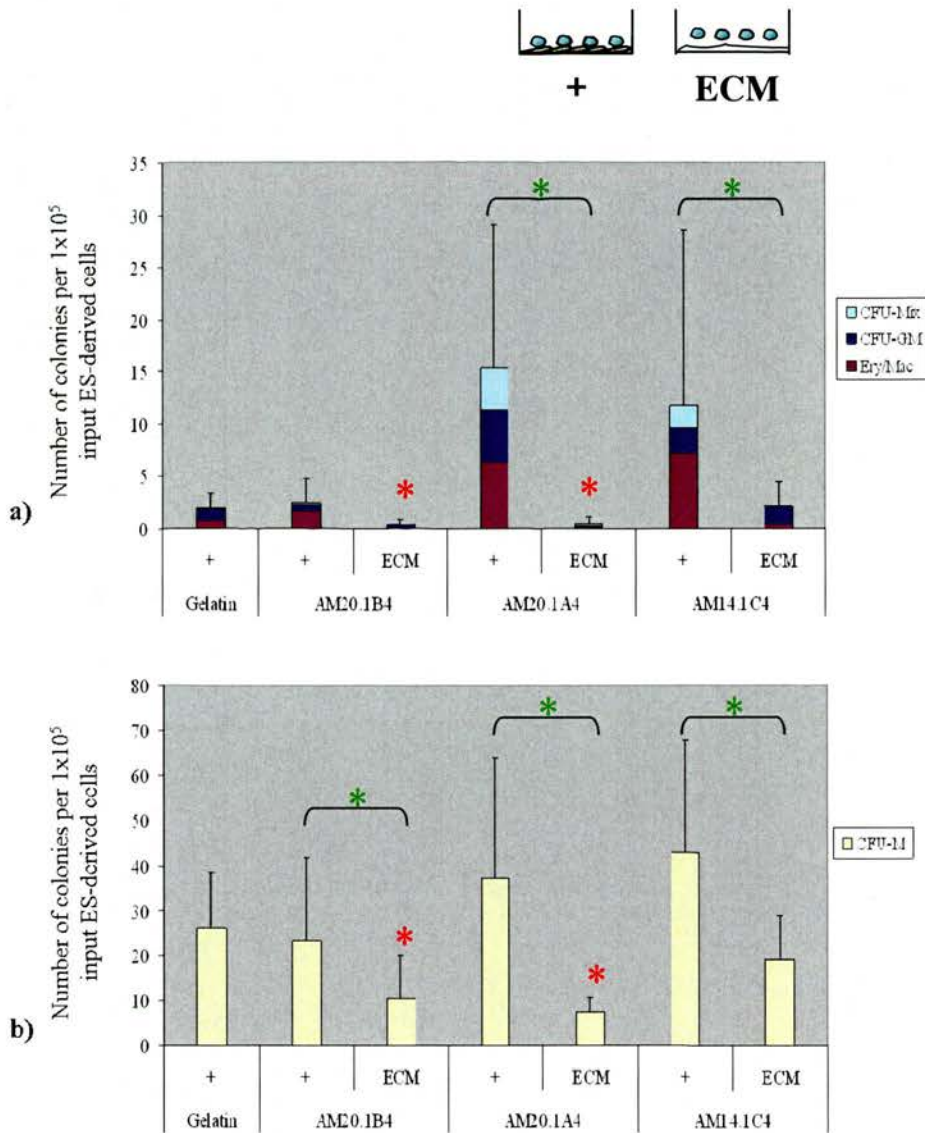


Figure 5.3 Haematopoietic activity in 7a-GFP EBs co-cultured on extracellular matrices (ECM) isolated from irradiated stromal cell layers. Shown are the frequencies of (a) multipotent CFU-Mix, CFU-GM and Ery/Mac and (b) CFU-M generated from EBs in culture with ECM or on intact stroma (+). Data represent 4 independent co-culture experiments. There was a reduction in haematopoietic activity in EBs differentiated on ECMs compared to the corresponding co-cultures ( $*p < 0.05$ ) and some ECMs inhibited CFU compared to gelatin controls ( $*p < 0.014$ ).



In these experiments, AM20.1B4 co-culture did not enhance haematopoietic differentiation in EBs compared to gelatin control cultures; however, it is interesting to note that the ECM isolated from AM20.1B4 stromal layers caused a significant reduction in EB-derived haematopoietic activity compared to gelatin controls ( $p < 0.014$ ) (Figure 5.3). This was also seen with ECMs isolated from AM20.1A4 stroma. While this could be due to residual deoxycholate detergent left behind after the ECM extraction, it is possible that some ECM components could have an inhibitory effect on haematopoietic differentiation. Stromal layers might secrete negative regulators of differentiation, which cell-cell interactions need to overcome in order to mediate haematopoietic enhancing effects. These data support the hypothesis that a balance between positive and negative regulatory signals determines the outcome of AM stromal/EB co-culture and slight changes in this balance might contribute to the variability between co-culture experiments.

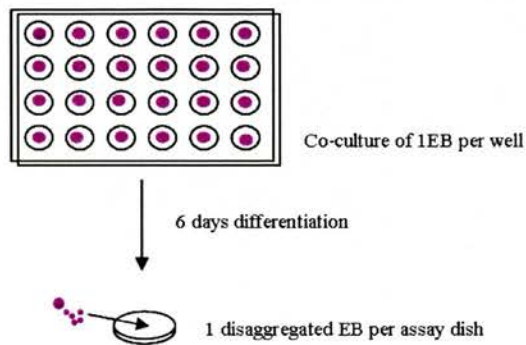
#### ***5.4.3 AM20.1B4 can have a de novo inductive effect on haematopoietic differentiation of ES cells***

In the E10-11 AGM region, *de novo* induction as well as proliferation of definitive LTR-HSCs takes place (Medvinsky and Dzierzak, 1996, de Bruijn et al., 2000a, 2000b, 2002). This suggests that the AGM provides a signals for both these processes. Therefore, it was of interest to determine whether AM-derived stroma had a proliferative effect on haematopoietic progenitors arising spontaneously upon ES cell differentiation or whether co-cultures could promote *de novo* induction of ES cells to haematopoietic fates. Single cell differentiation studies could distinguish between inductive and proliferative effects; however, this strategy could not be applied to the stromal/EB co-culture system used here. To date, there are no published reports which explicitly distinguish between haematopoietic induction of cells within EBs and proliferation of EB-derived haematopoietic progenitors. Yet, this is an important question to address if one is aiming to identify novel factors that can induce haematopoietic differentiation of ES cells and not simply expand haematopoietic progenitors in culture.

To distinguish between inductive and proliferative effects, individual 1 day old 7a-GFP EBs were co-cultured on stromal layers in wells of a 24 well plate to 6 or 10 days of differentiation. Individual EBs were disaggregated and subjected to colony assays to determine the proportion of EBs with haematopoietic activity and the number of haematopoietic CFU in each EB. It was hypothesised that an increase in the proportion of EBs with CFU activity compared to gelatin controls could imply that *de novo* induction had occurred; whereas an increase in the number of CFU per EB could be due to proliferation of haematopoietic progenitors (Figure 5.4).

The CFU-A assay was used to measure haematopoietic activity, as it lends itself to analysis of multiple samples. Table 5.1 summarises the data from the single EB analyses. In accordance with previous findings, UG26.1B6 and EL08.1D2 did not enhance CFU-A activity as compared to gelatin controls. In contrast, AM20.1B4 had a considerable proliferative effect on CFU-A progenitors at 6 days of differentiation. There was a significant increase in the number of CFU-A progenitors in each EB, with an average of 16.8 CFU-A/EB on AM20.1B4 compared to only 3.2 CFU-A/EB on gelatin, equating to a 5 fold increase ( $p < 0.001$ ,  $n = 201$ ). After 10 days of differentiation, there was a 3 fold increase in CFU-A per EB differentiated on AM20.1B4 compared to those cultured on gelatin (37 CFU-A/EB compared to 11 CFU-A/EB, respectively,  $p < 0.0001$ ,  $n = 48$ ). This correlates with the CFU-A activity observed in AM20.1B4 co-cultures carried out in 25cm<sup>2</sup> flasks, where there was a 3 fold increase at 6 days of differentiation and a 3.2 fold increase at 10 days, as compared to gelatin (Chapter 4, Figure 4.5).

Interestingly, AM20.1B4 co-culture also caused in a significant increase in the proportion of EBs with CFU-A activity, suggesting that this line could induce ES cells to CFU-A fates (Table 5.1). At 6 days differentiation, 64.2% of EBs co-cultured on AM20.1B4 generated 1 or more CFU-A colonies compared to 52.7% on gelatin ( $p = 0.02$ ,  $n = 201$ ). After 10 days of differentiation, 93.5% of AM20.1B4/EBs had CFU-A activity compared to only 68.3% of EBs on gelatin ( $p = 0.0044$ ,  $n = 48$ ); providing further evidence of the inductive effects of the AM20.1B4 cell line.



Score proportion of EBs with haematopoietic activity and number of colonies per EB

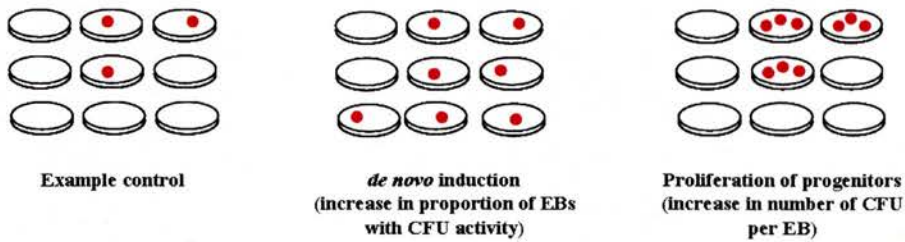


Figure 5.4 Strategy for analysis of single EBs. After co-culture in wells of a 24 well plate (with stroma or on gelatin), individual EBs were disaggregated and each EB was seeded into a separate colony assay dish. The proportion of single EBs (dishes) with colonies and the number of colonies in each EB was scored to determine whether haematopoietic induction of ES cells or proliferation of haematopoietic progenitors had occurred. Control EBs were cultured on gelatin alone.



Co-culture (n= number of EBs)		% EBs with haematopoietic activity	Haematopoietic colonies per EB
6 days	Gelatin/EB (n=207)	52.66%	3.15 (+/- 6.9) (range 0-62)
	AM20.1B4/EB (n=201)	64.18% (p=0.02)	16.78 (+/-33.7) (range 0-164) (p<0.001)
	UG26.1B6/EB (n=47)	42.55% (p=0.26)	1.68 (+/-3.7) (range 0-22) (p=0.14)
	EL08.1D2/EB (n=48)	27.08% (p=0.002)	0.38 (+/-0.7) (range 0-3) (p=0.0003)
10 days	Gelatin/EB (n=48)	68.3%	11.1 (range 0-45)
	AM20.1B4/EB (n=48)	93.5% (p=0.004)	36.98 (range 0-110) (p<0.0001)
	EL08.1D2/EB (n=48)	60.9% (p=0.51)	7.0 (range 0-40) (p=0.09)

Table 5.1 Co-culture of individual EBs and analysis of haematopoietic activity in each EB by CFU-A assay. The proportion of EBs with haematopoietic activity and the average number of CFU-A per EB was determined. Data represent EBs from 2 to 7 independent co-culture experiments and p-values were calculated by performing Fisher's exact tests on contingency tables, comparing stromal co-cultures to gelatin controls.

To confirm that the CFU-A enhancing effects were dependent on direct stromal cell-ES cell contact, single EBs were co-cultured per well or transwell insert to 6 or 10 days of differentiation (Table 5.2). At both time points, CFU-A activity was significantly reduced in EBs when contact with AM20.1B4 stroma was prevented; yet, the activity in contact and transwell gelatin control cultures was comparable. Again, these data demonstrate that the enhancing activity of AM20.1B4 is likely to be dependent on direct cell-cell contact. Furthermore, in non-contact AM20.1B4 cultures, there was a significant reduction in the proportion of EBs with CFU-A activity as well as the number of CFU-A progenitors per EB. Therefore, both the proliferative and inductive effects of AM20.1B4 appear to be contact-dependent.

When considering these data, it is important to note that two key assumptions have been made; namely, (i) each colony represents a single progenitor cell (CFU) and, (ii) every progenitor with CFU-A capability is detected. The latter point is pertinent, since if the number of progenitors seeded into the assay have to exceed a threshold before a CFU-A colony is likely to be detected, then one might argue that an increase in the proportion of EBs with CFU-A activity could also be due to proliferation of progenitors, provided that progenitor numbers in the cultured EBs were stimulated to expand beyond this threshold. However, even if the latter were the case, the data presented here demonstrate that AM20.1B4 exerted an enhancing effect on a significant proportion of co-cultured EBs, in that enhanced CFU-A activity did not arise from a small number of EBs that responded to co-culture. Another important point is that the growth rates of the differentiating ES cells were not significantly different between the co-cultures (Chapter 3, Figure 3.2). Therefore, the apparent inductive and proliferative effects of AM20.1B4 are unlikely to be due to a difference in the cell numbers within EBs differentiated on the stromal cell line or on gelatin.

Co-culture		% EB with CFU-A activity		Frequency of CFU-A per EB	
		Contact	Non-contact	Contact	Non-contact
6 day	Gelatin/EB (n=24)	50%	37.5% (p=0.56)	3.5 (range 0-18)	4.5 (range 0-38) (p=0.67)
	AM20.1B4 /EB (n=24)	75%	16.7% (p=0.0001)	4.0 (range 0-20)	0.6 (range 0-11) (p=0.0001)
10 day	Gelatin/EB (n=24)	54.2%	70.8% (p=0.37)	5.4 (range 0-30)	10.3 (range 0-38) (p=0.29)
	AM20.1B4 /EB (n=24)	87%	47.8% (p=0.01)	46.9 (range 0-110)	11 (range 0-43) (p=0.0001)

Table 5.2 CFU-A activity in single EBs in contact and non-contact (transwell insert) cultures was determined after 6 days and 10 days differentiation. CFU-A activity in AM20.1B4/ EBs was reduced in non-contact (□) compared to contact (■) cultures. Data represent EBs from 2 independent experiments. P-values calculated using Fisher's exact tests on contingency tables comparing non-contact cultures with corresponding contact cultures.



It has been shown previously (Chapter 4, Figures 4.8 and 4.9) that AM20.1A4, AM14.1C4 and OP9 co-culture do not promote CFU-A activity in EBs, but do significantly enhance the frequencies of multipotent progenitors such as CFU-Mix, CFU-GM and Ery/Mac, as well as CFU-M. Therefore, in order to assess whether these stromal lines have inductive or proliferative effects, it will be necessary to repeat the single EB analyses using methylcellulose-based colony assays which can detect these haematopoietic progenitors.

#### ***5.4.4 Kinetics of Brachyury expression during co-culture***

To gain further insight into the cellular interactions playing a role in the enhancing AM stromal microenvironments, it was considered important to identify which ES-derived population was responsive to the haematopoietic enhancing effects of co-culture. This has been addressed by co-culturing an ES cell line, Bry-201, which expresses eGFP under the control of endogenous *Brachyury* (mesoderm-specific) promoter elements (Fehling et al., 2003). The targeted allele is disrupted, as approximately two-thirds of the first exon is replaced by the eGFP expression cassette. Fehling et al (2003) demonstrated that the heterozygosity of the Bry-201 ES cells does not affect the *in vitro* expression pattern of *Brachyury* nor the haematopoietic specification or development within EBs. In addition, Herrmann et al (1991) reported that *Brachyury* heterozygosity had no significant impact on mesoderm development *in vivo* and did not affect the viability of mice.

Since *Brachyury* is an early mesoderm marker, flow cytometry analysis of the kinetics of GFP<sup>+</sup> cell emergence during co-culture should give an indication of the proportion of cells committing to mesodermal cell fates. To ensure that the proportion of GFP<sup>+</sup> ES-derived cells could be accurately measured by flow cytometry, stromal layers were stained with Vybrant DiD labelling solution (Invitrogen) prior to irradiation and co-culture (Figure 5.5). This enabled GFP<sup>+</sup> and GFP<sup>-</sup> ES cell populations to be readily distinguished from stained stromal cells.

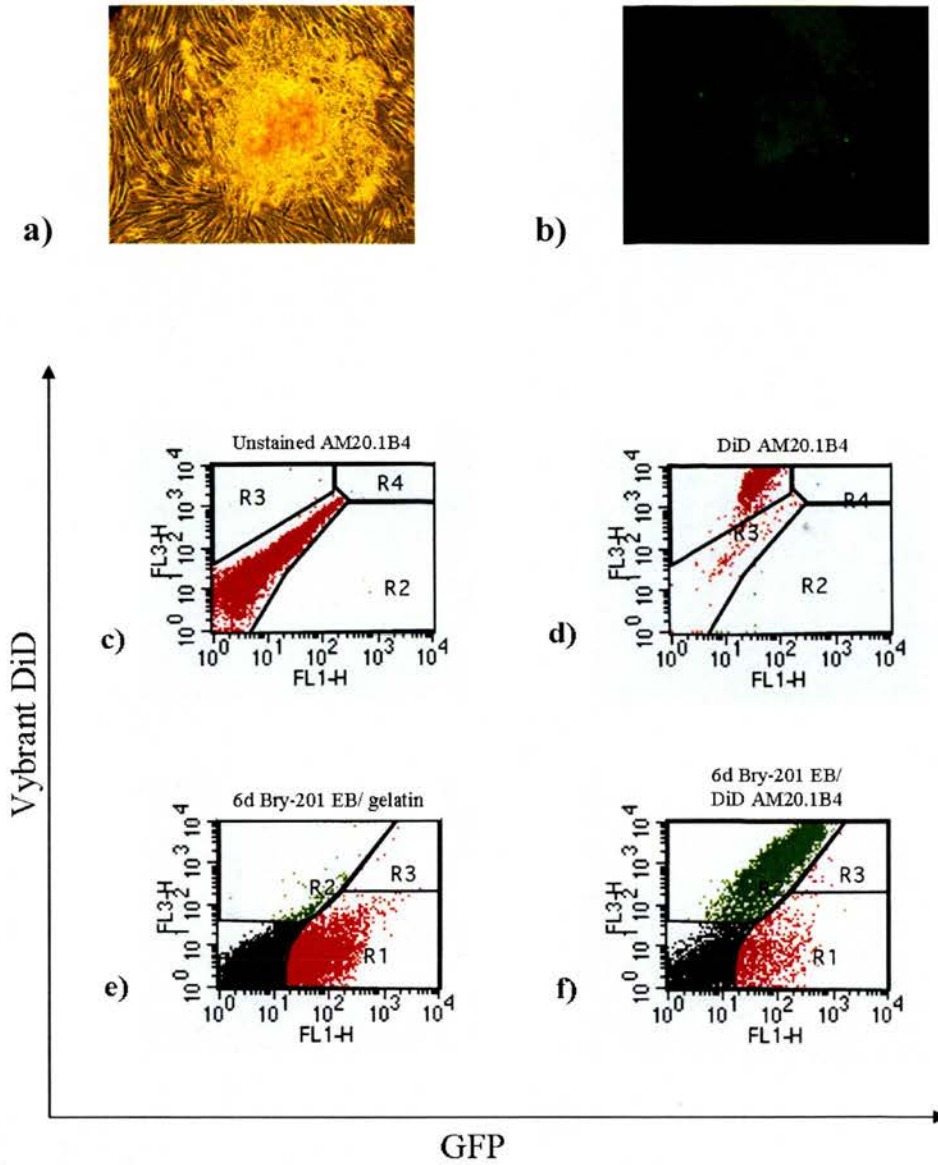


Figure 5.5 Co-culture of Bry-201 EBs on DiD stained stromal layers. (a) Light and (b) fluorescence microscopy images of a 4 day Bry-201 EB on AM20.1B4 stromal layer, showing some *Brachyury*-GFP positive ES cells. Flow cytometry of (c) unstained AM20.1B4 cells, (d) AM20.1B4 cells stained with DiD. (e) 6 day Bry-201 EBs differentiated on gelatin containing GFP+ and GFP- cells. (f) 6 day Bry-201 EBs co-cultured on DiD labeled AM20.1B4. Flow cytometry plots (c) to (f) were prepared by Kay Samuel.

Flow cytometric analysis demonstrated that in all the co-cultures, *Bry*-GFP expressing cells were first detected at 2 days of differentiation. Numbers of *Bry*-GFP<sup>+</sup> cells were increased by 4 days and peaked at 5 days of differentiation, after which the numbers were reduced (Figure 5.6). Comparisons of *Bry*-GFP kinetics revealed that there were no significant differences in the proportions of *Bry* expressing cells generated in AM20.1B4, AM20.1A4 AM14.1C4 or OP9 stromal co-cultures compared to gelatin controls,  $p=0.08$  (Figure 5.6a). Non-enhancing UG26.1B6, UG26.2D3 and EL08.1D2 co-cultures were also comparable to gelatin controls,  $p=0.19$  (Figure 5.6b). It was determined that the growth rates of the *Bry*-201 ES cells in these co-cultures were not significantly different,  $p=0.054$  (Figure 5.7). Taken together, these data suggest that AM lines did not promote numbers of cells expressing *Bry*<sup>+</sup> and non-enhancing UG-derived and EL08.1D2 stroma did not block haematopoietic differentiation by reducing numbers of (*Bry*<sup>+</sup>) cells committing to mesoderm. Therefore, all the stromal lines are likely to mediate their haematopoietic enhancing effects after *Brachyury* is expressed in EBs. In conjunction with flow cytometric analyses, *Bry*-GFP expression was visualised by fluorescence microscopy, which showed that similar numbers of *Bry*-GFP<sup>+</sup> EBs were present in the different co-cultures (data not shown). Thus, the *Bry*-GFP<sup>+</sup> cells enumerated by flow cytometry did not originate in only a small number of responsive EBs; thereby supporting the notion that the stromal lines do not enhance or block the emergence of *Bry*-GFP expressing ES cells. *Bry*-GFP<sup>+</sup> cell numbers peaked at 5 days of differentiation in all the co-cultures in 3 independent experiments (Figure 5.8). Comparison of the co-cultures at this time point showed no significant differences,  $p=0.25$ . Yet, when these cultures were analysed by methylcellulose colony assay at 6 days of differentiation, the haematopoietic activity in AM and OP9 co-cultures was significantly higher than in gelatin controls ( $p<0.002$ ) (Figure 5.8b). Interestingly, in these experiments UG26.1B6 co-culture also resulted in increased CFU output ( $p=0.006$ ). Regression analysis showed no correlation between the proportion of *Bry*-GFP<sup>+</sup> cells present at 5 days and the haematopoietic CFU output at 6 days,  $r^2=0.0273$  (Figure 5.9); thus confirming that the AM lines do not mediate their haematopoietic enhancing effects by promoting the numbers of ES cells committing to mesodermal (*Brachyury*<sup>+</sup>) cell fates.



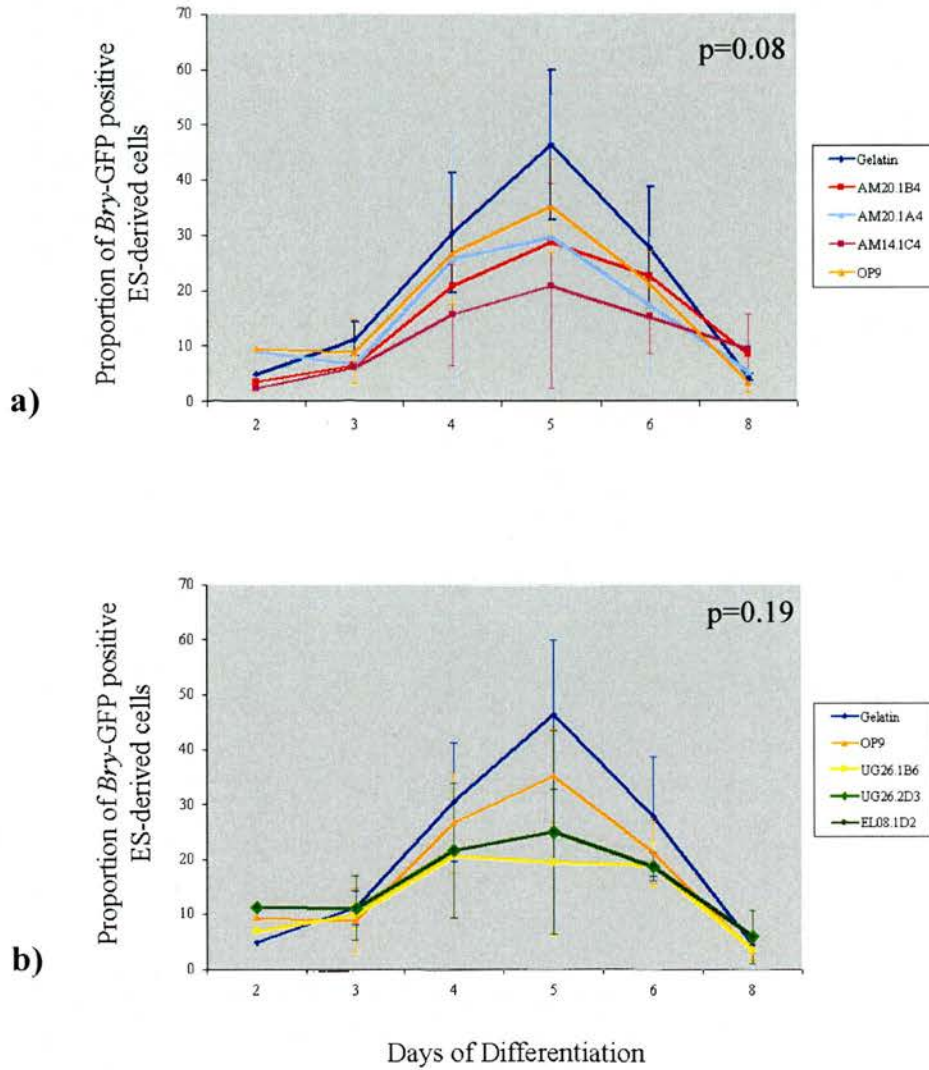


Figure 5.6 Kinetics of *Brachyury*-eGFP expression during co-culture. Co-cultured *Bry*-201 EB cells were analysed by flow cytometry at days 2, 3, 4, 5, 6 and 8 of differentiation. (a) Comparison of the enhancing AM-derived stroma with OP9 and gelatin ( $p=0.08$ ). (b) Comparison of non-enhancing UG-derived and EL08.1D2 stroma against OP9 and gelatin ( $p=0.19$ ). Data represent 3 independent experiments. P-values were calculated using paired one way ANOVA statistical tests.

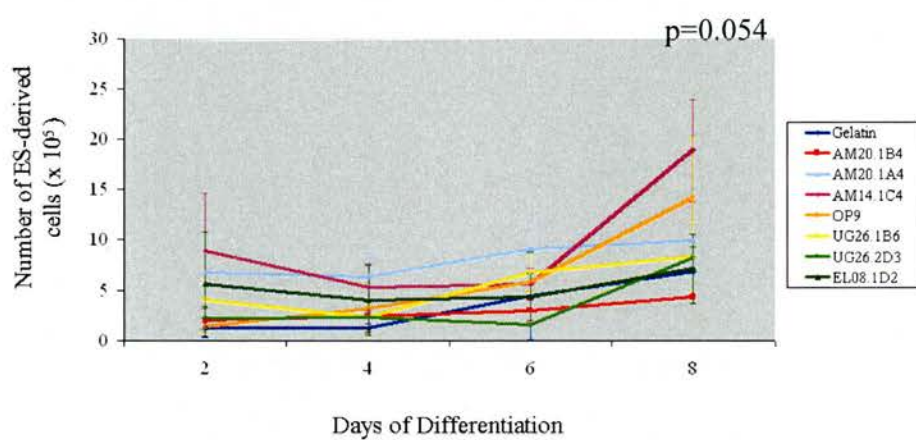


Figure 5.7 Growth rates of Bry-201 ES-derived cells differentiated in co-cultures. There were no significant differences in ES cell growth rates in the different co-cultures,  $p=0.054$ , 2 independent experiments.

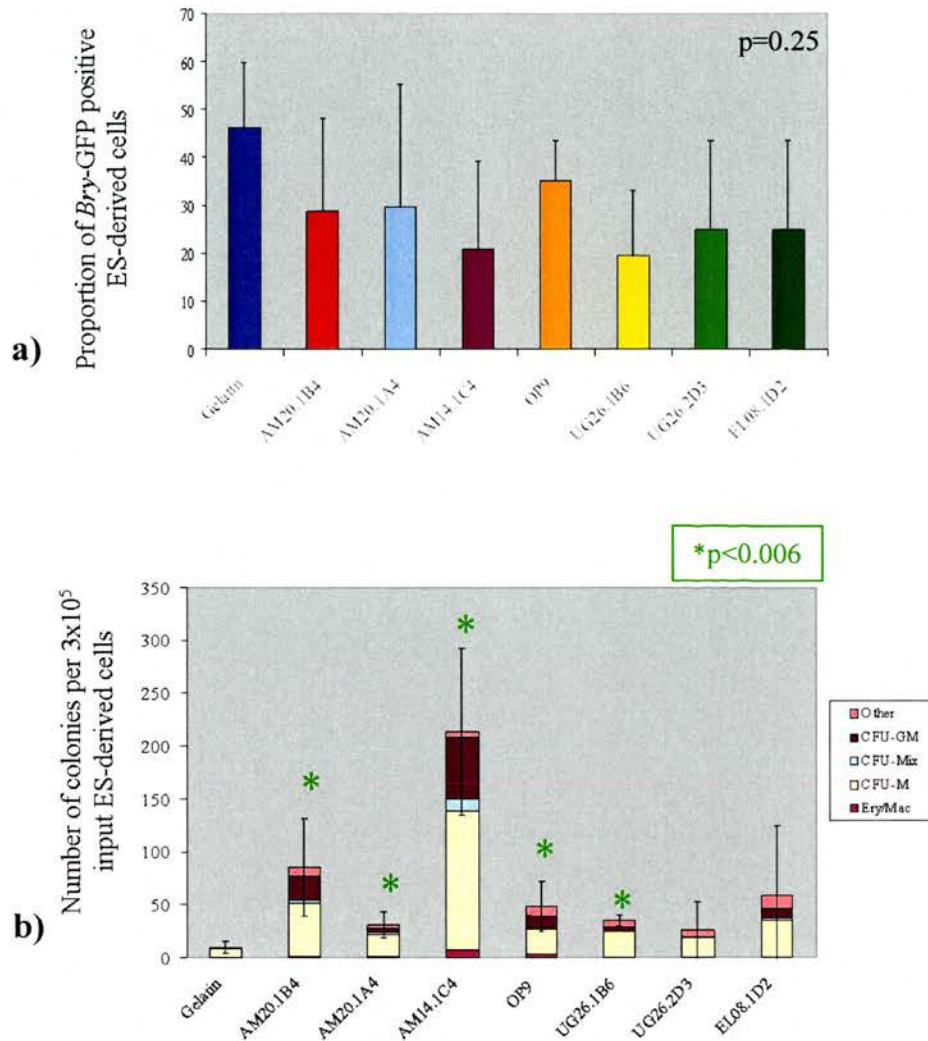


Figure 5.8 Enhancing stromal co-cultures did not contain increased proportions of *Brachyury*-eGFP positive cells. (a) Proportion of ES-derived *Bry*-GFP positive cells in co-cultures at 5 days differentiation. There were no significant differences when stromal co-cultures were compared with gelatin controls,  $p=0.25$ . (b) The haematopoietic readout of *Bry*-201 EBs in co-cultures at 6 days differentiation. Paired Mann Whitney U tests were used to calculate p-values.

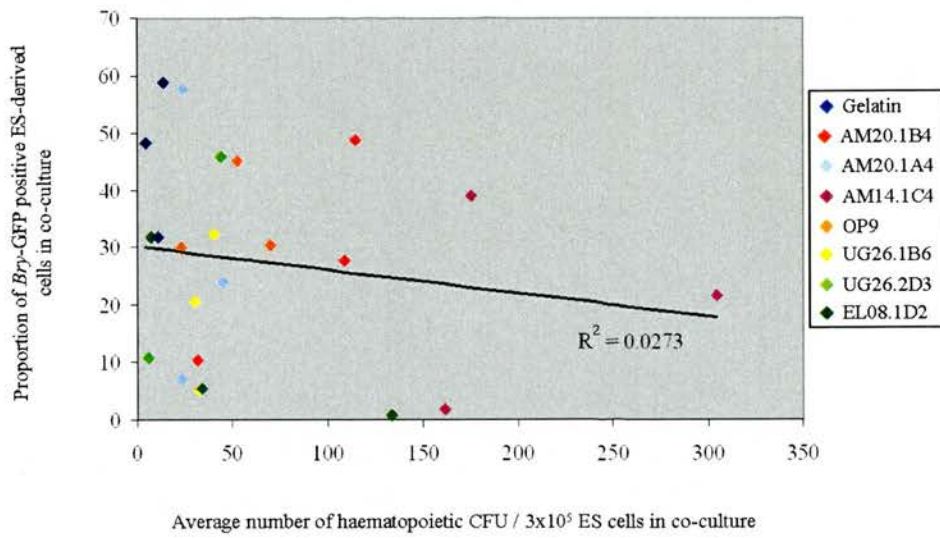


Figure 5.9 Regression analysis of *Brachyury*-eGFP positive cells in co-culture at 5 days against the haematopoietic CFU output at 6 days. There was no correlation ( $r^2=0.0273$ ) between the proportion of *Brachyury*-GFP expressing ES-derived cells present in co-cultures at 5 days differentiation and the resultant haematopoietic CFU readout at 6 days differentiation.

#### 5.4.5 Progeny of brachyury-GFP expressing cells respond to the haematopoietic enhancing effects of AM14.1C4

It was of interest to determine whether the stromal cells act after mesoderm (*Brachyury*) specification by increasing the numbers of *Brachyury*-expressing cells that go on to commit to haematopoietic fates. To address this, *Bry*-GFP positive and negative cells were sorted by FACS from 4 day suspension EBs and co-cultured for a further 6 days of differentiation (Figures 5.10 and 5.11). Due to limited cell numbers, only AM14.1C4, OP9 and gelatin conditions were tested. The sorted cell populations were co-cultured per 25cm<sup>2</sup> flask as follows: (i) 1x10<sup>5</sup> *Bry*<sup>+</sup> cells, (ii) 1x10<sup>5</sup> *Bry*<sup>-</sup> cells and (iii) 0.5x10<sup>5</sup> *Bry*<sup>+</sup> cells mixed with 0.5x10<sup>5</sup> *Bry*<sup>-</sup> cells. To control for the sorting step, intact 4 day suspension EBs (no FACS) were co-cultured (Figure 5.12a).

Haematopoietic CFU were only detected when *Bry*<sup>+</sup> cells were present in the starting co-cultured population (Figure 5.12b). Little or no CFU activity arose from *Bry*<sup>-</sup> populations. These data elegantly demonstrate that the haematopoietic activity in co-cultures was indeed derived from cells that had expressed *Brachyury* and therefore ES cells likely followed a pathway of haematopoietic lineage specification which included a *Bry*<sup>+</sup> stage. It is interesting that in AM14.1C4 co-cultures, haematopoietic activity did not appear to be further enhanced by culturing 1x10<sup>5</sup> sorted *Bry*<sup>+</sup> cells alone, compared to culturing a mixture of 0.5x10<sup>5</sup> *Bry*<sup>+</sup> and 0.5x10<sup>5</sup> *Bry*<sup>-</sup> cells (p=0.06). This suggests that, though *Bry*<sup>-</sup> cells and their progeny may not contribute directly to the haematopoietic CFU readout, these cells may contribute to the supportive co-culture niche *in vitro*. This might be in the form of other differentiated cell types with supportive abilities, or simply by acting as a carrier cell population during the co-culture procedure. The latter could be tested by co-culturing *Bry*<sup>+</sup> cells along with fibroblast cells instead of *Bry*<sup>-</sup> ES cells. This observation highlights the complexity of the co-culture microenvironment and underscores the importance of determining which other ES-derived cell types are generated in co-cultures.

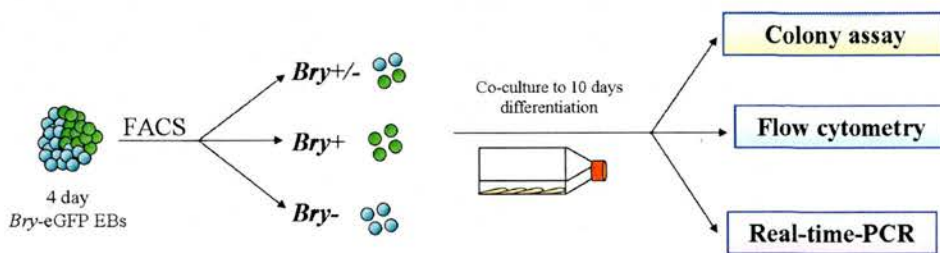


Figure 5.10 Strategy to assess whether *Brachyury* expressing cells and their progeny are the populations which respond to the haematopoietic enhancing effects of the stromal co-culture. *Bry*-eGFP positive and negative cells were sorted from 4 day old suspension EBs.  $1 \times 10^5$  *Bry*<sup>+</sup>,  $1 \times 10^5$  *Bry*<sup>-</sup> or a mixture of  $5 \times 10^4$  *Bry*<sup>+</sup> and  $5 \times 10^4$  *Bry*<sup>-</sup> cells were co-cultured on DiD labeled stromal layers or gelatin for a further 6 days differentiation. Colony assays and RT-PCR were carried out to assess haematopoietic activity. Flow cytometry enabled normalisation of colony data.



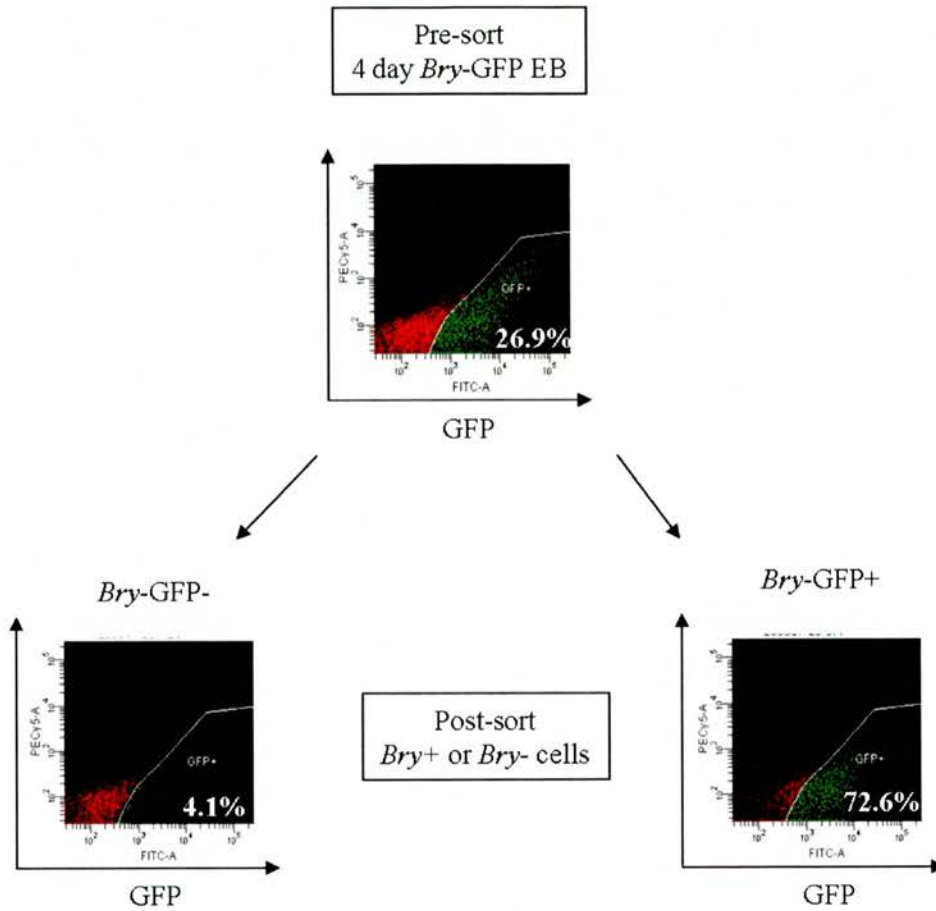


Figure 5.11 Fluorescence activated cell sorting (FACS) of *Brachyury*-eGFP positive and *Brachyury*-eGFP negative cells from 4 day old suspension EBs. The sorted populations were highly enriched. The data shown are from one representative sort.

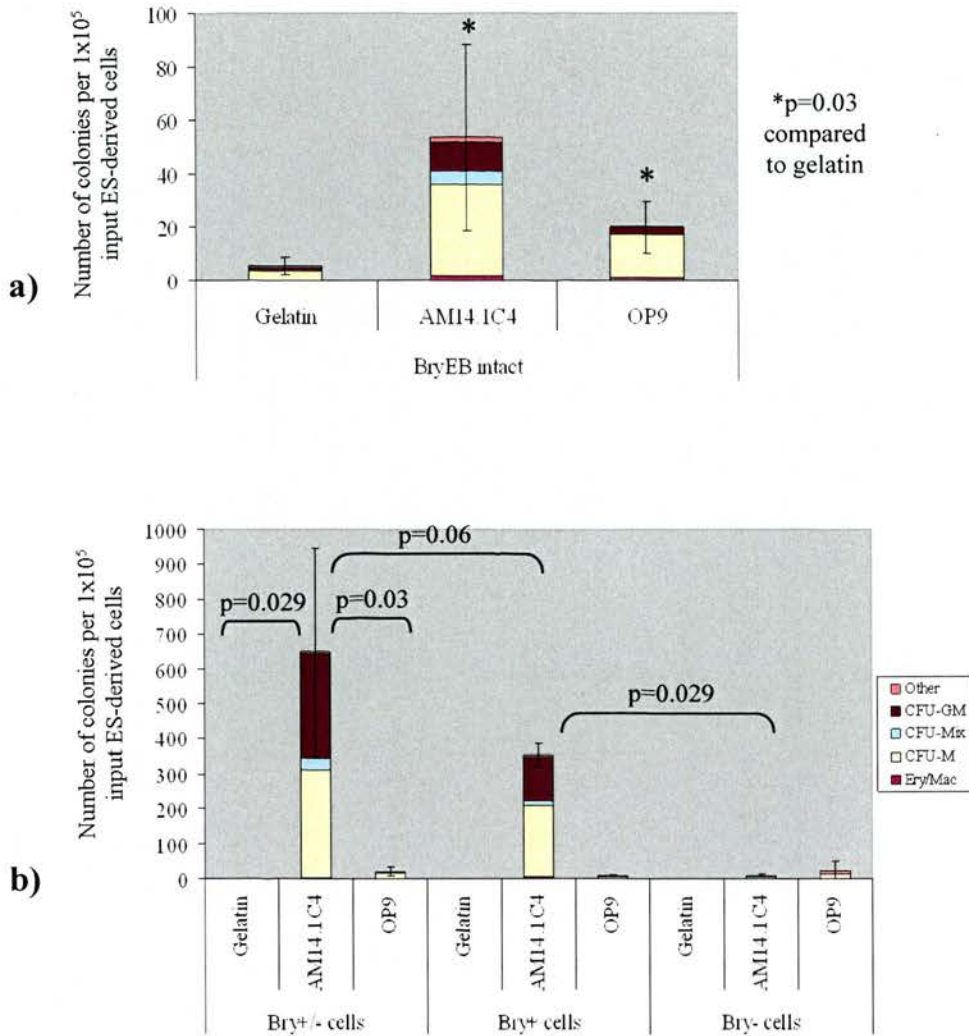


Figure 5.12 Co-culture of sorted *Bry*-GFP+ or *Bry*-GFP- sorted ES cells. (a) Intact 4 day old suspension EBs were co-cultured on gelatin, AM14.1C4 or OP9 stromal layers for a further 6 days differentiation and assessed by colony assay for haematopoietic activity. (b) *Bry*-GFP+ or *Bry*-GFP- cells were sorted from 4 day suspension EBs and co-cultured separately, or as a 50:50 mixture of positive and negative cells. The data shown represent two independent co-culture experiments. P-values calculated using paired Mann Whitney U tests.

Another possibility is that the presence of Bry-GFP<sup>+</sup> cells in AM14.1C4 co-cultures could stimulate Bry-GFP<sup>-</sup> cells to express *Brachyury*, thus allowing them to contribute to the haematopoietic CFU readout. This would explain why there was no significant difference between co-culturing  $10^5$  sorted Bry-GFP<sup>+</sup> cells or a mixture of  $0.5 \times 10^5$  Bry-GFP<sup>+</sup> and  $0.5 \times 10^5$  Bry-GFP<sup>-</sup> cells. This hypothesis could be tested by labeling Bry-GFP<sup>-</sup> cells (perhaps with DiD) prior to mixing with Bry-GFP<sup>+</sup> cells and monitoring the cells during co-culture to determine whether they become GFP<sup>+</sup> and whether they contribute to the haematopoietic readout.

In AM14.1C4 cultures, the frequency of haematopoietic CFUs was significantly increased when sorted (disaggregated) EB cells were co-cultured compared to control cultures carried out with intact 4 day old EBs (Figure 5.12). An average of  $648 \pm 298$  CFU colonies was generated when a mixture of *Bry*<sup>+/-</sup> cells were co-cultured with AM14.1C4 compared to an output of only  $53 \pm 35$  CFU when 4 day old intact EBs were co-cultured,  $p=0.03$ . Since *Bry*<sup>+</sup> cells and their progeny are the populations which give rise to the haematopoietic CFU readout and the enhancing effects of AM14.1C4 appear to be contact-dependent, it is perhaps not surprising that the CFU output is further enhanced by disaggregating EBs to release the responsive *Bry*<sup>+</sup> population such that more of these cells can be in direct contact with stroma. The responsive *Bry*<sup>+</sup> derived populations might display specific surface phenotypes which facilitate their ability to respond to enhancing effects of co-culture. Therefore, when novel factors involved in the enhancing activity are identified, one could narrow the search by looking for the corresponding surface receptors on the responsive ES derived population. In contrast to AM14.1C4, co-culture of sorted *Bry*<sup>+/-</sup> ES cells with OP9 stroma did not result in an increase in haematopoietic colony activity compared to culturing intact 4 day EBs,  $p=0.7$  (Figure 5.12). These data indicate that it was not simply the presence of a stromal layer which supported haematopoietic differentiation of sorted ES cells, but that the effects were specific to the AM14.1C4 co-cultures. Furthermore, it could suggest that the OP9 stromal line mediates enhancing activity via different mechanisms to AM-derived stroma. This is not unexpected, given that OP9 stromal cells are derived from newborn bone marrow, while AM stroma are derived from the mid-gestational AGM region; and

that *in vivo*, these two stromal cell types would interact with haematopoietic cells at different stages of development.

#### ***5.4.5 ES cells differentiated in contact with AM14.1C4 express the definitive marker, Lmo2***

When sorted *Bry*<sup>+</sup> or *Bry*<sup>-</sup> cells were co-cultured with AM14.1C4, resultant cells were also assessed by reverse transcriptase quantitative PCR to determine whether the *Bry* expressing population go on to generate cells which display the molecular characteristics of definitive haematopoietic cell types. Expression of *Lmo2* gene transcript was found to correlate closely with haematopoietic CFU activity in AM14.1C4 co-cultures after 10 days of differentiation (Figure 5.13). *Lmo2* expression also corresponded with co-culture of cells that had expressed *Bry*<sup>+</sup>. These preliminary data suggest that definitive haematopoietic cell types might have been generated. The RNA extractions and qPCR analyses depicted in Figure 5.13b were carried out by Caixin Huang as part of her Master's project in the JHBL.

### **5.5 Discussion**

It is well established that the stem cell niche plays a vital role in the induction, maintenance, expansion and differentiation of HSCs and haematopoietic progenitor cells. A complex network of interactions controls these processes *in vivo*, both within the haemogenic endothelium of the dorsal aorta when HSCs first emerge and in the adult bone marrow stem cell niche. However, the exact combination of cellular interactions, ECM components and secreted factors involved in these processes have not been elucidated. Results presented in Chapter 4 provided evidence that three clonal stromal cell lines derived from the AM subregion of the E10-11 AGM are capable of significantly enhancing haematopoietic differentiation of mouse ES cells. The aim of the experiments described here was to gain mechanistic insight into the cellular interactions which underlie these haematopoietic enhancing effects.

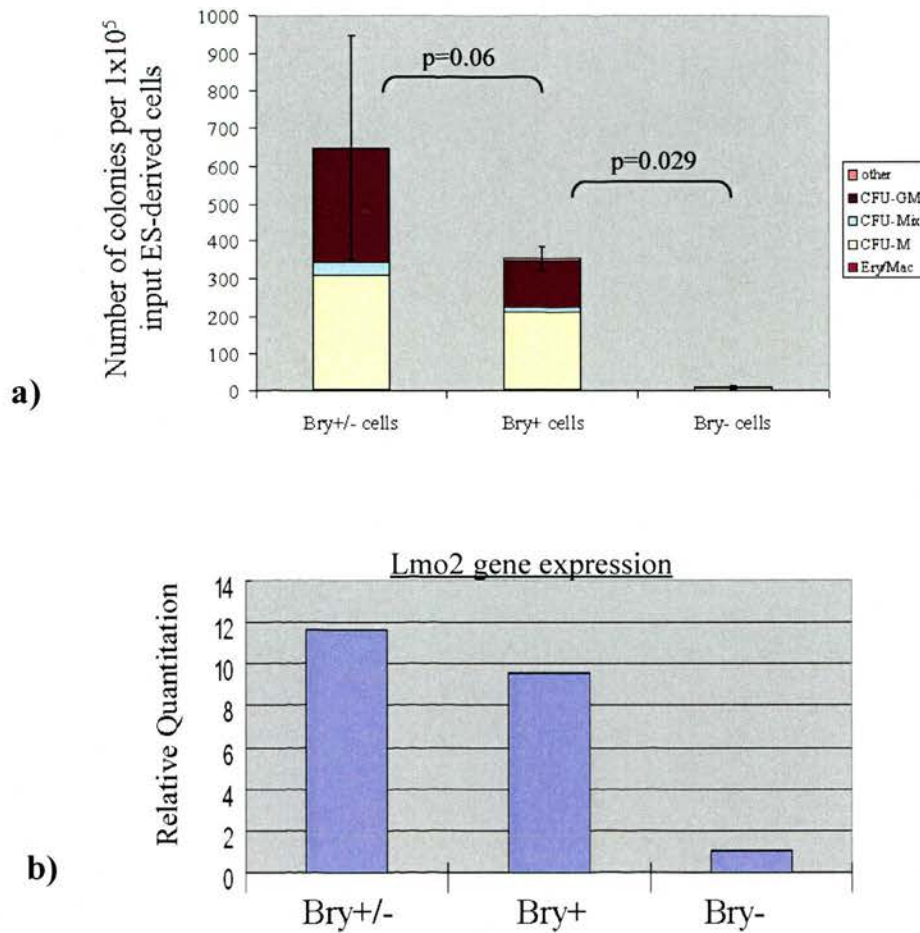


Figure 5.13 *Bry*-GFP+ or - cells sorted from 4 day suspension EBs co-cultured with AM14.1C4 to a total of 10 days differentiation. (a) Colony readout at 10 days differentiation, 2 independent experiments; (b) quantitative RT-PCR for *Lmo2* gene expression in 1 co-culture experiment. *Lmo2* gene expression correlated with co-culture of cells that had expressed *Bry*-GFP+ and with haematopoietic CFU activity. RNA extractions and quantitative RT-PCR shown in (b) were carried out by Caixin Huang.

### *5.5.1 Enhancing effects of AM-derived stroma are dependent on contact or short range secreted factors*

It has been shown that AM20.1B4 conditioned medium did not retain the CFU-A enhancing capacity of stromal co-culture. In addition, haematopoietic activity was not enhanced when direct cell-cell contact was prevented between EBs and AM20.1B4, AM20.1A4 or AM14.1C4. The data do not rule out the involvement of short-range secreted factors, but they suggest that direct cell-cell interactions play a major role in promoting haematopoietic differentiation of ES cells in the co-culture system. Furthermore, ECMs isolated from AM stroma did not retain the enhancing effects of stromal/EB co-culture and some ECMs had inhibitory effects on haematopoietic differentiation; therefore, it is possible that a balance between positive and negative regulatory signals determines the outcome of stromal/EB co-culture. Complex signalling pathways, cellular and ECM interactions can govern cell fate decisions in ES differentiation, therefore combinations of a number of mechanisms could be involved in the enhancing effects mediated by AM stromal cell lines.

These observations corroborate published findings, since the dependence of HSC/HPC maintenance on direct contact with stromal cells is a well documented phenomenon. Harvey and Dzierzak (2004) reported that contact was essential in the support and expansion of E11 aorta-derived (long-term repopulating) HSCs by UG26.1B6 and 2 additional UG-derived cell lines. In another study, Xu et al (1998) reported that proliferation of HPCs (e.g. CFU-Mix) from CD34<sup>+</sup> human cord blood cells by the AGM-S3 stromal cell line was also dependent on contact. Ohneda et al (1998) found that DAS104-4, an endothelial cell line derived from AGM, was able to significantly expand E13 foetal liver-derived HSCs (cKit<sup>+</sup> Sca1<sup>+</sup> CD34<sup>+</sup>lin<sup>-</sup>). In only 7 days of co-culture with DAS104-4, numbers of these cells in contact cultures were increased by 2,600 fold, compared to only 70 fold amplification in non-contact cultures. Importantly, it was demonstrated that contact was required to maintain the long-term repopulating abilities of the foetal liver HSCs.



It is interesting to note that, in contrast to the studies which used embryo-derived HSCs, Oostendorp et al (2005) have reported that cell-cell contact was not necessary for the maintenance of repopulating adult BM HSCs. In their experiments, cKit<sup>+</sup>Ly-6C<sup>-</sup> Hoechst33342 side population HSCs were sorted from adult BM and were maintained in contact and non-contact cultures with UG26.1B6 or EL08.1D2 for up to 6 weeks without loss of repopulating ability. This was a surprising finding, as contact mediated mechanisms are believed to be important for HSC regulation *in vivo*. Once more, these data highlight that there may be mechanistic differences between the regulation of adult HSCs, embryonic HSCs and ES cell-derived HSC/HPCs.

### ***5.5.2 AM20.1B4 can have de novo haematopoietic inductive effects on ES cells***

*De novo* induction and proliferation of LTR-HSCs take place in the E10-11 AGM region. Analysis of individual EBs co-cultured on stromal lines showed that the AM20.1B4 stromal line had a considerable proliferative effect on CFU-A activity of EBs and was also able to induce ES cells to haematopoietic fates (Table 5.1 and Table 5.2). Neither the inductive nor the proliferative effects of AM20.1B4 were observed when direct stromal/EB cell contact was prevented (Table 5.3).

The CFU-A is believed to represent amongst the earliest multipotent haematopoietic progenitors detectable by *in vitro* colony assay; one caveat is that the conditioned medium used in the assay is a source of GM-CSF and CSF-1, which can stimulate macrophages. Therefore, it is possible that the assay detects cells of the macrophage lineage. Nonetheless, CFU-A activity in single EBs is a reflection of the commitment of ES cells to haematopoietic fates, be they early multipotent progenitors or more mature macrophage progenitors.

It is interesting that AM20.1B4 can induce ES cells to haematopoietic fates and enhance proliferation of progenitors, yet it is non-supportive to adult BM-derived HSCs (Oostendorp et al., 2002a). This suggests that the signals required for the induction of HPC/HSCs from ES cells differ from the signals governing the proliferation and/or the maintenance of adult BM HSCs. Another AGM derived

stromal line, AGM-S3, has been reported to be able to induce repopulating activity in pre-HSCs derived from E8.5 yolk sac and para-aortic splanchnopleura tissues (Matsuoka et al., 2001). Given that AM20.1B4 has haematopoietic inductive effects on ES cells, it would be interesting to determine whether this line can support or induce yolk sac or AGM pre-HSC populations.

### ***5.5.3 Enhancing stromal lines do not act by promoting numbers of cells expressing *Brachyury****

Co-culture of Bry-201 ES cells expressing GFP targeted to the mesoderm-specific *Brachyury* gene demonstrated that all the stromal lines mediate their haematopoietic enhancing activity after *Brachyury* has been expressed in EBs (Figure 5.6). Regression analysis confirmed that haematopoietic enhancement in AM and OP9 co-cultures did not correlate with an increase in the proportion of cells expressing *Bry*-GFP (Figure 5.9).

Fehling and colleagues (2003) assessed *Bry*-GFP expression in Bry-201 EBs differentiated in suspension cultures and detected *Bry*-GFP<sup>+</sup> cells between days 2 and 4 of differentiation. 65% *Bry*<sup>+</sup> cells were present at day 3, this peaked at 85% on day 4 and by day 6 *Bry*<sup>+</sup> cell numbers were reduced to undetectable levels. Similar kinetics were observed by Robertson et al (2000). In Figure 5.6, the proportions of *Bry*-GFP<sup>+</sup> cells on gelatin were 12, 30, 50 and 30% on days 3, 4, 5 and 6, respectively. Thus, the pattern of *Bry* kinetics broadly correlates with that described by Fehling et al (2003), but the peak is delayed by 1 day and expression may be slightly prolonged. In the experiments described here, Bry-201 EBs were prepared in hanging drops and plated down onto gelatin or stroma on day 1 of differentiation; in contrast, Fehling et al (2003) prepared and differentiated EBs in suspension cultures. Therefore, the difference in the timing of *Bry* expression could reflect differences between these two culture methods. In addition, according to a study by Dang et al (2002), plating down EBs on gelatin prior to 4 days differentiation impairs Flk-1 induction, which can in turn impair haematopoietic differentiation and CFU activity (compared to suspension EBs). This might also account for the delay in the peak of *Bry* expression. Interestingly, this may explain the low CFU activity when 1

day old EBs are plated down on gelatin, but it suggests that the AM and OP9 stromal lines are able to overcome the haematopoietic drawbacks associated with plating down 1 day old EBs, such that they can still significantly promote haematopoietic differentiation.

When *Bry*<sup>+</sup> and *Bry*<sup>-</sup> cells were sorted from 4 day Bry-201 EBs and co-cultured, it was found that *Bry*<sup>+</sup> cells and their progeny are the populations which respond to the haematopoietic signals in AM14.1C4 co-cultures (Figure 5.12). Interestingly, disaggregation of 4 day EBs prior to co-culture significantly improved the enhancing effects of AM14.1C4 compared to culture of intact EBs, perhaps due to the release of responsive *Bry*<sup>+</sup> cells such that higher numbers came in direct contact with the stromal layer. This supports the hypothesis of a contact-dependent mechanism. Expression of *Lmo2* gene transcript was shown to correlate with haematopoietic CFU activity when sorted *Bry*<sup>+</sup> cells were co-cultured on AM14.1C4 (Figure 5.13). During mouse embryogenesis, *Lmo2* is required for establishing primitive haematopoiesis in the yolk sac and *Lmo2* null embryos die at E9-10 from anaemia due to a lack of yolk sac erythropoiesis (Warren et al., 1994). *Lmo2* is also known to be important in definitive haematopoiesis (Yamada et al., 1998) and has been shown to interact with *Scl* such that definitive haematopoiesis can occur (Lecuyer et al., 2007). Therefore, expression of *Lmo2* in AM14.1C4 co-cultures could indicate that definitive haematopoietic cells were generated. However, this is not conclusive and further RT-PCR analyses need to be carried out.

In light of the *in vivo* expression pattern of *Brachyury* and the role of the AM subregion as the site of HSC emergence, it is not surprising that AM stroma act on cells that are downstream of *Brachyury* expression. During mouse embryogenesis, *Brachyury* is first expressed in the primitive streak at gastrulation (E6.5). Expression persists in early mesoderm and epiblast cells for a short time, but is down-regulated when paraxial mesoderm cells undergo lateral migration (Herrmann et al., 1991). Later at E10, HSCs are believed to bud from intra-aortic clusters which form on the ventral wall of the dorsal aorta in the AM subregion of the AGM. These aortic clusters consist of haemogenic endothelium, which expresses both endothelial-

specific markers (VE-cadherin), as well as markers that are common to endothelial and haematopoietic cells. It is not clear whether HSCs have endothelial origins or whether they emerge from a precursor at the level of the mesenchyme and migrate through the aorta wall (Bertrand et al., 2005; Taoudi et al., 2005, 2007, 2008). The bi-potency of cell populations in E8.5 P-Sp and E10-12.5 AGM tissue has been demonstrated by *in vitro* colony assay after removing the cells from the *in vivo* microenvironment (Yao et al., 2007; Huber et al., 2004). Thus, it remains unclear whether cells with haemangioblast potential indeed give rise to both endothelial and haematopoietic lineages *in vivo*. However, during ES cell differentiation *in vitro*, haemangioblast cells have been identified; they arise in EBs at day 3.25 to 3.75 of differentiation and they have been shown to co-express Brachyury and Flk-1 (Fehling et al., 2003). Since AM stroma do not enhance numbers of ES cells expressing *Brachyury*, they are likely to act on a cell type that is down stream of the haemangioblast. It is possible that the AM lines act on an *in vitro* ES-derived cell type that shares characteristics with pre-HSCs present in aortic clusters or their progeny.

#### **5.5.4 Surface phenotype and cytokine expression by stromal lines**

The stromal cell lines used in this thesis could express a variety of factors which underlie their effects on ES cells and embryo-derived and adult HSCs. In attempt to identify novel factors involved in the haematopoietic supportiveness of the AGM- and foetal liver -derived stromal cell lines, Oostendorp and colleagues have characterised a large panel of these cell lines (Oostendorp et al., 2002a; Oostendorp et al., 2002b; Charbord et al., 2002; Oostendorp et al., 2005).

Oostendorp et al (2002a; 2002b) reported that the stromal lines used in this thesis express a number of haematopoietic cytokines; such as TPO, SCF, Flt3-L, G-CSF, IL-1 $\beta$ , IL6 and IL11. However, there was no correlation between expression of known haematopoietic cytokines by the stroma and their haematopoietic supportive abilities. AM14.1C4, UG26.1B6, UG26.2D3 and EL08.1D2 cells all expressed SCF and TPO transcripts. AM20.1B4 cells express surface bound SCF, which is in agreement with the contact-dependent effect of this stromal line. SCF is reportedly involved in the migration, proliferation and/or differentiation of HPC/HSCs

(Driessen et al., 2003) and TPO may support or regulate HSCs, as they express TPO receptor c-mpl (McKinstry et al., 1997; Yoshihara et al., 2007). Expression of IL11 and IL1 transcripts was variable in the stroma and IL3 and Oncostatin M were not detected. This expression profile is similar to the AGM derived lines used by Ohneda et al (1998) and Xu et al (1998), though the latter lines do not express G-CSF.

Since the effects of AM-derived stromal cells on ES cells were likely to be dependent on contact, it is of particular interest to compare the surface phenotypes of the enhancing and non-enhancing stromal cells. However, to date, no clear correlation has been identified between surface antigen expression and haematopoietic supportiveness of the stromal lines. Comparisons of surface phenotypes by Charbord and others (2002) revealed that none of the stromal clones express surface markers characteristic of endothelial cells or haematopoietic surface antigens (including VE-cadherin). Krassowska et al (unpublished observations) confirmed that AM20.1B4, UG26.1B6 and EL08.1D2 cells do not express CD31, CD45, c-Kit or Flk-1. Krassowska found that these stromal lines expressed comparable levels of CD29 (integrin  $\beta$ 1), CD44 (polymorphic glycoprotein 1) and VCAM-1. Interestingly, AM20.1B4 cells expressed higher levels of CD49e (integrin subunit  $\alpha$ 5) compared to UG26.1B6 and EL08.1D2; however, it is unlikely that this is wholly responsible for the differential effects of these lines on haematopoietic differentiation of ES cells. It is more likely that slight differences in expression levels of positive and negative regulators underlie the differential effects that the stromal lines have on haematopoiesis.

Charbord et al (2002) reported that, in addition to VCAM-1, CD44 and integrin subunit  $\alpha$ 5, the stroma also express integrin subunits  $\alpha$ 6 and  $\beta$ 1, as well as Sca-1, Thy1 and CD34. Sca-1 is well known to be associated with stroma that have haematopoietic supportive abilities. Furthermore, this marker is expressed on adult HSCs and it is thought that a homotypic adhesive pathway (i.e. Sca-1 on HSCs to bone marrow Sca-1) could play a role in homing of HSCs to the bone marrow niche (Remy-Martin et al., 1999; Charbord et al., 2002). The expression of integrin

subunits on the stromal cell lines could indicate that interactions mediated by very late activating antigens (VLA) might play roles. VLA-4, VLA-5 and VLA-6 antigens consist of integrin subunits  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$  and  $\alpha 6\beta 1$ , respectively. The ligands for these receptors are VCAM-1, fibronectin and laminin, respectively. VLA-4/VCAM-1 interactions are thought to be important for haematopoietic development and cell cycle regulation by facilitating HSC anchorage to stromal cells in the bone marrow (Funk et al., 1995; Hurley et al., 1997; Oostendorp et al., 1997; Peled et al., 1999). CD44 (polymorphic glycoprotein 1) is also thought to facilitate HSC interaction with the stromal cells in the bone marrow niche (Nilsson et al., 2005; Chan and Watt, 2001). Interestingly, VLA-4, VCAM-1, VLA-5 and CD44 are also expressed by AGM-S3 stroma (Xu et al., 1998).

When Charbord and colleagues (2002) compared a panel of stromal cell lines with different anatomic and developmental origins, they found that foetal liver derived stroma express higher levels of ECM markers compared to BM- and AGM-derived stroma; these included osteopontin, laminin and fibronectin (perhaps involved in HSC homing to the foetal liver). BM-derived stromal lines expressed higher levels of Sca-1 and less VCAM-1 compared to the other stromal lines, consistent with a role for HSC anchorage in the bone marrow niche. Assessment of AGM-derived stroma revealed that these cell lines express higher levels of vascular smooth muscle cell (VSMC) markers such as alpha-smooth muscle actin (ASMA), SM actinin and endoglin. Interestingly, endoglin has been shown to be required for haemangioblast specification and early haematopoietic development from murine ES cells (Perlingeiro et al., 2007). Endoglin is a TGF $\beta$  associated (non-signalling accessory) receptor and the expression of this antigen could indicate that this transduction pathway might play a role in co-cultures with AGM-derived lines.

In collaboration with members of the John Hughes Bennett Laboratory (JHBL) in Edinburgh, Ledran and colleagues (2008) investigated the effects of the clonal stromal lines on human ES cells. They reported that haematopoietic differentiation of hES cells was enhanced by co-culture on AM20.1B4, UG26.1B6 and EL08.1D2 stroma. Importantly, intra-femoral transplantation of hES cells differentiated on



AM20.1B4 resulted in long term engraftment in immuno-compromised adult NOD/SCID-IL2R $\gamma$ <sup>null</sup> mice. Up to 16% donor contribution (human CD45<sup>+</sup> cells) was observed, which is higher than that previously described for human ES-derived cells. Transcriptional analysis demonstrated that AM20.1B4, UG26.1B6 and EL08.1D2 all express TGF $\beta$ 1 and TGF $\beta$ 3, but AM20.1B4 stroma expressed the highest levels of TGF $\beta$ 1. Blocking studies revealed that TGF $\beta$ 1 and TGF $\beta$ 3 acted as positive regulators in the haematopoietic enhancing activity of stromal co-culture. In contrast, TGF $\beta$ 2, BMP4 and ActivinA appeared to be negative regulators of haematopoietic differentiation of hES cells in the co-culture system. In the murine ES cell differentiation system, Park et al (2004) and Pearson et al (2008) have demonstrated that BMP4, bFGF, ActivinA, VEGF and TGF $\beta$ 1 signalling can play roles in mesoderm, haemangioblast and subsequent haematopoietic differentiation. In light of these reports and the observations by Ledran et al (2008), experiments will be undertaken to test the role of TGF $\beta$ 1 and TGF $\beta$ 3 in the AM stroma/ murine EB co-culture system established here.

A complex network of cellular and ECM interactions and signals controls the induction, proliferation and differentiation of HSC/HPCs during development *in vivo*. Therefore, it is doubtful that a single factor or interaction is wholly responsible for the enhancing effects of AM-derived stromal cells. The data presented here suggest that subtle differences in the expression levels of positive and negative regulatory factors are likely to contribute to the differential haematopoietic enhancing activities of the stromal lines. Furthermore, there may be mechanistic differences between the regulation of adult HSCs, embryonic HSCs and ES cell-derived HSC/HPCs. Investigation into the differences and common characteristics of these panels of stromal cell lines could pave the way to identifying novel factors and interactions involved in the regulation of HSCs and HPCs derived from different sources.

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Chapter Six  
**Transplantation of co-cultured ES cells**

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## 6.1 Aim

To determine whether ES-derived cells generated in co-culture can achieve long term repopulation of the haematopoietic system in immuno-compromised adult recipients.

## 6.2 Introduction

*In vitro* analyses have demonstrated that the AM-derived stromal cell lines are comparable to OP9 stroma in their ability to promote haematopoietic differentiation of murine ES cells. AM stroma /EB co-culture significantly enhanced the frequencies of CFU-Mix colonies, which are thought to represent early multipotent haematopoietic progenitor cells and might reflect the presence of a cell type that has *in vivo* repopulating potential. The experiments reported in this chapter aimed (i) to test whether ES cell -derived adult repopulating LTR-HSCs were generated in co-culture and (ii) to assess the possible reasons for the difficulties in achieving haematopoietic repopulation with ES-derived cells, by testing the ability of ES-derived cells to home and by testing different routes of injection. In the experiments described here, Kay Samuel (SNBTS) carried out the work on live animals and also helped with tissue harvesting and setting gates on flow cytometric plots.

## 6.3 Experimental approach

- To establish a suitable molecular assay to quantify male donor cells in female recipient tissues, by performing *Sry* quantitative PCR on genomic DNA samples comprising known dilutions of male C57/Bl6 cells in a female background.
- To determine the *in vivo* repopulating potential of co-cultured cells, 7a-GFP EBs were cultured with irradiated stromal layers for up to 10 days differentiation and transplanted into sublethally irradiated NOD/SCID adult female mice. Secondary and tertiary transplantations were carried out where possible.

- To determine the number of HSCs required to achieve repopulation in the NOD/SCID mouse model, limiting dilutions of Rosa26 BM cells were transplanted into adult mice. Intra-venous, intra-splenic and intra-peritoneal injections were carried out to test the most efficient route of transplantation.
- To test the homing abilities of ES-derived cells, DiD stained E14 and 7a-GFP ES cells, EBs and C57/Bl6 BM cells were injected intra-venously into NOD/SCID recipients. Tissues were harvested 1 hour and 24 hours post-transplantation to assess donor contribution.

## 6.4 Results

### 6.4.1 Establishing a quantitative PCR strategy to detect male donor cells

The 7a-GFP ES cells used in co-cultures for transplantation experiments constitutively expressed eGFP and were karyotypically male (Gilchrist et al., 2003). This should allow donor cells to be distinguished from host tissues by flow cytometry for GFP and by PCR for the male Y chromosome-specific genes. To establish a reliable molecular assay for detection and quantification of male donor cells present in female host tissues, quantitative PCR was performed to detect the Y chromosome-specific *Sry* gene. In the first instance, Y chromosome PCR was carried out on genomic DNA isolated from samples containing known numbers of male C57/Bl6 bone marrow cells in a female C57/Bl6 cell background. It was found that the fold increase in *Sry* target signal incremented appropriately in the presence of increasing male cells (Figure 6.1 and 6.2). *βactin* was used as the endogenous control to account for the amount of DNA loaded per well. Validation of these primer sets confirmed that they were equally efficient and could be used together in this way (Appendix 4). The relative quantitation of *Sry* target was determined by the *delta delta* Ct Method in ABI 7500FAST software. Briefly, to quantify the *Sry* target, this signal was first normalised against *βactin* and then expressed as fold change relative to the chosen calibrator (1 in 300 male:female dilution) which was assigned a value of 1.

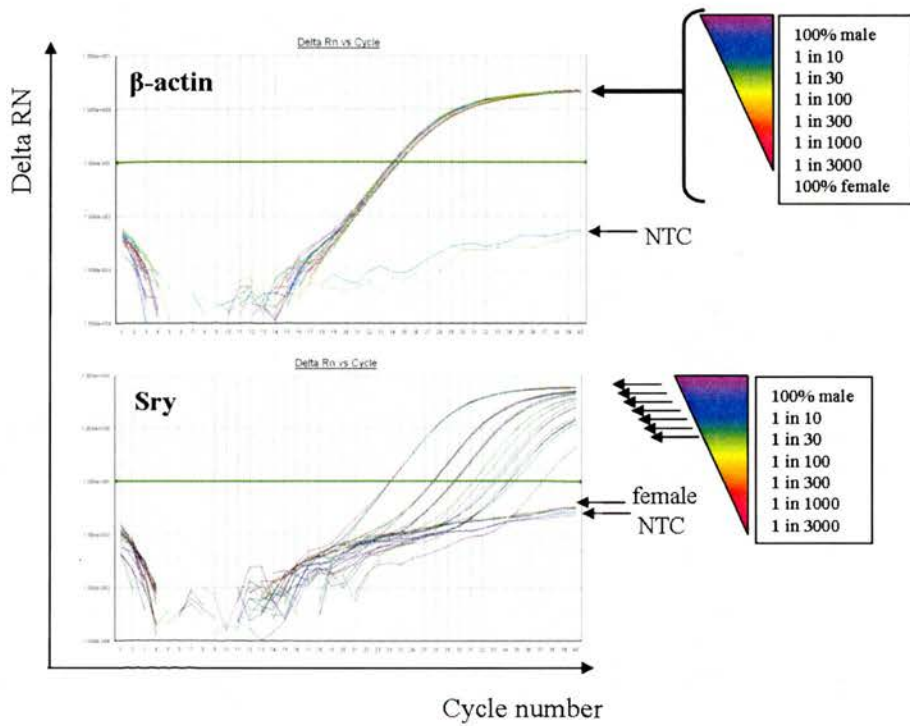


Figure 6.1 Establishing a quantitative PCR Y chromosome detection assay. Shown are the fluorescence amplification plots for  $\beta$ actin and Sry targets in a dilution series of male C57 bone marrow cells in a female cell background. The raw fluorescence measurement (delta RN) in each sample is plotted against the cycle number. The green line represents the threshold at which the Ct values were determined. NTC, no template control.

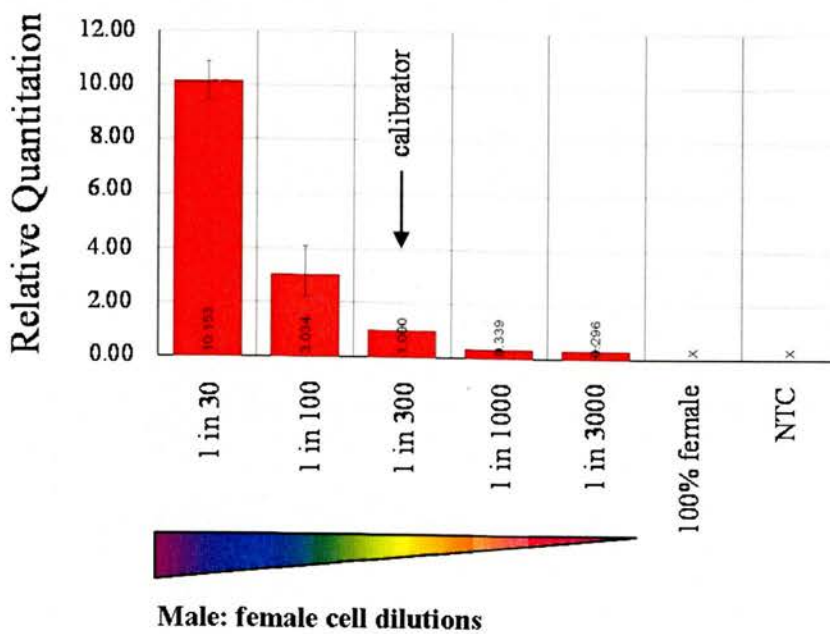


Figure 6.2 Establishing a quantitative PCR Y chromosome detection assay. qPCR for Sry performed on serial dilutions of male C57 bone marrow cells in a female cell background. Using the delta delta Ct method in ABI 7500FAST software, Sry has been normalised against corresponding  $\beta$ actin endogenous controls and calibrated relative to the 1 in 300 dilution sample. Relative quantitation incremented appropriately according to the number of male cells present in a sample. NTC, no template control.



As seen in Figure 6.2, the *Sry* target in the 1 in 100 male:female dilution sample was approximately 3 fold higher than the calibrator and the 1 in 30 sample was 10 fold higher. Similarly, the 1 in 1,000 dilution was 0.3 fold of the 1 in 300 calibrator. These data demonstrate that the Y chromosome qPCR assay established here could reliably quantify the number of male donor cells present in recipient tissues.

#### ***6.4.2 Transplantation of ES-derived cells differentiated in the novel stromal cell/EB co-culture system to assess their in vivo potential***

To determine the *in vivo* potential of co-cultured cells, 1 day old 7a-GFP EBs were co-cultured with irradiated stromal cell layers for up to 10 days of differentiation. Co-cultures were transplanted into sublethally irradiated adult female NOD/SCID mice. Cells were injected by intra-venous or intra-splenic routes and serial transplantations into secondary and tertiary recipients were carried out where possible (Figure 6.3, Table 6.1). When 7a-GFP ES-derived cells were sorted from 4 day EB/AM14.1C4 co-cultures (using GFP as a marker for ES cells) were injected by the intra-splenic route, teratomas formed at the injection site within 4 weeks, resulting in mortality (Experiment 2, Table 6.1). This was likely due to residual undifferentiated ES cells being lodged in the spleens at the site of injection. Tumours only formed when ES derived cells were injected intra-splenically and were not observed in intra-venous recipients.

Genomic DNA was extracted from recipient tissues for Y chromosome qPCR analysis. When 10 day sorted or unsorted co-cultures were transplanted into NOD/SCID, no male donor cells were detected in peripheral blood of recipients after 3 or 6 months (Experiments 3, 4, 5 and 6, Table 6.1). Bone marrow and spleens of these animals were not tested; though one recipient of cKit<sup>+</sup> cells isolated from 10 day EB/AM20.1B4 co-cultures had a very low level (less than 0.1%) of male donor cells in its spleen and bone marrow (Experiment 5, data not shown). This suggested that the cells present in co-cultures at 10 days differentiation did not have long term repopulating potential in adult recipients; though these experiments do not rule out the presence of short term repopulating CFU-S.

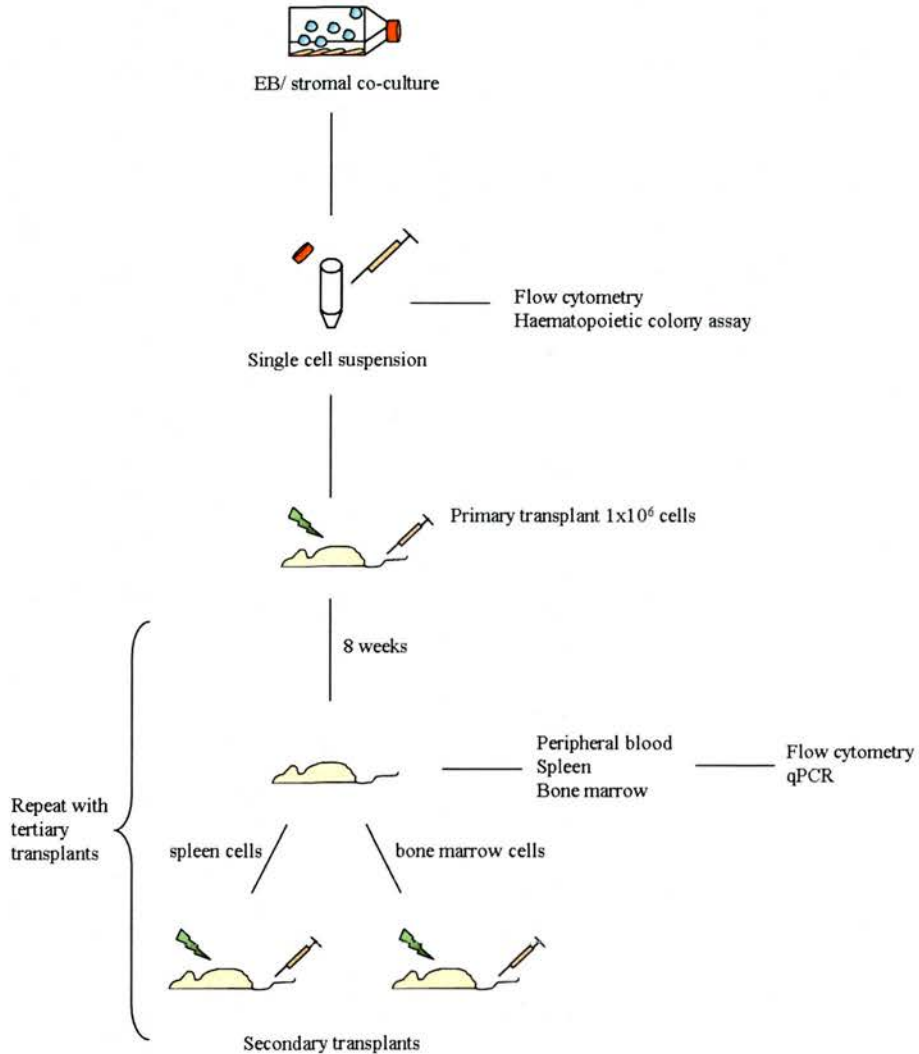


Figure 6.3 Transplantation strategy. 7a-GFP EBs were co-cultured for up to 10 days differentiation. Cells were disaggregated to a single cell suspension for *in vitro* analysis and the equivalent of  $1 \times 10^6$  ES-derived cells were transplanted into sub-lethally irradiated (250rad) female NOD/SCID adult recipients. Secondary and tertiary transplantations of bone marrow and spleen cells were carried out where possible. Half the bone marrow and spleen cells were kept for molecular analysis and the other half (on average  $35 \times 10^7$  cells) injected per secondary or tertiary recipient.

Co-culture			FACS	Control	Route	Recipients	1°, 2°, 3° transplant
1	4d	AM20.1B4	N/A	EB suspension	i.v	NOD/SCID	1°, 2°, 3°
2	4d	AM14.1C4	GFP+	EB suspension	i.s./i.v	NOG/SCID	1° only
3	10d	AM20.1B4	N/A	EB/gelatin	i.v	NOD/SCID	1° only
4	10d	UG26.1B6	N/A	EB/gelatin	i.v	NOD/SCID	1° only
5	10d	AM20.1B4	GFP+ cKit+	EB/gelatin cKit+	i.v	NOD/SCID	1° only
6	10d	AM20.1B4	GFP+ cKit-	EB/gelatin cKit-	i.v	NOD/SCID	1° only

Table 6.1 Summary of transplantations carried out with co-cultured cells. In experiments 2, 5 and 6 GFP+ 7a-GFP ES cells were sorted from the stromal layer by FACS. Cells were transplanted by intra-venous (i.v) or intra-splenic (i.s) routes. When only primary transplantations were carried out, the mice were killed approximately 6 months post-transplantation to assess donor contribution. When serial transplantations were carried out, primary mice were killed 8-12 weeks post-transplantation, secondary recipients were injected on the same day and killed approximately 8 weeks later. This was repeated for tertiary transplantation.



Intra-venous injection of EBs co-cultured with AM20.1B4 to 4 days of differentiation led to low levels of male donor cell engraftment in primary, secondary and tertiary recipients (Experiment 1, Table 6.1). Approximately 8-12 weeks post-injection, 3 out of 4 primary recipients had 0.1% to 0.6% male donor cell engraftment in their spleens and 1 spleen had <0.1%; though, no donor contribution was detected in the bone marrow at this time (Figure 6.4a). Low levels of donor cells must have been present in the bone marrow, however, since secondary transplantation of primary bone marrow led to some donor engraftment. In spleens of secondary recipients of primary bone marrow, between 0.65 and 1.8% donor contribution was detected in all 3 surviving recipients (1 died)(Figure 6.4b). Again, low levels of donor cells were detected in the bone marrow of these recipients (0.08 to 0.17%)(Figure 6.4b). Interestingly, these donor cells appeared to have long term self-renewal potential, as tertiary transplantation of bone marrow from secondary recipients resulted in 0.001 to 13.55% contribution to tertiary spleens and bone marrow (in 3 recipients)(Figure 6.4d). Similarly, transplantation of secondary spleens led to 0.001 to 10.2% donor contribution in tertiary bone marrow and spleens of 2 surviving recipients (1 died)(Figure 6.4e).

EB/AM20.1B4 co-cultured cells that engrafted in the spleens of primary recipients did not appear to have long term self-renewal potential. Transplantation of primary spleen cells (Figure 6.4a) into secondary recipients resulted in 0.4 to 3.7% donor contribution in spleens (in 4 of 4 recipients) and 0.07 to 0.33% contribution to secondary bone marrow (in 4 of 4 recipients) (Figure 6.4c). However, all the tertiary recipients of these tissues had less than 0.1% donor contribution (Figure 6.4e), suggesting that these populations had exhausted their self-renewal capacity. Only 3 of the 8 tertiary recipients survived (a 37.5% survival rate), suggesting that the frequency of long term repopulating cells was low (Figure 6.4e). By contrast, tertiary recipients of primary bone marrow cells (Figure 6.4d) had better survival rate, as 5 of 6 tertiary recipients survived (83% survival rate); despite donor cells being undetectable in the primary bone marrow.

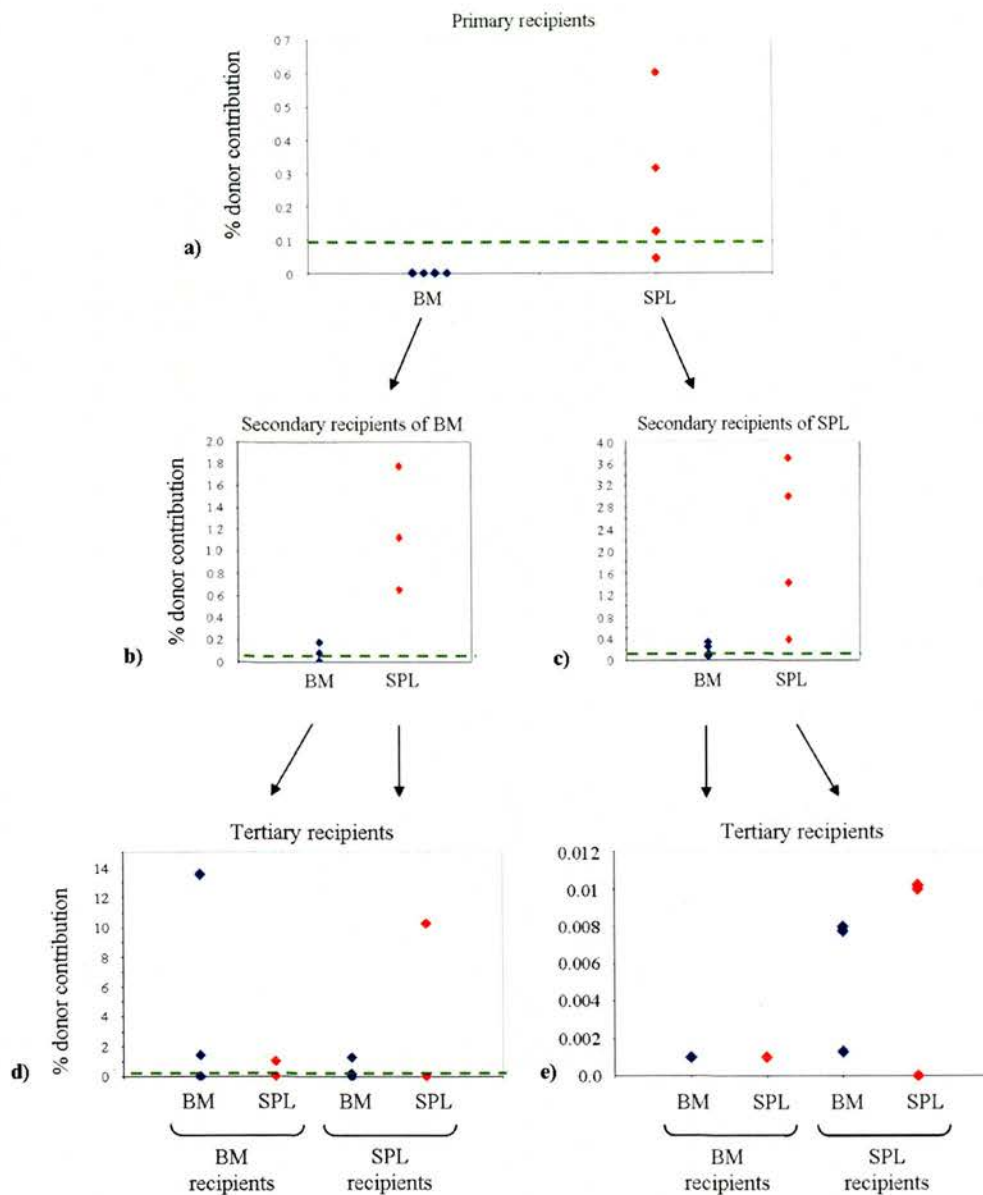


Figure 6.4 Serial transplantation of 4 day 7a-GFP EB/AM20.1B4 co-cultures. Y chromosome qPCR was used to measure male donor contribution to bone marrow (BM) and spleens (SPL) of (a) primary recipients (n=4); (b) secondary recipients that received primary BM (n=3); (c) secondary recipients that received primary SPL (n=4); (d) tertiary recipients that received either BM (n=3) or SPL (n=2) from secondary mice in (b); and (e) tertiary recipients that received either BM (n=1) or SPL (n=2) from secondary mice in (c). The green line represents a threshold of 0.1% donor cell contribution.

These data suggest that co-cultured EB cells that are able to home to and engraft in the adult bone marrow after intra-venous transplantation are more likely (than cells that home to the spleen) to have long term self-renewal potential and radioprotective abilities upon serial transplantation. In addition, these cells expanded in numbers with each transplantation.

In the experiment described above (Figure 6.4), the proportion of male donor cells in recipient tissues was determined by the Y chromosome qPCR assay and the *Sry* target was calibrated against a sample known to consist of 0.1% male 7a-GFP ES-derived cells in a background of female NOD/SCID bone marrow. The raw Y chromosome qPCR data is provided in Appendices 5 to 9. These data demonstrate that 4 day EB/AM20.1B4 co-cultured cells could achieve low levels of engraftment for >6 months in serial recipients, and that cells expanded in numbers upon serial transplantation (Figure 6.4). However, the contribution of donor cells to different haematopoietic compartments (lymphoid, myeloid) could not be determined, as flow cytometry analysis did not show any 7a-GFP<sup>+</sup> cells in recipient tissues. This could have been due to silencing of the GFP transgene in donor cells.

#### ***6.4.3 Silencing of the eGFP transgene in 7a-GFP ES cells in vivo***

Flow cytometry analysis of co-cultured 7a-GFP EBs confirmed that eGFP was constitutively expressed by the differentiating ES cells and 7a-GFP ES cells formed GFP<sup>+</sup> haematopoietic colonies in methylcellulose-based colony assays (Chapter 3). Furthermore, it has been shown that 7a-GFP ES cells can contribute to haematopoietic tissues in chimaeras when microinjected into blastocysts, where they continued to express GFP (Gilchrist et al., 2003).

To assess the stability of GFP expression when differentiated 7a-GFP ES cells were injected into adult NOD/SCID recipients, undifferentiated 7a-GFP ES cells and 4 day 7a-GFP EBs were stained with Vybrant DiD cell labelling solution (Invitrogen) and subsequently injected intra-venously into NOD/SCID recipients, which were killed 24 hours later (Figure 6.5).



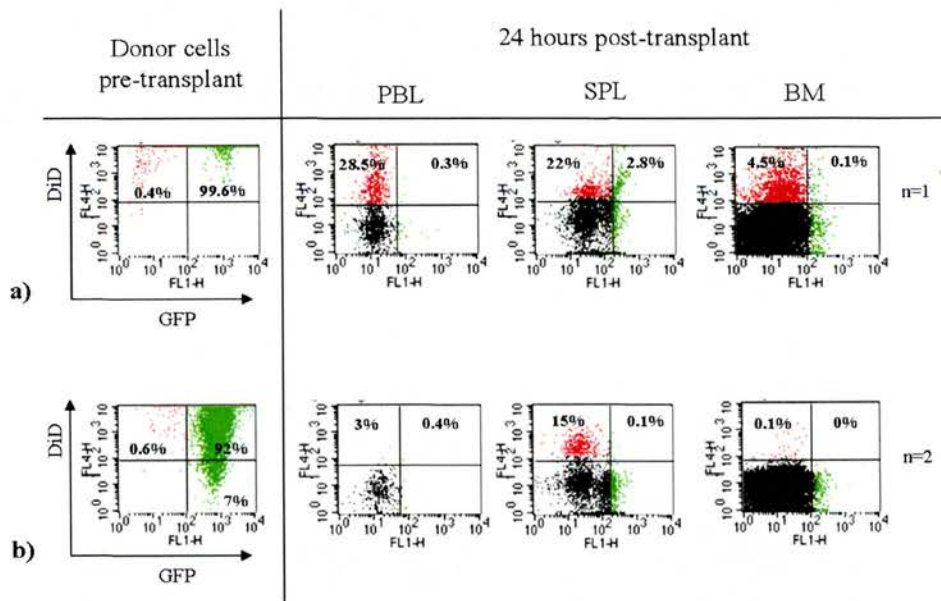


Figure 6.5 eGFP transgene in 7a-GFP ES cells is silenced within 24 hours after transplantation. (a) DiD stained 7a-GFP ES cells and (b) DiD stained 4 day 7a-GFP EB cells were injected intra-venously into female NOD/SCID. Peripheral blood (PBL), spleen (SPL) and bone marrow (BM) were harvested 24 hours after injection and analysed by flow cytometry for donor cell contribution. Gating was carried out by Kay Samuel.

Flow cytometry confirmed that the donor cells were double positive for DiD and GFP before injection. Peripheral blood, spleen and bone marrow samples were harvested 24 hours post-transplantation. Even though donor cells were GFP<sup>+</sup>DiD<sup>+</sup> prior to transplantation, only DiD<sup>+</sup> cells were detected in host tissues after transplantation (n=3) (Figure 6.5). This was also observed when DiD labelled 7 day old 7a-GFP EB cells were injected (n=10, data not shown). Y chromosome qPCR confirmed that male donor cells were indeed present in these tissue samples (data not shown). Thus, these data indicate that the eGFP transgene was silenced when 7a-GFP ES-derived cells were transplanted into adult recipients. In light of this, it was not possible to determine which haematopoietic compartments were repopulated in recipient tissues, as GFP expression in donor cells could not be correlated with surface markers specific to cells of the myeloid (CD11b, Gr1) or lymphoid (B220, CD4) lineages. Therefore, engraftment of non-haematopoietic EB-derived cells could not be ruled out.

Naturally occurring GFP from jellyfish contains only 12 CpG sites; however, enhanced GFP (eGFP) contains 60 CpG sites as a result of changes made at specific residues to optimise the stability and fluorescence characteristics of the protein. One explanation for the silencing of eGFP in donor cells could be that the high CpG dinucleotide content within its 720bp sequence becomes a high-density target of DNA methylation, thereby triggering silencing of nearby promoter elements (PGK in 7aGFP ES cells). Dalle and colleagues (2005) highlighted this as a possible silencing mechanism by synthesising and testing a CpG-free eGFP variant against the effects of eGFP in transgenic mice, ES cells and mouse bone marrow CFU-S progenitors. The CpG-free eGFP variant was silenced less frequently than eGFP; therefore, it is possible that a CpG-mediated silencing pathway could contribute to the effects observed here, likely in addition to other silencing mechanisms.

#### ***6.4.4 Assessing possible reasons for difficulties in achieving repopulation with ES-derived haematopoietic cells***

##### *6.4.4.1 Could ES-derived HSC be present in too few numbers to achieve high levels of repopulation in the NOD/SCID mouse model?*

It was possible that low numbers of ES-HSCs could account for the low levels of repopulation observed; therefore, it was of interest to determine the number of bone marrow cells required to achieve repopulation in the NOD/SCID mice. It was also considered important to test whether the NOD/SCID mouse model used in these transplantation studies could be successfully repopulated by adult BM derived HSCs.

Limiting dilutions of Rosa26-LacZ BM cells were transplanted into NOD/SCID adult mice. Four groups of 5 mice received (A)  $5 \times 10^6$ , (B)  $5 \times 10^5$ , (C)  $5 \times 10^4$  or (D)  $5 \times 10^3$  bone marrow mononuclear cells per recipient (Figure 6.6). LTR-HSCs (SP cells) represent approximately 0.1% of mouse BM mononuclear cells (Goodell et al., 1996). Therefore, approximately 5,000 LTR-HSCs would be present in the  $5 \times 10^6$  BM cells injected into the mice of group A. Likewise, each recipient in groups B, C and D received 500, 50 and 5 LTR-HSCs, respectively.

The recipients were killed 8 weeks post-transplantation and spleen and bone marrow samples were analysed for donor Rosa26 cell contribution. Rosa26 mice express  $\beta$ -galactosidase under the control of Rosa26 promoter elements, which are expressed constitutively and ubiquitously. Rosa26 donor cells could therefore be detected by assessing  $\beta$ -galactosidase activity; thus, recipient tissues were treated with non-fluorescent fluorescein-di- $\beta$ -galactosidase (FDG) substrate.  $\beta$ -galactosidase enzyme metabolises FDG, causing the release of a fluorescent by-product which can be readily detected in donor Rosa26 cells by flow cytometry. Untreated Rosa26 cells were used as controls.

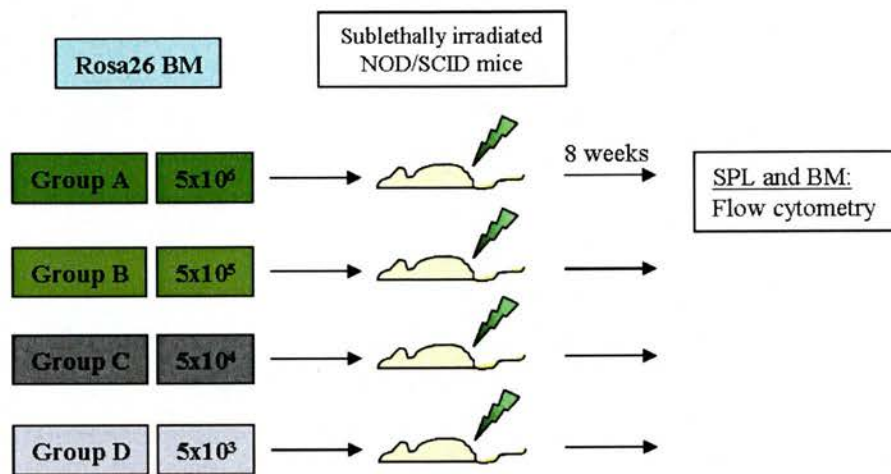


Figure 6.6 Testing the ability of bone marrow cells to reconstitute NOD/SCID mice.



It was found that spleens of mice that received higher numbers of Rosa26 BM cells ( $5 \times 10^6$  and  $5 \times 10^5$ ) were highly repopulated (Figure 6.7 and Table 6.2). The bone marrow of mice that received  $5 \times 10^6$  cells showed higher repopulation than those that received  $5 \times 10^5$  cells ( $93.1 \pm 5.9$  compared to  $32.7 \pm 2.9\%$ , respectively). Transplantation of lower numbers of BM cells ( $5 \times 10^4$  and  $5 \times 10^3$ ) also led to high levels of bone marrow repopulation (approximately 86%), but lower levels of engraftment were observed in recipient spleens (approximately 25%). Interestingly, recipients of  $5 \times 10^4$  and  $5 \times 10^3$  BM cells had higher bone marrow engraftment compared to those that received  $5 \times 10^5$  cells (approximately 86% compared to 33%, respectively). This suggests that donor cell contribution to recipient bone marrow after intra-venous injection can be variable. Furthermore, injection of low numbers of HSCs (5 to 50 HSCs per recipient) led to lower spleen repopulation compared to injecting 500 to 5,000 HSCs. This could suggest that HSCs required more time to self-renew in the bone marrow before they would undergo multilineage differentiation to repopulate other haematopoietic tissues such as the spleen. It is possible that secondary transplantations could stimulate repopulation by BM-HSCs, as the NOD/SCID mice were kept in individually vented cages and were not subject to immune challenges which could promote differentiation of HSCs.

Transplantation of HSCs directly into haematopoietic niches could result in higher (or less variable) levels of engraftment when HSC numbers are low (Burt et al., 2004). In order to test the most efficient route of transplantation,  $5 \times 10^4$  Rosa26 BM cells were injected intra-venously or directly into the spleen or peritoneal cavity (Table 6.3). Recipients were killed 8 weeks post-transplantation. Bone marrow, spleen and peripheral blood samples were harvested, treated with FDG and analysed by flow cytometry to quantify donor cell contribution. When BM cells were injected intra-venously, only 3 of 5 recipients had high levels of donor contribution to the bone marrow ( $88.3 \pm 3.9\%$ ). These recipients also had consistently high levels of engraftment in the spleen and peripheral blood. By contrast, two intra-venous recipients showed low levels of engraftment in the bone marrow ( $5.6 \pm 0.01\%$ ) and the contribution of donor cells to spleen and peripheral blood was highly variable ( $50 \pm 46.2$  and  $51.9 \pm 47.5\%$ , respectively).

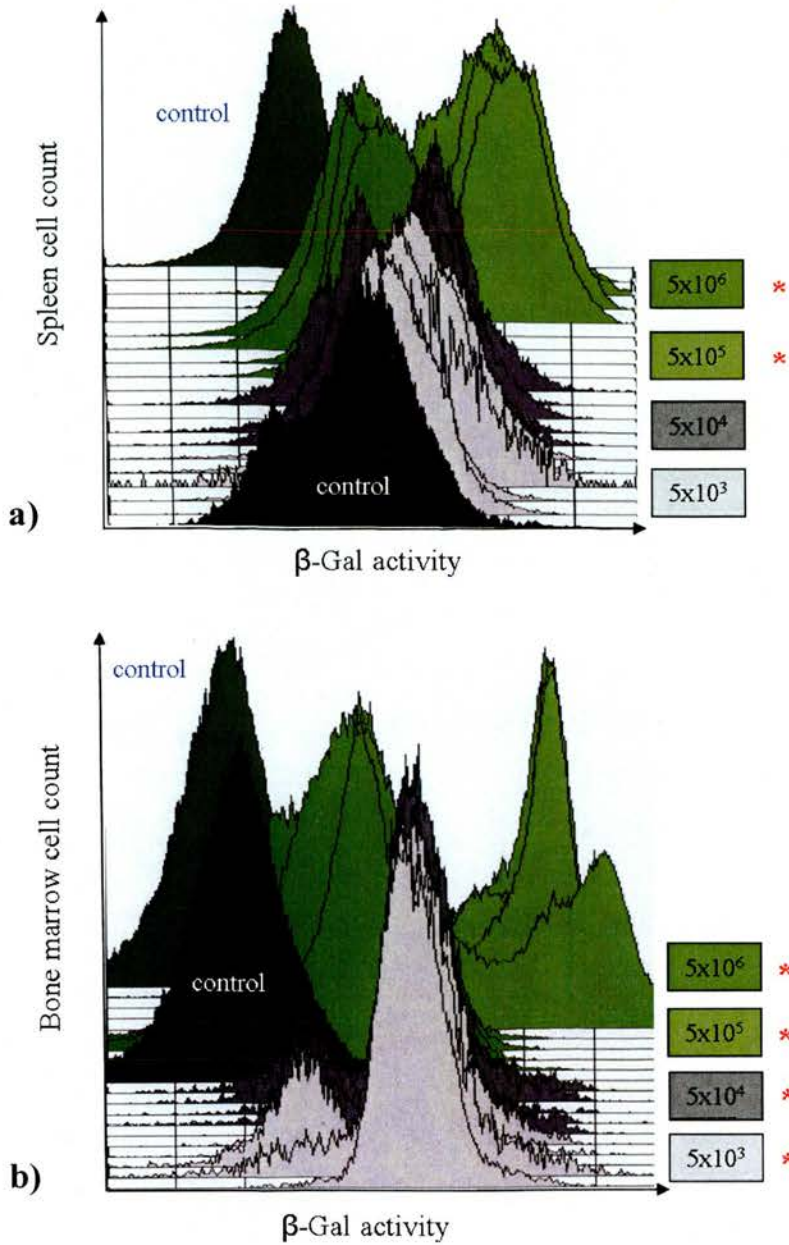


Figure 6.7 Intra-venous transplantation of Rosa26 bone marrow cells in limiting dilutions into NOD/SCID recipients. Donor cell contribution was determined by measuring  $\beta$ -galactosidase activity in (a) spleens and (b) bone marrow of recipients 8 weeks after injection. Each peak represents a replicate recipient from one transplantation experiment. Untreated spleen or bone marrow cells were used as controls (no FDG added). Control peaks in black are representative controls for the samples depicted in grey (recipients of  $5 \times 10^4$  and  $5 \times 10^3$  donor cells); control peaks in dark green relate to samples shown in green (recipients of  $5 \times 10^6$  and  $5 \times 10^5$  donor cells). \*denotes high levels of repopulation. Histograms prepared by Kay Samuel.



Rosa26 BM donor cells	HSCs	Number of recipients	Average donor cell contribution (%)	
			Spleen	Bone Marrow
A $5 \times 10^6$	5,000	4	68.5 ± 0.03	93.1 ± 5.9
B $5 \times 10^5$	500	4	58.9 ± 5.9	32.7 ± 2.9
C $5 \times 10^4$	50	5	24.8 ± 4.5	85.9 ± 4.6
D $5 \times 10^3$	5	5	24.7 ± 1.7	85.5 ± 7.4

Table 6.2 Limiting dilutions of Rosa26 BM cells injected intra-venously into NOD/SCID recipients. Average contribution of donor cells to recipient tissues was assessed 8 weeks after injection by measuring  $\beta$ -galactosidase activity.

Route	Recipients	Donor contribution to BM	Average donor cell contribution (%)			
			BM	SPL	PBL	PEC
Intra-venous	n=5	Low n=2	5.6 ±0.1	50 ±46.2	51.9 ±47.5	9.6 ±2.8
		High n=3	88.3 ±3.9	85.1 ±0.8	75.6 ±31.3	7.7 ±2.2
Intra-peritoneal	n=5	High n=5	69.8 ±18.9	63.4 ±19.3	5.2 ±3.1	71.5 ±21.2
Intra-splenic	n=5	High n=5	72.2 ±4.7	52.8 ±16.4	88.5 ±5.9	38.6 ±27.6

Table 6.3 Transplantation of Rosa26 BM cells by different routes. Intra-venous, intra-peritoneal and intra-splenic routes were tested. Donor cell contribution to bone marrow (BM), spleen (SPL), peripheral blood (PBL) and peritoneal exudate cells (PEC) was determined by measuring  $\beta$ -galactosidase activity.

Intra-peritoneal and intra-splenic injection of Rosa26 BM cells led to high levels of engraftment in the bone marrow of all the recipients ( $69.8 \pm 18.9$  and  $72.2 \pm 4.7\%$  respectively). High numbers of donor cells were also detected in the spleens of these recipients ( $63.4 \pm 19.3$  and  $52.8 \pm 16.4\%$  respectively). However, in intra-peritoneal recipients, repopulation of peripheral blood was low ( $5.2 \pm 3.1\%$ ), though high levels of donor cells were detected in the peritoneal cavity ( $71.5 \pm 21.2\%$ ). By contrast, intra-splenic injection led to high levels of contribution to peripheral blood ( $88.5 \pm 5.7\%$ ). Thus, transplantation of BM cells by the intra-splenic route reproducibly resulted in high levels of donor engraftment in the bone marrow, spleen and peripheral blood. Donor cells were also detected in the peritoneal cavity, probably due to the transplant procedure. These data demonstrate that intra-splenic transplantation of low numbers of BM cells results in the highest levels of engraftment when recipients were assessed 8 weeks post-transplantation. This supports the notion that if only low numbers of ES-derived HSCs were present, they would achieve higher repopulation of spleen and peripheral blood upon direct injection into a haematopoietic microenvironment.

#### 6.4.4.2 Do ES-derived cells have aberrant homing capabilities?

ES-derived HSCs might have the potential to repopulate immuno-compromised adult recipients, but could be defective in homing to appropriate adult niches to carry out their functions (Burt et al., 2004). In order to test whether ES-derived cells were defective in homing to the haematopoietic niches of adult recipients, undifferentiated ES cells and 4 or 7 day old EB cells (differentiated in suspension) were injected intra-venously into sublethally irradiated NOD/SCID recipients ( $1 \times 10^6$  donor cells per mouse). 7a-GFP or wild-type E14 ES cells were used. C57 BM cells were injected as positive controls. All donor cells were stained with Vybrant DiD prior to transplantation to enable detection by flow cytometry and recipient tissues were harvested 1 hour and 24 hours post-transplantation to assess donor contribution (Figure 6.8). C57 BM cells were able to rapidly home to the bone marrow, as donor cells were detected 1 hour after injection ( $23.4 \pm 5.8\%$ ) (Table 6.4). Lower numbers of C57 donor cells were present in the spleen after 1 hour ( $3.3 \pm 0.5\%$ ), but numbers were increased after 24 hours ( $34.3 \pm 9.3\%$ ).

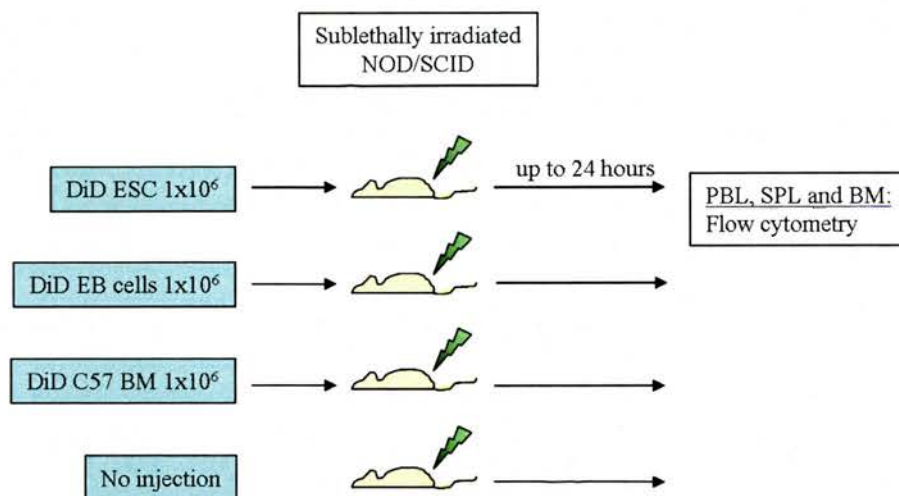


Figure 6.8 Assessing the homing capabilities of ES derived cells. Undifferentiated ES cells, differentiated EB cells and C57 bone marrow cells were injected intra-venously into NOD/SCID recipients ( $1 \times 10^6$  cells per recipient). Donor cells were stained with Vybrant DiD prior to transplantation to enable donor cells to be distinguished from unstained host tissue by flow cytometry. Mice were killed 1 hour and 24 hours later to assess donor cell contribution to spleens (SPL) and bone marrow (BM).

Donor cells (DiD <sup>+</sup> )	Number of recipients	Tissue harvest	Average donor cell contribution (%)	
			SPL	BM
ES cells	n=5	1 hour	2.0 ±0.4	22.3 ±1.3
	n=7	24 hours	8.7 ±7.3	23.3 ±14.6
4 day EBs	n=3	24 hours	14.8 ±9.5	0.2 ±0.02
7 day EBs	n=5	1 hour	1.7 ±0.2	24.4 ±4.3
	n=5	24 hours	7.8 ±1.3	27.9 ±5.7
C57 BM	n=5	1 hour	3.3 ±0.5	23.4 ±5.8
	n=8	24 hours	34.3 ±9.3	35.0 ±9.3

Table 6.4 Homing capabilities of ES derived cells in NOD/SCID recipients. Undifferentiated ES cells, 4 day or 7 day old suspension EBs and C57/B16 bone marrow cells were injected at  $1 \times 10^6$  cells per recipient.

When undifferentiated ES cells or 7 day old EB cells were injected, the pattern of donor contribution after 1 hour was broadly comparable with that of control C57 BM cells, with  $22.3\pm 1.3$  and  $24.4\pm 4.3\%$  donor contribution to the bone marrow, respectively. Again, low donor contribution was observed in recipient spleens ( $2.0\pm 0.4\%$  donor ES cells and  $1.7\pm 0.2\%$  7 day EBs cells). After 24 hours, however, the numbers of ES-derived cells in the spleens did not reach the level observed for C57 BM donor cells. By contrast, 4 day EB cells were able to home to and accumulate in the spleen, as there was  $14.8\pm 9.5\%$  donor contribution after 24 hours, but only low numbers of donor cells were detected in the bone marrow ( $0.2\pm 0.002\%$ ). This implies that ES cells at different stages of differentiation vary in their homing capabilities in an adult microenvironment. These data are also consistent with the 4 day EB/AM20.1B4 transplantation study, where 4 day EB donor cells engrafted in the spleens of primary recipients, but numbers were undetectable in bone marrow (Figure 6.4a).

Y chromosome qPCR analysis of genomic DNA isolated from selected tissues in Table 6.4 confirmed that male donor cells were present (data not shown); therefore, it was unlikely that the DiD stain leaked from donor cells and marked host tissues. Though, fusion of donor and host cells could not be ruled out; however, this could still indicate that the donor cells had homed to the niches in which their markers were detected.

It has been suggested that full body irradiation of recipients prior to transplantation can affect the SDF-1/CXCR4 homing signal pathway in adult bone marrow niches. The data described here demonstrate that C57 BM cells were able to home to the bone marrow niche after intra-venous injection into sublethally irradiated NOD/SCID recipients, suggesting that full body irradiation does not disrupt the signalling pathways required for these cells to home. It is possible, however, that irradiation could disrupt the pathways on which ES-HSCs rely for homing to adult niches.



## 6.5 Discussion

### 6.5.1 Derivation of transplantable EB-derived cells

To date, there has been only limited success in generating HSCs with long term repopulating capabilities from mouse ES cells which have not been genetically manipulated to over-express haematopoietic genes. Furthermore, there have been no reports that ES cells differentiated on OP9 stroma generate LTR-HSCs which can repopulate recipients, without over-expressing haematopoietic genes in culture (Ji et al., 2008; Kyba et al., 2002; Wang et al., 2005). Muller and Dzierzak (1993) reported that EB cells differentiated in suspension for 5-22 days were able to achieve 0.1-6% engraftment (mainly lymphoid) in recipients for over 6.5 months. Repopulation activity peaked between days 11 and 13 of differentiation. This contrasts with the transplantation experiments described here, where no repopulation activity was present in 10 day EB/AM stromal co-cultures. This could be a reflection of the different methods used for ES differentiation. Hole et al (1996) reported that 4 day EB cells differentiated in suspension cultures could achieve long term repopulation in irradiated adult recipients, though donor contribution was low and variable (less than 5% in most recipients). The experiments described in this chapter were designed to determine whether HSCs with long term repopulating potential were generated in EBs differentiated in AM stromal co-cultures. The data confirm the observation by Hole et al (1996) that ES derived cells able to engraft adult recipients are present in 4 day EBs (also reported by Miyagi et al., 2002). However, the strategy used here differs from that of Hole et al (1996), as no irradiated spleen carrier cells were co-injected with donor EB cells. In Hole's experiments, no repopulation was achieved by EB cells in the absence of spleen carriers (Kay Samuel, personal communication).

EBs that were co-cultured with AM20.1B4 stroma to 4 days differentiation were able to engraft in spleens and bone marrow of primary, secondary and tertiary recipients and were detected for >6 months (Figure 6.4). The ES-derived donor cells were found to expand in numbers when serially transplanted into secondary and tertiary recipients. Donor contribution of between 0.1% and 13.55% was detected in spleens and/or bone marrow in 13 out of 18 recipients (Figure 6.4). Starting populations of

only  $1 \times 10^6$  co-cultured EB cells were injected and, on average,  $54 \times 10^7$  and  $85 \times 10^7$  cells were recovered from NOD/SCID recipient spleens and bone marrow, respectively. This equates to an expansion of up to 115 fold in donor cell numbers from the time of primary injection to tertiary recipients >6 months later.

In primary recipients, higher numbers of donor cells engrafted in the spleens, compared to the bone marrow. This is consistent with the data presented in Table 6.4, where 4 day disaggregated EB cells were detected at higher levels in spleens compared to bone marrow 24 hours after intra-venous injection into NOD/SCID recipients. Interestingly, serial transplantation of EB/AM20.1B4 donor cells that had engrafted into primary bone marrow (at levels undetectable by qPCR) had higher self-renewal/proliferation potential compared to those that engrafted in the spleens of primary recipients. The highest levels of donor chimaerism (10.2 and 13.55%) were detected in two tertiary recipients of primary bone marrow cells more than 6 months after the primary injection of EB/AM20.1B4 co-cultures. By contrast, spleen-derived repopulating donor cells had only short term engraftment capabilities, as higher levels of donor cells were detected in secondary recipients, but the levels reduced to <0.1% in tertiary recipients. Furthermore, only 37.5% of tertiary recipients survived when spleen repopulating cells were serially transplanted, suggesting that these donor cells had only short term radioprotective abilities. Conversely, a high tertiary survival rate (83%) was associated with serial transplantation of donor cells that homed to and engrafted in primary bone marrow. This suggested that these cells had long term self-renewal capabilities and also conferred radioprotection. It is important to note that in this transplantation experiment, it was not possible to confirm that haematopoietic compartments were repopulated, as the GFP transgene in donor cells was silenced (Figure 6.5). Thus, it cannot be ruled out that the male donor contribution was due to engraftment of non-haematopoietic ES derived cells. It will therefore be important to repeat these transplantation experiments using another ES cell line, such as C57/BL6 ES cells, which would be distinguishable from host tissues by differential expression of Ly5 alleles that can be detected by flow cytometry. Alternatively, haematopoietic

populations (e.g. CD45<sup>+</sup>) could be isolated by FACS from recipient tissue samples prior to genomic DNA extraction and Y chromosome qPCR analysis.

One could argue that engraftment of donor ES-derived cells with high tumour forming ability could also expand upon serial transplantation. However, transplantation of undifferentiated ES cells leads to teratoma formation and 100% mortality within 8-13 days (Burt et al., 2004). In addition, Muller and Dzierzak (1993) reported that injection of 3 day EB cells led to teratoma formation and mortality within 6 weeks after transplantation; while injection of EBs on or after 5 days of differentiation did not lead to tumour formation. In the transplantation experiment described in Figure 6.4, no tumour formation was observed, even though donor cells were serially transplanted every 8-10 weeks and recipients monitored for longer than 6 months. Therefore, it is unlikely that tumour cells were responsible for the engraftment observed. Furthermore, serial transplantation and expansion of tumour-like cells would not correlate with an increased recipient survival rate or radioprotective abilities, which were specifically observed in donor cells that engrafted in primary bone marrow. It is possible that these observations are a reflection of a clonal dominance effect in a small proportion of karyotypically abnormal (but non-malignant) ES-derived cells that have been selected for by serial transplantation. This phenomenon has been observed in retrovirally marked mouse HSCs, whereby retroviral integrations occurring near loci involved in self-renewal or cell survival conferred a selective advantage by promoting non-malignant expansion of HSC clones in long-term serial transplantation experiments (Kustikova et al., 2005).

The correlation between spleen colonisation by donor cells and short-term self-renewal abilities is perhaps not surprising (Figure 6.4a, c, e); since CFU-S cells, known to confer short term radioprotection, home to the spleen upon intra-venous injection and form macroscopic colonies comprising of myeloid cells that have short term self-renewal capabilities when excised and serially transplanted into new recipients (Till and McCulloch, 1961). In light of the data presented here, it would

be interesting to assess the CFU-S potential of 4 day EB/AM20.1B4 co-cultured cells.

It is enticing that a small number of donor cells that home to and engraft in the adult bone marrow (though undetectable by qPCR), are apparently able to confer radioprotection and persist on serial transplantation (Figure 6.4a, b and d). It is possible that host bone marrow and spleen cells co-injected with donor cells into secondary and tertiary recipients could act as carriers to aid the survival and homing of donor cells, thereby facilitating the expansion of donor cell numbers. In addition, serial transplantation of the primary bone marrow could have selected for a rare EB-derived cell type that has LTR-HSC adult homing characteristics. In support of this hypothesis, Krause et al (2001) used a selection strategy, whereby they labelled donor BM cells and injected them intra-venously into lethally irradiated adult mice. 48 hours later, labelled donor cells were recovered (by FACS) from the recipient bone marrow. By limiting dilution, a single labelled donor cell was transplanted into each new recipient. It was found that a single HSC, selected on the basis of its ability to home to the adult bone marrow, could be serially transplanted without loss of self-renewal or repopulating ability.

It should be noted that the donor contribution reported in Figure 6.4 is consistent with results recently published by Ledran and colleagues (2008), where AM20.1B4 stroma were able to promote haematopoietic repopulating activity in human ES cells. In collaboration with my colleagues in Edinburgh, Ledran and colleagues (2008) investigated the effects of the AM20.1B4, UG26.1B6 and EL08.1D2 stromal lines on human ES cells. The authors differentiated human ES cells on AM20.1B4 stroma for 12 days and transplanted  $5 \times 10^5$  co-cultured cells directly into the femoral cavities of irradiated NOD/SCID-IL2R $\gamma^{\text{null}}$  adult recipients. 8-12 weeks post-transplantation, 2.06-16.26% of recipient bone marrow and peripheral blood was donor derived (measured by expression of human CD45 $^+$ ) and there was donor contribution to myeloid and lymphoid compartments. Higher levels of repopulation were achieved with AM20.1B4 co-cultures compared to UG26.1B6, EL08.1D2 and primary AGM derived monolayers, suggesting that AM20.1B4 was particularly potent in enhancing

repopulating activity in human ES cells. Importantly, these data represent the highest levels of human ES cell derived repopulation achieved to date. Therefore, it will be extremely useful to further characterise the effects and properties of the AM20.1B4 stromal cell line.

### ***6.5.2 Reasons for difficulties in achieving repopulation with ES-derived cells***

A number of reasons have been put forward to account for the difficulties in achieving high levels of repopulation with ES cell-derived HSCs in adult recipients. For instance, ES-HSCs could be developmentally too immature to function in or respond to an adult microenvironment. Prior to E10.5, CD34<sup>+</sup>cKit<sup>+</sup> cells derived from the yolk sac or P-Sp region can repopulate neonatal, but not adult, recipients (Yoder et al., 1997a, 1997b). This supports the notion that a pre-HSC exists that can repopulate only when transplanted into a developmentally appropriate microenvironment. In light of this, it would be interesting to determine whether the ES-HSCs generated in AM stromal co-cultures are able to provide long-term multilineage repopulation in newborn recipients, as newborn mice still have a functional foetal liver haematopoietic niche.

Another explanation could be that ES-HSCs have a tendency to terminally differentiate in culture and might therefore only be present in a small time window. Low frequencies of ES-HSCs could make these cell populations less likely to achieve repopulation by intra-venous injection within the time frame of the LTR assay. In support of this hypothesis, it has been demonstrated that intra-venous injection of fewer than 50 Rosa26 BM-HSCs can achieve high levels of repopulation in recipient bone marrow, but lower repopulation of the spleens after 8 weeks (Table 6.2). Increased numbers of BM-HSCs (500 to 5,000) were required to achieve high levels of repopulation in both spleen and bone marrow. These data suggest that when cells are injected intra-venously into NOD/SCID, the equivalent of 500 to 5,000 ES-HSCs would need to be injected per recipient to achieve high levels of repopulation after 8 weeks in primary recipients, provided that the cells were fully competent in homing and responding to the adult bone marrow niche. *In vitro*, CFU-Mix colonies might represent cells that are functional *in vivo*, or could represent CFU

down-stream of cells which may have repopulating abilities (Kerk et al., 1985; Nakahata et al., 1982; Humphries et al., 1981). In 6 day EB/AM20.1B4 and AM14.1C4 co-cultures, CFU-Mix were detected at frequencies of 42.9 and 36.8 per  $3 \times 10^5$  ES-derived cells, respectively (Chapter 4). Starting populations of  $1 \times 10^6$  EB-derived co-cultured cells were transplanted into each NOD/SCID recipient, equating to only 123 to 143 CFU-Mix progenitors per recipient. Presuming that each CFU-Mix represented a cell with repopulating potential, then low numbers of ES-HSCs could account for the low levels of engraftment observed in recipients. In order to transplant the equivalent of 500 ES-HSCs, more than  $4 \times 10^6$  EB-derived cells would need to be injected into each recipient. In the experiments here, only 4 day and 10 day co-cultures were assessed. Since CFU-Mix numbers in EB/stromal co-cultures peaked at 6 days of differentiation, it would be important to transplant the cells at this time point.

Retroviral marking studies have shown that a single adult BM-derived definitive HSC is able to achieve long-term repopulation of an immuno-compromised adult recipient when injected intra-venously (Osawa et al., 1996). This relies on the cell surviving the injection and successfully homing to the appropriate adult bone marrow niche, evading any residual host immunity. Subsequently, the HSC must respond to the niche by undergoing self-renewal to expand the HSC pool and some of these HSCs have to undergo multilineage differentiation to provide repopulation of the blood and immune system. In light of all these requirements, it follows that the more HSCs transplanted, the more likely it is that high levels of haematopoietic repopulation will be achieved within the time frame of the experiment.

It is possible that ES-HSCs are unable to home appropriately to adult haematopoietic niches upon intra-venous transplantation, which could account for the low levels of engraftment observed. In support of this hypothesis, after intra-venous injection 4 day EB cells were defective in their ability to home to the adult bone marrow, compared to 7 day EBs and C57 BM cells (Table 6.4). Thus, the stage of EB differentiation appears to influence the homing capabilities of the ES-derived cells. It would be useful to assess expression of SDF-1 and CXCR4 receptor on



differentiating EB cells in co-culture to determine which time point would be amenable to intra-venous injection into adult recipients. Furthermore, a study by Burt and colleagues (2004) showed that injection of ES-derived haematopoietic cells (CD45<sup>+</sup>cKit<sup>+</sup>) sorted from 7 day suspension EBs directly into the femoral cavity resulted in significantly higher levels of repopulation compared to intra-venous injection. When different routes of injection were tested with Rosa 26 BM cells (Table 6.3), it was found that injection of the cells directly into recipient spleens was more efficient at achieving repopulation compared to intra-venous injection. In light of all these observations, it would be interesting to test the ability of the EB/AM co-cultured cells to repopulate recipients when injected directly into the femoral cavity. This would also be in accordance with the strategy of Ledran and colleagues (2008), where repopulation was achieved with human ES/AM20.1B4 co-cultures by using the intra-femoral route.

### **6.5.3 Summary**

It is promising that 4 day EB/AM20.1B4 stromal co-cultures generate cells that are able to achieve long term engraftment in bone marrow and spleen niches of adult recipients; though, engraftment of non-haematopoietic donor cells has not been ruled out. It will be important to repeat these transplantation experiments with another ES cell line, such as C57/Bl6 derived ES cells, that will allow donor and host cells to be distinguishable by flow cytometry, so that haematopoietic repopulation can be demonstrated. The data presented in this chapter have highlighted ways in which the transplantation experiments with murine EB/stromal co-cultures can be improved. For instance, transplantation of higher numbers of EB-derived cells (at least  $4 \times 10^6$ ) directly into the intra-femoral cavity, could improve repopulation. In addition, co-cultures could be transplanted at 6 days differentiation when CFU-Mix activity is highly enriched and CFU-S assays could be performed to assess the short-term repopulating potential of the co-cultured cells. Alternatively, cells could be injected into newborn recipients to test their repopulating potential in an embryonic niche.

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Chapter Seven  
**Summary and prospective**

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## 7.1 Summary

A novel co-culture system has been established using AGM-derived clonal stromal cell lines to reliably and efficiently enhance haematopoietic differentiation of mouse ES cells. It has been demonstrated that stromal cell lines derived from the AM subregion of the E10.5-11 AGM, but not the UG subregion, potently promote haematopoietic differentiation. In future, this could be an important factor to consider when deriving AGM stromal cell lines for the purpose of differentiating ES cells. Furthermore, the enhancing activity of AM stroma was comparable or better than that of the widely used OP9 stromal cell line (Nakano et al., 1994). Importantly, EBs co-cultured with AM20.1B4 were apparently capable of low levels (0.1 to 13.55%) of long-term engraftment in NOD/SCID mice, though contribution of donor cells to haematopoietic compartments in recipient animals is yet to be confirmed.

Co-culture of sorted ES cell populations isolated from EBs revealed that the stromal lines mediate their haematopoietic enhancing effects after mesoderm (*Brachyury*) specification in EBs (after day 4 of differentiation). Such sorting experiments will contribute to the understanding of which ES-derived cell populations are responsive to haematopoietic regulation by the stromal cells. It was also found that the enhancing activity of co-culture is dependent on direct contact between EBs and the stromal cells, as demonstrated in experiments using transwell inserts to inhibit contact. Extracellular matrices (ECMs) isolated from stromal cell layers did not retain the enhancing activity of intact stromal co-cultures. These data strongly support a contact-dependent mechanism of action. Interestingly, some ECMs had inhibitory effects on haematopoietic differentiation of EBs, as compared to those cultured on gelatin alone. Thus, it is possible that a balance between positive and negative haematopoietic regulators underlie the effects of co-culture. It will be important to keep this in mind when investigating the factors involved in the haematopoietic enhancing effects of the AM-derived stromal cell lines.

## 7.2 Ongoing work

A master's project was undertaken by Caixin Huang, under my supervision, to further the investigations described in this thesis. In an important development, Huang has shown that AM stroma are able to efficiently enhance haematopoietic differentiation of EBs under serum-free conditions. The serum-free differentiation medium was modified from a protocol described by Gouon-Evans and colleagues (Gouon-Evans et al., 2006) and supplemented with SCF and BMP4. Importantly, comparable levels of haematopoietic activity were observed in serum-containing and serum-free EB/AM co-cultures (observed in 3 independent experiments). The use of serum-free media in the stromal co-culture system should reduce the variability in haematopoietic enhancement between experiments, which can be augmented by differences between batches of foetal calf serum. A highly defined differentiation protocol such as this will also facilitate future investigations into the molecular signalling underlying the enhancing activity of AM stroma.

Quantitative RT-PCR analysis of the stromal cell lines revealed that they all express Notch1 receptor and its ligands Jagged1 and Delta-like4 (Huang, unpublished observations). Notch1 and Jagged1 were also expressed by 5 day EB cells sorted from (serum-containing) co-cultures (Gordon-Keylock, unpublished). Notch signalling has been implicated in the regulation of haematopoiesis *in vivo* (Stier et al., 2002; Calvi et al., 2003). Therefore, experiments were designed to test whether the enhancing activity of the stroma was dependent on Notch signalling. To block Notch signalling during EB/stromal co-culture, a gamma-secretase inhibitor (Calbiochem)(Lowell et al., 2006) was added to (serum-containing) co-cultures between 4 and 6 days of EB differentiation. In the presence of the inhibitor, the frequency of multipotent haematopoietic progenitors (CFU-Mix, CFU-GM and Ery/Mac) was reduced by 57, 63 and 56% in AM20.1B4, AM14.1C4 and OP9 co-cultures, respectively, as compared to the corresponding co-cultures where only diluent was added ( $p < 0.05$ , 3 independent experiments). By contrast, the inhibitor did not significantly affect haematopoietic differentiation of EBs cultured on gelatin, as compared to the diluent control ( $p = 0.61$ ). Thus, blocking Notch signalling significantly reduced the haematopoietic enhancing activity of EB/stromal co-

culture, suggesting that the effects of these stroma are at least partially dependent on Notch signalling pathways. This is in accordance with a recent publication by Cheng et al (2008) showing that Notch signalling is required for definitive haematopoietic differentiation of mouse ES cells. In future experiments, Huang will ectopically express Notch ligands, such as Jagged1, in UG26.1B6 stromal cells to determine if these ligands can confer haematopoietic promoting activity on a non-supportive cell line.

To date, there have been no reports of long-term engraftment by ES-derived cells differentiated on OP9 stroma without over-expressing haematopoietic genes in culture (Kyba et al., 2002; Wang et al., 2005; Ji et al., 2008). A tamoxifen inducible HoxB4-ERT ES cell line has been established by Dr Melany Jackson and Dr Richard Axton in the JHBL. The induction of HoxB4-ER<sup>T2</sup> in EBs cultured on gelatin significantly enhances haematopoietic differentiation by 6 days (on average, there is a 7-fold increase in total haematopoietic CFUs generated from induced EBs compared to non-induced EBs). However, when HoxB4-ER<sup>T2</sup> EBs were differentiated in the AM stromal co-culture system (in the presence of serum), it was found that induction of HoxB4 did not further enhance the haematopoietic activity in EBs co-cultured with AM14.1C4, suggesting that these two approaches did not have additive effects (3 independent experiments, Gordon-Keylock, unpublished). One interpretation is that AM co-culture and HoxB4 induction mediate their effects on EBs through overlapping signalling pathways. It has been reported that Notch1 is up-regulated when HoxB4 is over-expressed in differentiating ES cells (Schiedlmeier et al., 2007) and Notch signalling has already been shown to be involved in the haematopoietic enhancing activity of AM stroma (Huang et al, unpublished). Therefore, it is possible that both these systems utilise the Notch pathway. To test this hypothesis, experiments are underway to determine whether HoxB4-ER<sup>T2</sup> induction can still enhance haematopoietic activity in EBs (on gelatin) when Notch signalling is blocked by the gamma-secretase inhibitor.

### 7.3 Prospective

The work described in this thesis has already been translated into the human ES cell system. As stated previously, Ledran and colleagues (2008) have demonstrated that co-culture of human ES cells with AM20.1B4 for 12 days can promote multilineage repopulating HSC activity. The authors reported the highest levels of repopulation described for human ES cells to date (up to 16% donor contribution to peripheral blood and bone marrow of NOD/SCID-IL2R $\gamma^{\text{null}}$  recipients). Therefore, further investigations into the molecular mechanisms underlying the effects of AM20.1B4 on murine ES cells will be directly applicable to the human ES cell differentiation system. These studies could ultimately facilitate the generation of transplantable human ES cell derived HSCs for autologous cell replacement therapies, for use in drug discovery or to identify novel factors able to expand or maintain HSCs in culture.

It has recently been shown that functional enucleated definitive erythrocytes can be derived from human ES cells on a large scale (Lu et al., 2008). Lu and colleagues (2008) demonstrated, for the first time, that the oxygen transport capabilities of ES-derived erythrocytes were comparable with that of normal adult erythrocytes and they responded appropriately to environmental changes (such as pH levels). Another key aspect of this publication was that the cells were derived under highly defined serum-free conditions and it was the first time erythrocytes were produced in large enough numbers to validate their potential for use in the clinic. The work is ongoing, but it is a promising demonstration of how ES cells could provide a limitless source of blood for transfusions, which is often in short supply. If the erythrocytes were derived from ES cells of blood group O negative, they would be compatible with any patient. The advantages of this “universal” blood supply would include the absence of blood pathogens such as HIV and hepatitis. Since erythrocytes do not have nuclei, this removes the possibility of uncontrolled growth upon transfusion, provided that a pure population of donor cells can be isolated from the starting undifferentiated ES cell population. This study represents a promising advance towards the clinical application of differentiated ES cells.



Recently, there have been two significant developments in the wider stem cell field; namely, the generation of induced pluripotent stem (iPS) cells from human fibroblasts and the generation of animal-human hybrid embryos to establish human ES cell lines. The UK human embryology and fertilisation association (HEFA) recently approved the generation of bovine-human hybrid embryos to derive disease-specific human ES cells (February 2008). The work will be carried out at King's College London and the University of Newcastle Upon Tyne. Nuclei from skin cells of individuals carrying genetic mutations for human neurodegenerative diseases (such as Parkinson's and Alzheimer's disease) will be introduced into enucleated bovine oocytes by somatic cell nuclear transfer. Disease-specific human ES cell lines will be derived from resultant hybrid embryos, which will be incubated for no longer than 14 days before being destroyed. The ES cells will be 99.9% human and will only contain 0.1% bovine DNA derived from oocyte mitochondria. Directed differentiation of disease-specific hES cells will provide novel insights into the pathogenesis, genetic and cellular factors underlying these diseases. Importantly, this approach will generate cellular tools for the study of diseases which are not well understood and thus will facilitate the development of novel therapies. These types of investigation represent an exciting new application for ES cells, but they will largely depend on efficient differentiation protocols. Even though the initial studies will focus on neurodegenerative disorders, work will also be carried out to elucidate the molecular pathways underpinning somatic cell nuclear reprogramming events. The use of animal-human hybrid embryos will facilitate this research, as human oocytes are often in short supply. This work could also pave the way to deriving better quality human ES cell lines for use in research, as the human ES cell lines to date have been generated from surplus embryos from *in vitro* fertilisation clinics and are therefore not of the highest quality. Thus, this work will have a positive impact on all human ES cell differentiation applications; including differentiation into haematopoietic lineages.

Yamanaka and colleagues (Takahashi et al., 2007) and Yu and colleagues (Yu et al., 2007) have demonstrated that transduction of four specific transcription factors into human fibroblasts results in somatic cell reprogramming such that the cells revert to

a pluripotent state. These induced pluripotent stem (iPS) cells display many characteristics associated with human ES cells; including morphology, cell surface phenotype, gene expression and telomerase expression. Importantly, the iPS cells were able to differentiate into lineages of all three germ layers *in vitro* and after transplantation into immuno-compromised mice (teratomas formed). The advent of human iPS cells is an exciting breakthrough for autologous cell replacement therapies, as the use of somatic cells bypasses the ethical problems associated with embryonic stem cells. Interestingly, the two groups used different cocktails of transcription factors to generate the iPS cells. Takahashi et al (2007) used Oct3/4, Sox2, Klf4 and c-Myc, whereas Yu et al (2007) used Oct4, Sox2, Nanog and LIN28. However, the molecular mechanisms underlying the reprogramming are unknown. The authors noted that the iPS clones contained a number of retroviral integrations for each transcription factor. This feature and the use of oncogenes raises safety concerns for the use of these cells in humans. This highlights a need for novel reprogramming methods that do not use retroviruses or oncogenes and, ideally, do not require permanent genetic modification of the cells. This technology is still in its early stages, but there is clearly enormous research potential. For instance, Park et al (2008) have recently generated patient-specific iPS cells, which could serve as new disease models for research and drug discovery. The differentiation of disease-specific and normal human ES cells will function as a key model system to complement these studies.

It has been only 27 years since the isolation of the first mouse ES cells in 1981 (Evans and Kaufman, 1981; Martin, 1981), which dramatically altered the way in which gene function can be studied in developmental biology. Since the derivation of the first human ES cell lines in 1998 (Thomson et al., 1998), the field of stem cell research has rapidly progressed as promising new technologies have emerged, including the generation of iPS cells and new human ES cell lines from animal-human hybrid embryos. The success of these technologies will rely heavily on efficient, reliable and well-defined large-scale culture methods to direct differentiation into lineages of interest. In terms of haematopoietic differentiation, the large-scale production of human ES cell derived erythrocytes by Lu et al (2008)

has brought the field a step closer the clinic. The success of this study has highlighted the importance of projects such as the one described in this thesis. Importantly, the work presented here has already been translated into the human ES cell system by Lako and colleagues in Newcastle (Ledran et al., 2008). This collaboration will continue as further investigations are made into the murine AM stromal co-culture system.

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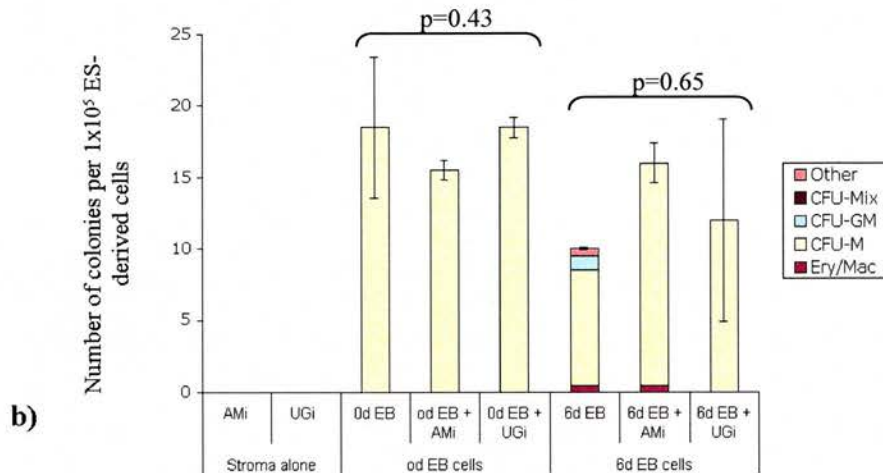
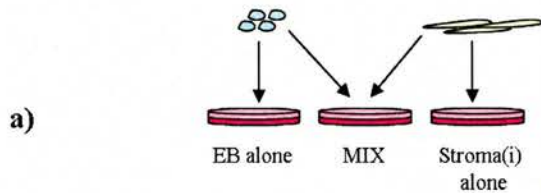
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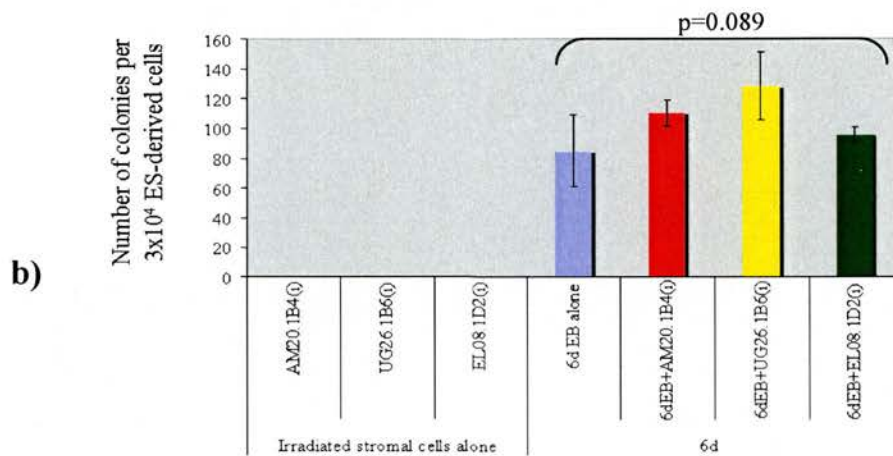
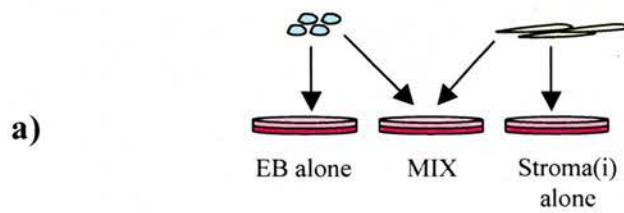
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## Appendix

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Appendix 1 The presence of irradiated stromal cells did not affect haematopoietic activity of EB cells in methylcellulose-based colony assays. (a) Diagrammatic summary of experiment. (b) Colony assays set up with 0 day or 6 day suspension EB cells alone or mixed with irradiated stromal cells. Irradiated AM20.1B4 and UG26.1B6 cells did not generate any colonies in the assays. There was no significant difference in the numbers of colonies obtained from 0 day or 6 day EB cells seeded alone or when they were seeded along with irradiated stromal cells ( $p=0.43$  and  $p=0.65$ , respectively). Friedman tests were carried out (non-parametric one-way ANOVA with matched pairs).



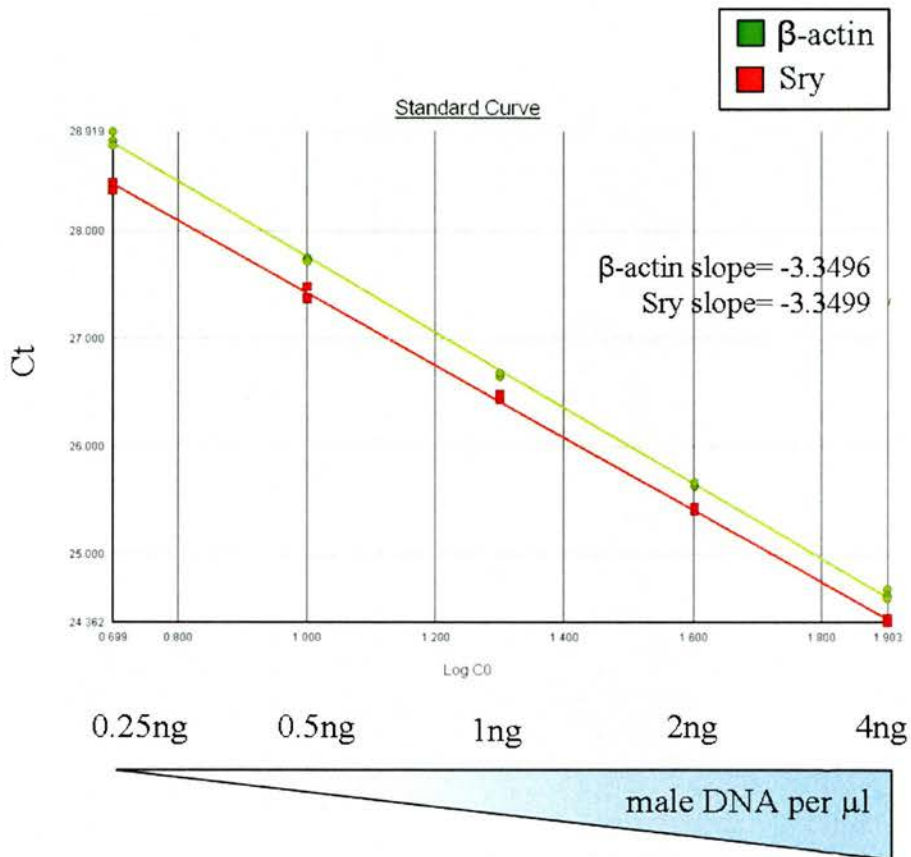
Appendix 2 The presence of irradiated stromal cells did not significantly affect CFU-A activity of EB cells in the assays. (a) Diagrammatic summary of experiment. (b) Testing the effects of stromal cells present in the CFU-A assay with disaggregated 6 day EBs. Non-parametric one-way ANOVA showed that there was no difference in CFU-A colony numbers when 6 day old EB cells were seeded alone or when seeded together with irradiated stromal cells ( $p=0.086$ ).



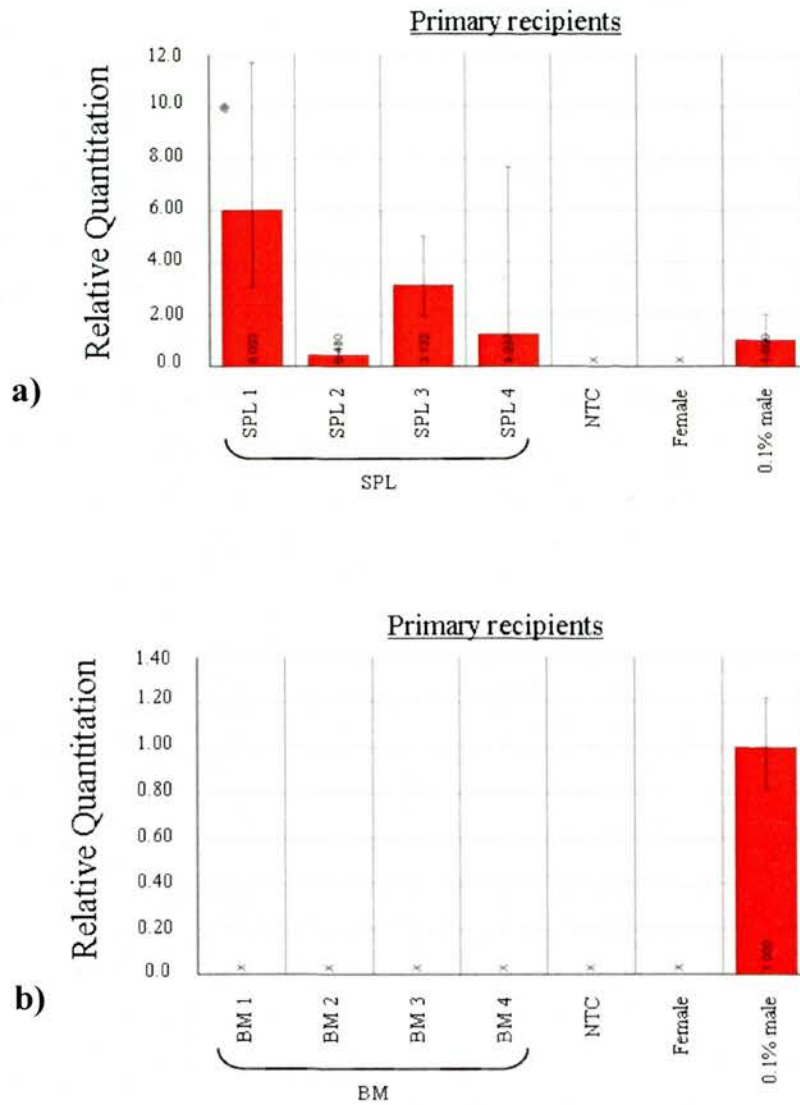
Surface marker	% Positive ES-derived cells							
	4 days differentiation				6 days differentiation			
	Gelatin	AM20.1B4	UG26.1B6	EL08.1D2	Gelatin	AM20.1B4	UG26.1B6	EL08.1D2
cKit	1.9 ± 1.9	0.6 ± 0.6	0.6 ± 0.6	0.3 ± 0.3	9.4 ± 4.9	7.0 ± 6.0	2.8 ± 2.1	1.5 ± 0.6
Sca1	nd	nd	nd	nd	3.9 ± 2.2	3.4 ± 2.5	3.7 ± 3.0	1.9 ± 1.2
CD45	nd	nd	nd	nd	0.1 ± 0.04	0.7 ± 0.5	0.1 ± 0.1	0.4 ± 0.3
Gr1	nd	nd	nd	nd	0.03 ± 0.04	0.1 ± 0.1	0.01 ± 0.02	0.2 ± 0.4
CD11b	nd	nd	nd	nd	0.12 ± 0.1	1.8 ± 1.6	0.15 ± 0.18	0.22 ± 0.38
CD49d	7.21	4.98	9.53	2.48	25.8 ± 17.2	18.1 ± 9.7	4.6 ± 0.6	0.8 ± 1.1
Ter119	nd	nd	nd	nd	0.04 ± 0.06	1.31 ± 2.06	0.01 ± 0.01	0.54 ± 0.77
B220	nd	nd	nd	nd	2.98 ± 0.3	1.57 ± 1.2	0.6 ± 0.14	0.14 ± 0.19
Gr1+ CD11b+	nd	nd	nd	nd	0.03 ± 0.04	0.1 ± 0.1	0 ± 0	0.22 ± 0.38
VCAM-1	0.42	0.02	0.33	0.36	3.5 ± 0.8	2.4 ± 2.1	1.4 ± 1.2	1.7 ± 1.3
CD31	0.86	4.75	4.32	2.37	25.8 ± 2.7	20 ± 14	16 ± 12	1.7 ± 1.9
Flk1	1.24	0	0.11	0.84	3.6 ± 1.0	2.0 ± 1.2	2.3 ± 1.01	0.6 ± 0.97
CD54	nd	nd	nd	nd	34.6 ± 3.2	40.1 ± 6.2	26.6 ± 1.8	17 ± 3.3
cKit+ Flk1+	0.54	0	0.11	0.29	2.1 ± 0.7	0.6 ± 0.5	0.6 ± 0.8	0.3 ± 0.4
VCAM+ CD31+	0.42	0	0.28	0.36	2.3 ± 1.8	2.1 ± 1.9	0.9 ± 0.9	1.4 ± 2.1

\* *nd* = not determined

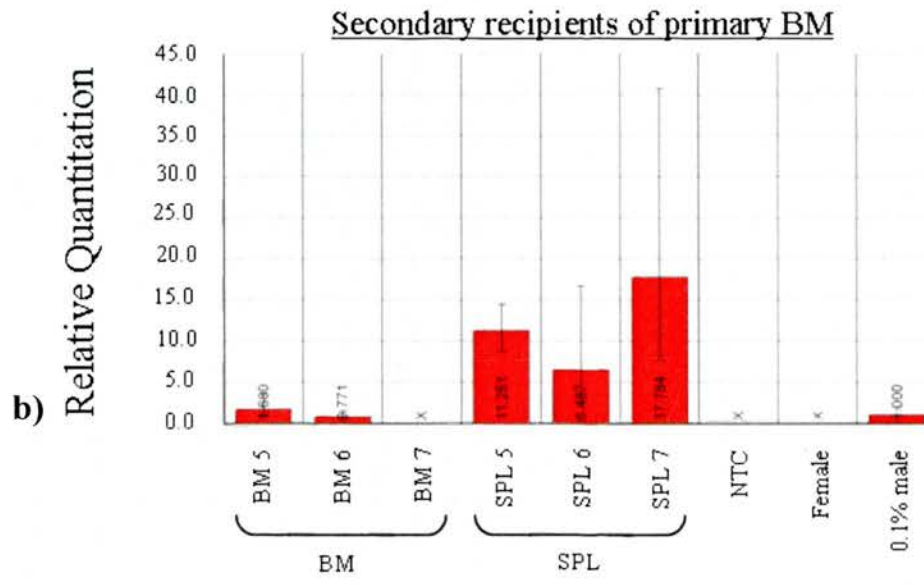
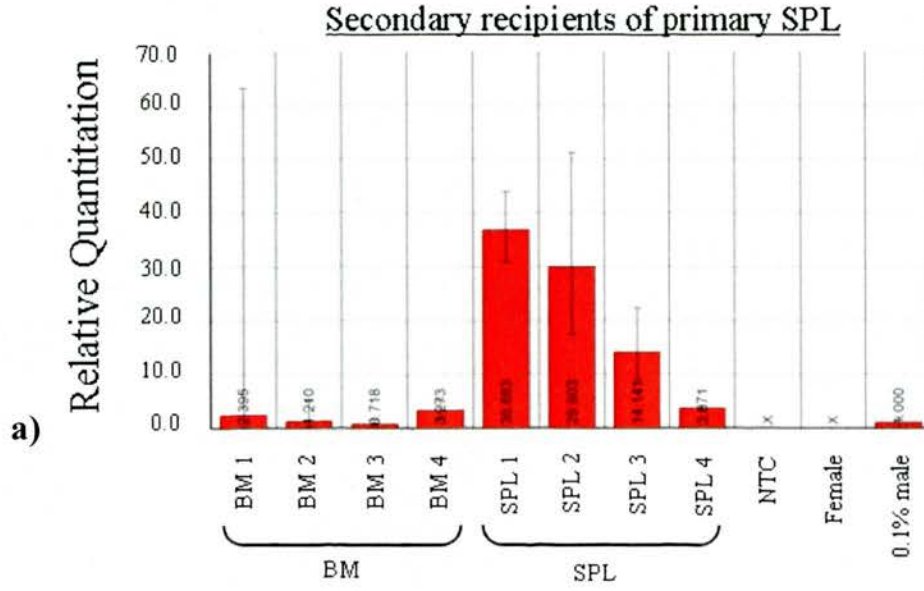
Appendix 3 Flow cytometry analysis of 7a-GFP EB/stromal co-cultures at 4 and 6 days differentiation. Shown are the proportions (%) of ES-derived cells expressing different haematopoietic surface markers (cKit, Sca-1, CD45, Gr-1, CD11b, Ter119, B220) and CD49d (alpha4 integrin). VCAM-1 and CD31 are endothelial markers, Flk-1 marks haemangioblasts. CD54 is an intercellular adhesion molecule. Data represent up to 3 independent co-culture experiments and in each condition, no less than  $1 \times 10^5$  cells were collected for analysis.



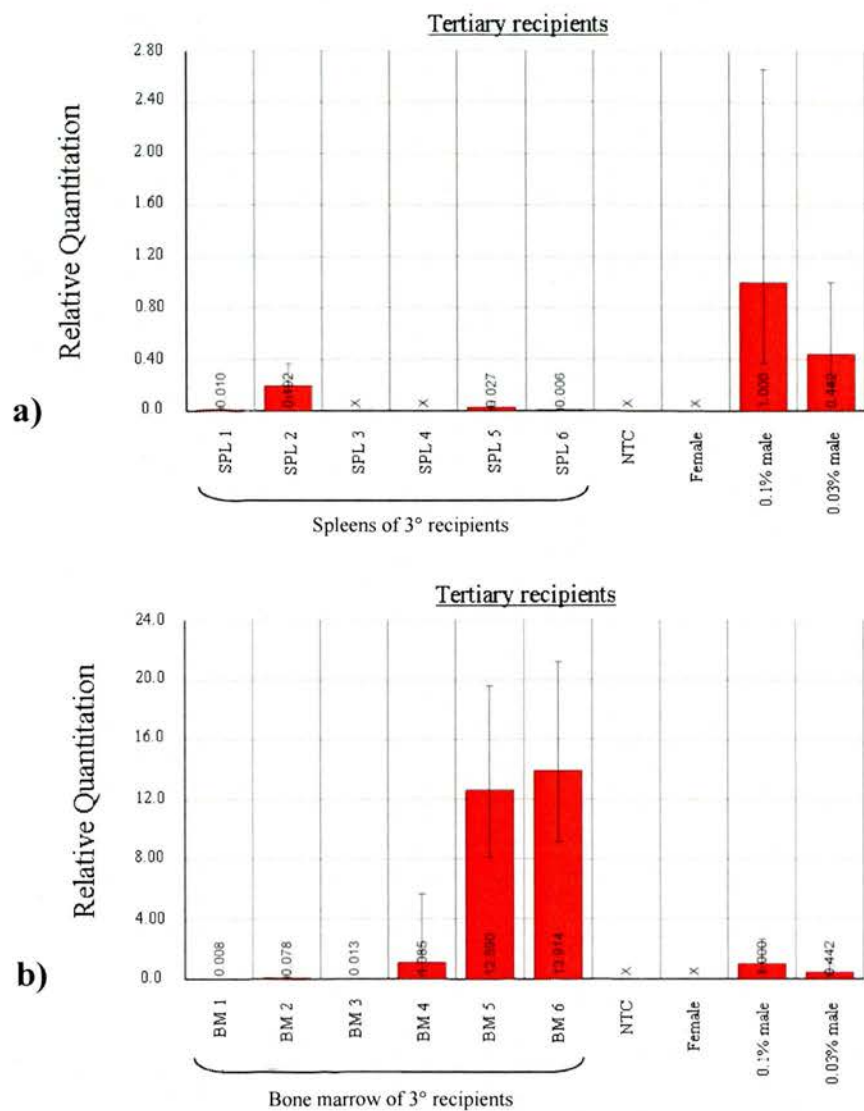
Appendix 4 Validation of Sry and  $\beta$ -actin primer sets. Standard curve of Ct values against increasing concentrations of male C57 bone marrow DNA loaded per well (reactions in triplicate). The slopes (efficiencies) for the primer sets were comparable, indicating that  $\beta$ -actin could be used as the endogenous control for the Sry detection assay. A slope of -3.34 denotes the most efficient reaction.



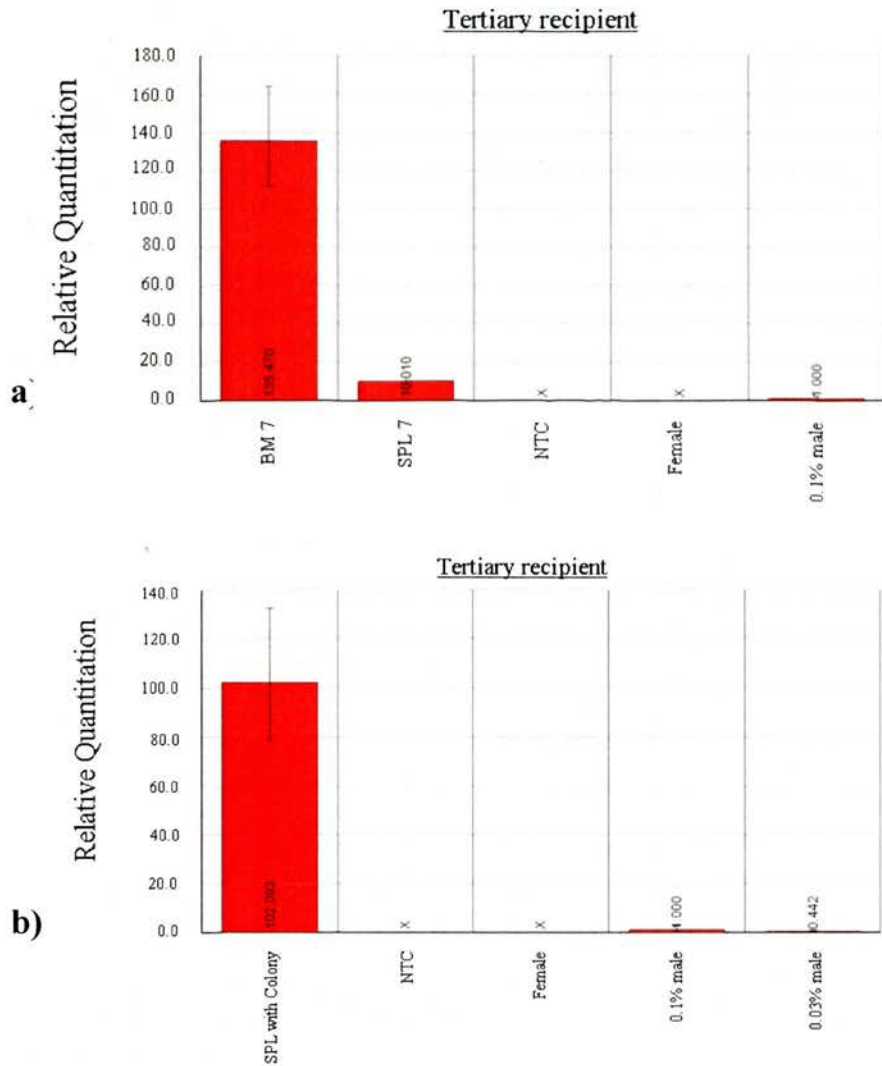
Appendix 5 Primary NOD/SCID recipients of 4 day 7a-GFP EB/ AM20.1B4 co-cultured cells. Raw data for Figure 6.4a. Mice were killed 8 weeks after intra-venous transplantation and tissues harvested. Y chromosome qPCR was carried out on genomic DNA to quantify the male donor ES-derived cells in (a) spleens and (b) bone marrow. A sample known to comprise of 0.1% male 7a-GFP ES cells in NOD/SCID bone marrow was used as the calibrator, which is assigned a value of 1. All samples are expressed as relative fold increase over the calibrator. Relative quantitation was determined by the delta delta Ct method in ABI 7500FAST software. NTC, no template control.



Appendix 6 Secondary NOD/SCID recipients of (a) primary SPL and (b) primary BM samples. Raw data for Figure 6.4 b and c. Primary recipients received 4 day 7a-GFP EB/ AM20.1B4 co-cultured cells. Secondary mice were killed 8 weeks after transplantation (i.v.) and tissues harvested. Y chromosome qPCR was carried out on genomic DNA to quantify male donor ES cell contribution.



Appendix 7 Tertiary recipients of bone marrow and spleen samples from secondary mice. Raw data for Figure 6.4 d and e. Primary recipients received 4 day 7a-GFP EB/ AM20.1B4 co-cultured cells. Tertiary mice received cells from secondary mice as indicated. Tertiary mice were killed 8 weeks after transplantation (i.v.) and tissues harvested. Y chromosome qPCR was carried out on genomic DNA to quantify male donor ES cell contribution in (a) spleens and (b) bone marrow of tertiary mice.



Appendix 8 Tertiary recipients in which the highest repopulation was seen. Y chromosome qPCR on tissues from tertiary mice that received (a) bone marrow and (b) spleen from a secondary mouse that received BM from the primary EB/AM20.1B4 recipient.



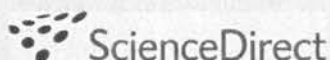
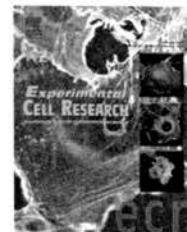
Primary Tissues		% Donor cells	Secondary Tissues	% Donor cells	Tertiary Tissues	% Donor cells
M1	BM	undetected	BM	0.17%	BM	nd
					SPL	nd
			SPL	1.13%	BM	nd
					SPL	nd
	SPL	0.6%	BM	0.24%	BM	0.001%
					SPL	0.001%
			SPL	3.69%	BM	0.001%
					SPL	undetected
M2	BM	undetected	BM	0.08%	BM	13.55%
					SPL	1%
			SPL	0.65%	BM	1.26%
					SPL	0.003%
	SPL	0.043%	BM	0.12%	BM	nd
					SPL	nd
			SPL	3.0%	BM	nd
					SPL	nd
M3	BM	undetected	BM	Undetected	BM	1.4%
					SPL	0.001%
			SPL	1.78%	BM	0.19%
					SPL	* 10.21%
	SPL	0.313%	BM	0.072%	BM	nd
					SPL	nd
			SPL	1.4%	BM	nd
					SPL	nd
M4	BM	undetected	BM	nd	BM	nd
					SPL	nd
			SPL	nd	BM	nd
					SPL	nd
	SPL	0.124%	BM	0.33%	BM	nd
					SPL	nd
			SPL	0.38%	BM	0.008%
					SPL	0.01%

Appendix 9 Serial transplantation of 4 day 7a-GFP EB/AM20.1B4 co-cultures injected intra-venously into NOD/SCID recipients. Donor contribution to host tissues was quantified by the Y chromosome qPCR assay. (\*spleen containing a small white colony).

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## Research Article

## Promotion of haematopoietic activity in embryonic stem cells by the aorta–gonad–mesonephros microenvironment

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## ABSTRACT

We investigated whether the *in vitro* differentiation of ES cells into haematopoietic progenitors could be enhanced by exposure to the aorta–gonadal–mesonephros (AGM) microenvironment that is involved in the generation of haematopoietic stem cells (HSC) during embryonic development. We established a co-culture system that combines the requirements for primary organ culture and differentiating ES cells and showed that exposure of differentiating ES cells to the primary ACM region results in a significant increase in the number of ES-derived haematopoietic progenitors. Co-culture of ES cells on the AM20-1B4 stromal cell line derived from the AGM region also increases haematopoietic activity. We conclude that factors promoting the haematopoietic activity of differentiating ES cells present in primary AGM explants are partially retained in the AM20.1B4 stromal cell line and that these factors are likely to be different to those required for adult HSC maintenance.

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### Introduction

Embryonic stem (ES) cells are able to differentiate into a wide variety of mature cells *in vitro* [1] including a range of haematopoietic cell lineages [2–7]. A number of studies indicate that haematopoietic commitment in differentiating ES cells parallels that found in early stage embryos, making this a suitable *in vitro* model system of haematopoietic development [8–10]. In most of these studies, haematopoietic differentiation is achieved after the initial generation of three-

dimensional embryoid bodies (EBs) and subsequent culture in classical haematopoietic progenitor colony assays. The differentiation of ES cells into haematopoietic lineages using a two dimensional culture system has also been achieved when; for example, ES cells were differentiated directly on the OP9 stromal cell line derived from the CSF-1-deficient adult bone marrow [4]. A recent study directly comparing haematopoietic differentiation between the EB and OP9 system has indicated that optimal haematopoietic cell differentiation occurs when the three-dimensional EB system is used [11].

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Several studies have shown that exposure of ES cells to embryonic tissue may have a significant effect on the differentiation of the cells. For example, an increase in cardiomyocyte differentiation was observed when human ES cells were cultured with visceral endoderm cells from the mouse embryo [12], and when mouse ES cells were cultured with explanted avian precardiac endoderm [13]. T lymphocytes were generated from ES cells in vitro by culture with foetal thymic rudiments [7], and pancreatic differentiation of ES cells in vitro was influenced by soluble factors produced from the developing pancreas [14]. We tested here whether exposure of differentiating ES cells to organ rudiments involved in lympho/haematopoietic development in vivo could stimulate haematopoietic differentiation of ES cells in vitro. Several embryonic organs have been implicated in haematopoietic development including the yolk sac [15], aorta-gonad-mesonephros (AGM) region [16], liver [17] and the placenta [18,19]. The AGM region plays an important role in initiating the generation of haematopoietic stem cells (HSCs) capable of long-term repopulation (LTR) of adult recipients [16,20]. In organ culture, the AGM demonstrated its autonomous capacity to expand LTR-HSCs [16,21], indicating that elements of their supporting microenvironment can be captured in vitro.

To investigate the impact of the AGM microenvironment on haematopoietic differentiation of ES cells, we established a co-culture system that combines the requirements for primary AGM organ culture with the defined conditions for ES cell embryoid body differentiation. We show that the number of haematopoietic progenitors arising from differentiating ES cells is significantly increased when they are co-cultured with the primary AGM region. We then co-cultured differentiating ES cells on stromal lines derived from the AGM and foetal liver that had previously been tested for their ability to support adult haematopoietic stem cells. Interestingly, the ability of the different stromal cell lines to promote haematopoietic activity in ES cells did not correlate with their supportive effect on adult bone marrow haematopoietic stem cells [22–24]. The UG26-1B6 and EL08-1D2 lines that had been shown to support adult haematopoietic stem cells (HSCs) did not promote ES cell haematopoietic activity whereas the number of haematopoietic progenitors generated from ES cells after co-culture on the AM20-1B4 cell line was significantly increased. As adult HSCs could not be maintained on the AM20-1B4 line, these data highlight important differences between the mechanisms involved in the induction and maintenance of HSC activity.

## Materials and methods

### Animals

C57Bl/6 mice were mated, the day of the vaginal plug designated as E0.5, and AGM regions were dissected from E10.5 and E11.5 embryos using fine tungsten needles [25]. Foetal liver was harvested from E13.5 embryos. All animal procedures were carried out according to the provisions of the Animals (Scientific Procedures) Act (UK) 1986.

### Cell culture

The ES cell lines which express GFP either constitutively (GFP#7a) [26] or driven by the *Brachyury* promoter (Bry-201) [27] were maintained on gelatin-coated tissue culture flasks as described previously and differentiated into embryoid bodies (EBs) using the *hanging drop* method to make undifferentiated ES cell aggregates of as uniform a size as possible [28]. Stromal cell lines AM20-1B4, UG26-1B6 and EL08-1D2 were maintained as described [22] on gelatin coated flasks in specialised stroma medium (50% MyeloCult long-term culture medium M5300 and 35%  $\alpha$ -minimal essential medium containing 15% FCS, 4 mM L-glutamine and 10  $\mu$ M 2-mercaptoethanol supplemented with 10–20% 0.2  $\mu$ m-filtered supernatant from the previous passage). All stromal cultures were maintained at 33°C due to the presence of tsA58 transgene encoding the temperature-sensitive SV40 large T antigen.

For AGM co-culture experiments, embryo tissue explants and EBs were placed in direct contact at the air/medium interface on semi-permeable hydrophilic 0.65  $\mu$ m Durapore membranes supported by stainless steel grids (Fig. 1) in ES medium (in the absence of LIF) and with no additional cytokines. Co-cultures were harvested at defined time points, and the number of ES-derived (GFP<sup>+</sup>) haematopoietic progenitors was determined using the agar-based CFU-A and/or HPP-CFC assays. In the co-cultures involving stromal cell lines, EBs were cultured directly on  $\gamma$ -irradiated (30 Gy) stromal cells or transparent Greiner Bio-one 24 well ThinCert-tissue culture inserts (membrane pore size of 0.4  $\mu$ m and pore density of  $2 \times 10^6$ ) were used to inhibit direct contact between the EBs and stromal cells. Cultures were harvested, digested with dispase II (1.2 U/ml) and DNase I (7  $\mu$ g/ml) in PBS for 1 h at 37°C then passed through a 23-gauge needle to generate single cell suspensions for analysis by haematopoietic colony assays and flow cytometry.

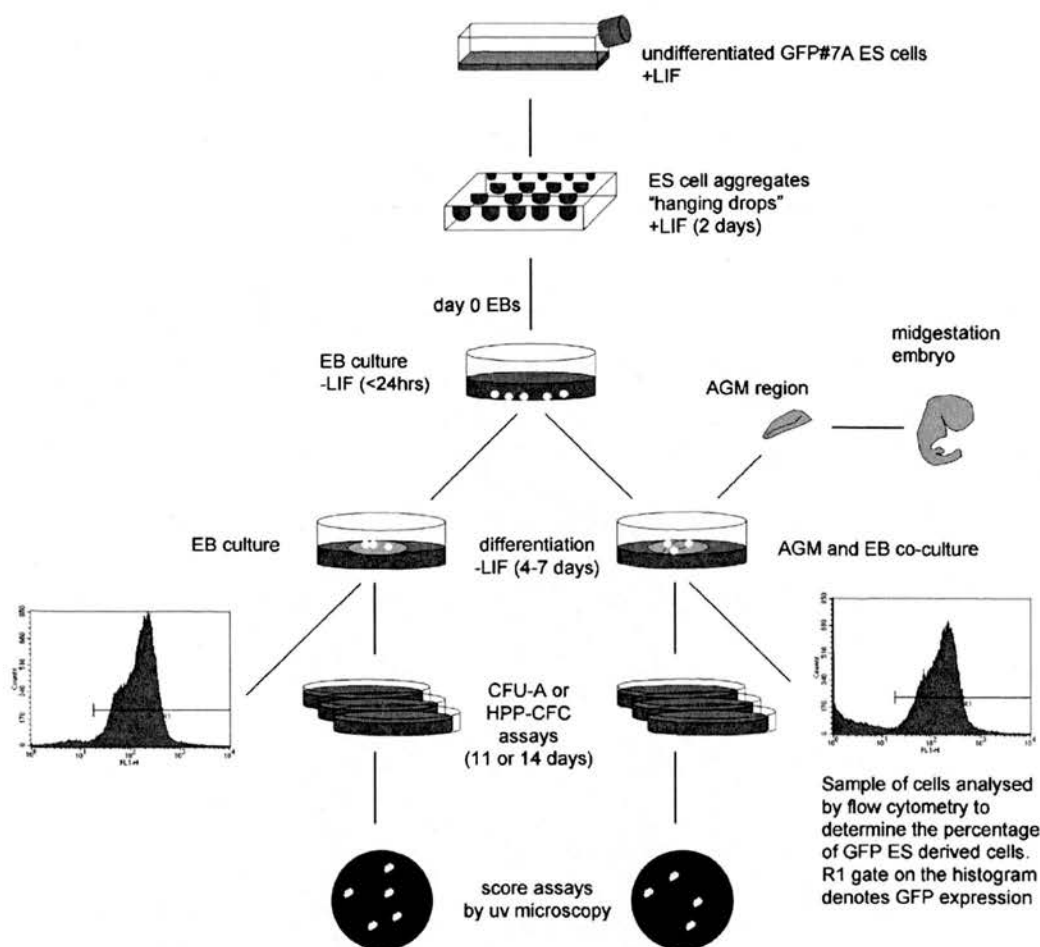
### Haematopoietic colony assays

#### Agar-based assays

The CFU-A assay was performed as described previously [29,30]. A feeder layer of 0.6% agar in a modified Eagle's medium (25%  $\alpha$ -MEM, 20% horse serum, 0.25% sodium bicarbonate and 4 mM L-glutamine) supplemented with 10% conditioned medium from each of two cell lines, L929 and AF1-19T (a source of M-CSF and GM-CSF, respectively), was poured into 3 cm diameter tissue culture grade dishes (1 ml per dish). Cells were resuspended in 0.3% agar in Eagle's medium at a density of  $3 \times 10^4$  cells/ml and plated onto the agar feeder layers in triplicate. After incubation at 37°C in a 5% O<sub>2</sub> and 10% CO<sub>2</sub> humidified atmosphere for 11 days, the colonies (>2 mm in diameter) that primarily consisted of myeloid cells were counted. The procedure for the HPP-CFC [31] assay was similar to the method for the CFU-A assay but conditioned medium from the L929 and WEHI 3b cell lines (a source of M-CSF and IL3, respectively) was added. Colonies were counted after 14 days and, like the CFU-A assay, consisted of myeloid cell types.

#### Methylcellulose-based assays

CFU-mix and CFU-GM assays were performed by plating  $1 \times 10^5$  test cells in 35 mm plates containing 1.5 ml 1% methylcellulose



**Fig. 1 – Co-culture procedure.** Flow diagram illustrating the air–medium interface co-culture of primary embryonic AGM region and EBs made by the *hanging drop* method and the subsequent analysis for haematopoietic differentiation.

in Iscove's Modified Dulbecco's Medium (IMDM) (Stem Cell Technologies (SCT)) supplemented with 10% foetal bovine serum (SCT); 340  $\mu$ M monothioglycerol; 3 Units/ml mouse Epo (Roche); 10  $\mu$ g/ml recombinant human insulin (Sigma) 10 ng/ml mlL3 (SCT); 10 ng/ml rhIL6 (SCT); 50 ng/ml recombinant mouse Stem Cell Factor (SCT), 2 mM L-glutamine and penicillin/streptomycin and incubated at 37°C. CFU-Mix and CFU-GM were identified morphologically and counted after 10 days. Statistical significance of the numbers of resulting colonies was assessed using the t test.

#### Analysis of GFP expression

Harvested co-cultures were analysed by a FACSCaliber equipped with a 488 nm laser (Becton Dickinson) to determine the percentage of GFP<sup>+</sup> (ES-derived) cells. CFU-A colony assays were scored by fluorescence microscopy to determine the number of ES-derived colonies expressing GFP, thus excluding the colonies derived from the non-GFP AGM region in the analysis. The number of GFP-expressing EB-derived CFU-A colonies from the co-cultures was then normalised to represent the number of colonies per  $3 \times 10^4$  GFP<sup>+</sup> (EB-derived) cells.

#### Flow cytometry

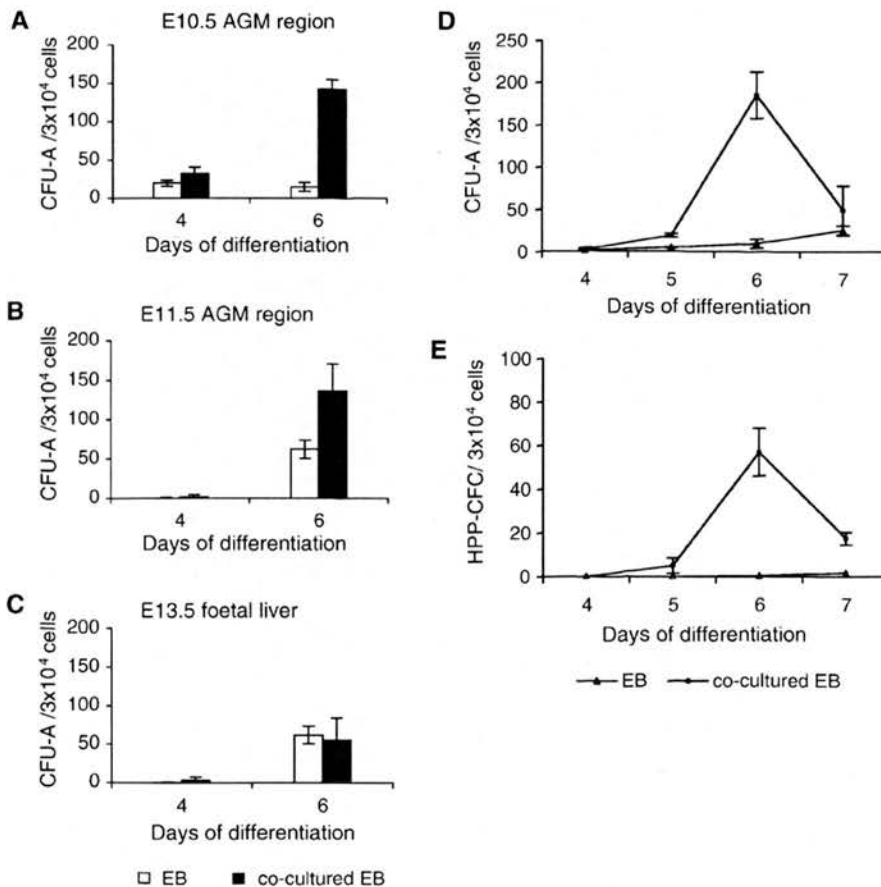
Single cell suspensions of harvested cultures were washed twice in FACS-PBS (PBS supplemented with 0.1% BSA and 0.1% sodium azide (Sigma, UK)) and resuspended at  $2 \times 10^7$  cells/ml.  $2 \times 10^5$  cells were incubated for 40 min at 4°C with optimum concentrations (determined by titration) of fluorochrome-conjugated anti-mouse monoclonal antibodies Sca-1-PE and CD45-APC, whereas c-Kit expression was detected using a biotinylated antibody and detected using APC-avidin (Caltag Medsystems Ltd, UK). Cells were washed twice to remove unbound antibody and resuspended in FACS-PBS for acquisition, unstained cells and cells labelled with APC-avidin alone were included as controls. Dead and apoptotic cells and debris were excluded from analysis using an electronic 'live' gate on forward scatter and side scatter parameters. Data for 10,000–100,000 'live' events were acquired using a FACSCaliber cytometer equipped with 488 nm and 633 nm lasers and analysed using CellQuest software (Becton Dickinson). Post-acquisition, EB-derived cells were identified by GFP expression and an electronic gate applied to exclude stromal cells from analysis for expression of markers.

## Results

### AGM region co-culture enhances haematopoietic differentiation of ES cells in vitro

EBs were co-cultured in direct contact with dissected primary embryonic tissues then assayed for haematopoietic activity (Fig. 1). In these experiments, it was necessary to differentiate between AGM- and ES-derived colonies using fluorescence microscopic detection of the GFP transgene that was constitutively expressed in the GFP#7a ES cell line [26]. We found that prolonged culture in standard methylcellulose-based assays resulted in a high level of auto-fluorescence of all cell types that precluded the detection of GFP expression in this standard haematopoietic assay. To overcome this problem, we used the agar-based CFU-A [29,30] and HPP-CFC [31] assays reported to detect early multi-lineage progenitor cells. Using these assays, we confidently distinguished between AGM- and ES-derived colonies that were GFP negative and

positive, respectively. We tested the haematopoietic activity of EBs after co-culture with E10.5 (Fig. 2A) and E11.5 AGM regions (Fig. 2B) or E13.5 foetal liver (Fig. 2C). After 6 days of co-culture, the E10.5 and E11.5 AGM regions significantly increased haematopoietic activity in EBs 10-fold ( $p < 0.001$ ) and 2-fold ( $p < 0.023$ ), respectively, compared to the equivalent control cultures. The data presented represent the results of one experiment performed in triplicate. In two additional experiments where we directly compared AGM derived from E10.5 and 11.5, we consistently observed a higher level of induction in the E10.5 (2.5–19 fold) compared to the E11.5 (1.5- to 2-fold) primary AGM tissue. E13.5 foetal liver co-culture had no significant effect ( $p < 0.72$ ) on ES-derived CFU-A formation in three independent experiments and co-cultures set-up with remnants of E10.5 embryos (limb buds or foetal head) similarly had no effect on haematopoietic activity of differentiating ES cells (data not shown). The variability in inductive activity observed between experiments with the primary AGM tissue could reflect the inherent variability in the organ culture system or the precise timing of embryonic



**Fig. 2 – Haematopoietic activity of differentiating ES cells is increased in AGM region co-cultures.** The number of CFU-A per  $3 \times 10^4$  input ES cells from EBs cultured alone and co-cultured with either E10.5 (A) or E11.5 AGM (B) region or E13.5 foetal liver (C) for 4 and 6 days. The number of CFU-A (D) and HPP-CFC (E) during EB differentiation when cultured with E10.5 AGM region as compared to control EBs cultured alone over a time course of up to 7 days. The number of ES cells plated in these assays was calculated by assessing the proportion of GFP positive cells by flow cytometry. Error bars represent SD of the mean number of colonies per  $3 \times 10^4$  cells plated in triplicate. Data from representative individual experiments are shown, but similar results were obtained from three independent experiments (see text).



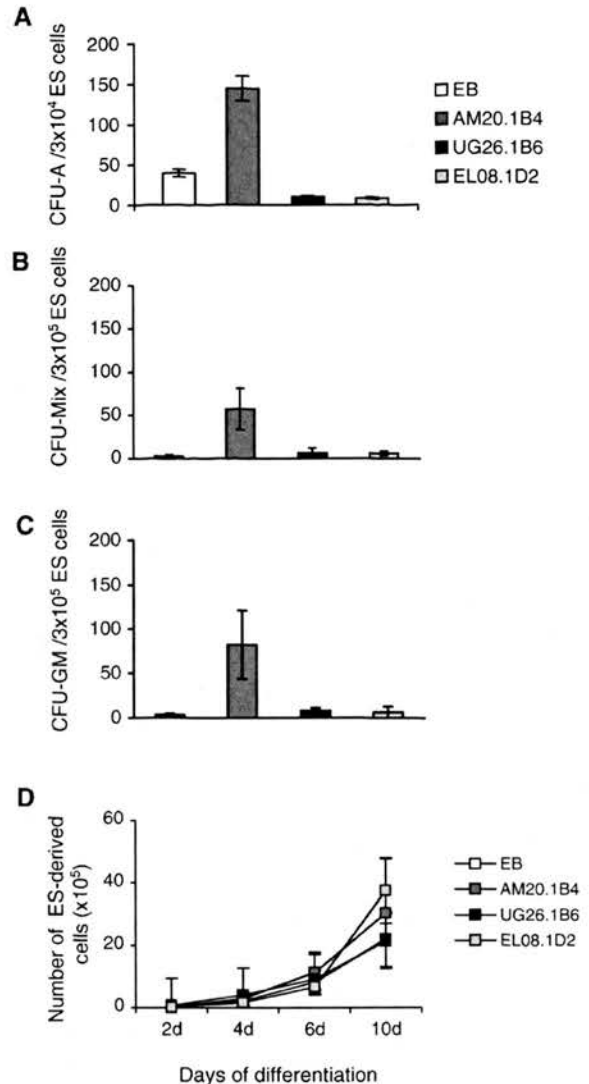
development in tissue collection. Despite this inherent variability, our data show that the primary AGM, but no other regions of the E10.5 embryo, can provide the necessary microenvironment to induce haematopoietic differentiation of ES cells and that the E10.5 AGM appears to be more potent than the AGM derived from embryos 1 day later.

We then concentrated our efforts on the E10.5 AGM tissue, and a time course analysis showed that the emergence of CFU-A from differentiating ES cells was maximal after 6 days of co-culture (Figs. 2D, E). We also assessed these cultures using the HPP-CFC assay that has also been reported to detect early haematopoietic progenitors [31]. The frequency of CFU-A and HPP-CFC colonies from control EBs was comparable to our own unpublished data and to published data [15], respectively. Co-culture of ES cells with E10.5 AGM region resulted in a highly significant ( $p < 0.0004$ ), 19-fold increase in CFU-A (Fig. 2D) and a 57-fold increase in HPP-CFC ( $p < 0.0009$ ) (Fig. 2E). The reduction in the numbers of colonies detected after this 6-day time point could indicate that the cells further differentiate into more mature cell types that are not detected by the CFU-A assay.

#### Co-culture of ES cells with the AM20-1B4 stromal cell line enhances haematopoietic differentiation

EBs were cultured on a panel of stromal cell lines derived from the haematopoietic tissues of the midgestation embryo [22,23]. Three cell lines (AM20-1B4, UG26-1B6 and EL08-1D2) derived from the dorsal aorta and surrounding mesenchyme, urogenital ridge and foetal liver, respectively, were chosen based on their ability to support bone marrow-derived HSCs. UG26-1B6 and EL08-1D2 lines had been shown to be supportive of adult bone marrow-derived HSCs, whereas the AM20-1B4 was shown to be non-supportive. To assay for haematopoietic commitment in this series of experiments, we initially used the CFU-A assay to directly compare our data with the primary AGM culture (Fig. 3A). We were also able to use the more standard methylcellulose based assays since no haematopoietic colonies were generated from the irradiated stromal cells alone (data not shown) so our analysis did not rely on the detection of the GFP transgene.

We observed a significant ( $p < 0.002$ ), 3.5-fold increase in the number of CFU-A when EBs were co-cultured on the AM20-1B4 cells, compared to EBs cultured alone, but this increase was not observed when EBs were co-cultured on UG26-1B6 and EL08-1D2 stromal cell lines (Fig. 3A). The AM20-1B4 stromal cell line also showed an inductive effect when haematopoietic activity was assessed using methylcellulose based haematopoietic colony assays (Figs. 3B, C). The type of haematopoietic progenitor detected in such assays can be inferred from the morphology of the resulting colony: we defined CFU-Mix colonies as consisting of erythroid cells and at least two white cell types and were presumably generated from a multilineage progenitor. There was a significant increase ( $p < 0.016$ ) in the number of CFU-Mix colonies generated after co-culture on the AM20-1B4 stromal cell line compared to control cultures, and this increase ranged from 10- to 54-fold compared to control cultures in three independent experiments (Fig. 3B). We also observed a significant increase ( $p < 0.023$ ) in the number of CFU-GM colonies when EBs were cultured on the AM20-1B4 cell line compared to control EBs (Fig. 3C). The fold increase



**Fig. 3 – Haematopoietic promoting activity is present in the AM20-1B4 stromal cell line derived from the AGM region. The number of CFU-A (A), CFU-Mix (B) and CFU-GM (C) colonies generated from control differentiating EBs or in EBs co-cultured on the AM20-1B4, UG26-1B6 or EL08-1D2 stromal cell lines to 6 days. The number of ES cells plated in each assay was calculated by assessing the proportion of GFP positive input cells by flow cytometry. For experiments involving CFU-A assays, error bars represent SD of the mean number of colonies per  $3 \times 10^4$  cells plated in triplicate. The data shown are from individual experiments, representative of results obtained from three independent experiments. In CFU-Mix and CFU-GM assays, the error bars represent the SD of the mean of three independent experiments. (D) Total number of EB-derived cells up to 10 days in control cultures or in co-cultures with the AM20-1B4, UG26-1B6 or EL08-1D2 stromal cell lines showing no significant difference in the overall growth rates.**

in this type of colony ranged from 17 to 37 in three independent experiments. No significant increase in either CFU-Mix or CFU-GM was observed when ES cells were co-cultured on the UG26-

1B6 and EL08-1D2 stromal cell lines (Figs. 3B, C). We observed no significant difference in the number of secondary EBs (presumably formed from undifferentiated ES cells) that were generated in the control and co-culture conditions (data not shown), and any haematopoietic activity associated with these secondary EBs (although rare) was not included in the true haematopoietic colony assays described above.

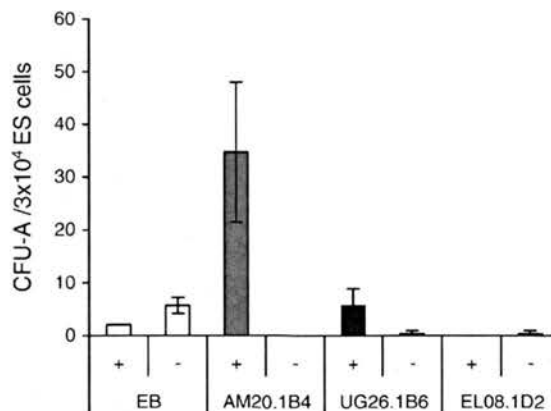
It is formally possible that the differences observed in the frequency of haematopoietic colonies in the differentiating ES cells grown on the different stromal cell lines could have been due to differences in the number of output ES-derived cells and not due to an increase in the efficiency of differentiation. For example, a relative reduction in the total number of ES-derived cells after co-culture compared to controls could result in an apparent increase in the frequency of ES-derived haematopoietic progenitor production. To address this point, we counted the total number of EB-derived cells in control cultures and in 2- to 10-day co-cultures and found no significant difference in growth rate (Fig. 3D) which indicates that co-culture on the AM20-1B4 cell line results in an increase in the frequency of haematopoietic progenitors produced.

We therefore conclude that the AM20-1B4 stromal cell line has an inductive effect on the differentiation of ES cells into haematopoietic lineages. The difference in the level of induction observed in the different progenitor assays possibly reflects the types of progenitors detected by each assay. The CFU-A is reported to assay for a more primitive progenitor [29,30] while the CFU-Mix and CFU-GM possibly represent progenitors at a later point in the haematopoietic hierarchy.

Co-culture of ES cells on the AM20-1B4 stromal cell line also increased the proportion of ES cells expressing the haematopoietic markers CD45, Sca-1 and c-Kit (Table 1), further supporting the haematopoietic promoting activity of this AGM-derived stromal cell line.

#### Cell contact is required for the inductive activity of the AM20-1B4 cell line

Conditioned medium from the AM20-1B4 had no consistent inductive effect on the haematopoietic activity of ES cells (data not shown), indicating that either cell contact or short range signals were responsible for the inductive effect. To assess whether cell contact was required, we co-cultured EBs with the stromal cell lines in transwell cultures (Fig. 4). In this experiment, we again observed a significant ( $P=0.006$ ) increase in the number of haematopoietic colonies when EB were cultured for 6 days on the AM20-1B4 stromal cell line compared to control EBs, but this increase in colony number



**Fig. 4** – Cell contact is required for the haematopoietic promoting activity of the AM20-1B4 stromal cell line. The number of CFU-A colonies per  $3 \times 10^4$  ES cells detected in EBs cultured in direct contact (+) with gelatin (EB) or with the AM20-1B4, UG26-1B6 or EL08-1D2 stromal cell lines or separated (-) from the gelatin and stromal cell lines by a porous membrane in a transwell culture. Error bars represent the SD of the mean of triplicate cultures.

was prevented by a membrane with a pore size of  $0.4 \mu\text{m}$  in the transwell culture conditions.

#### The AM20-1B4 stromal cell lines did not have an inductive effect on early mesoderm commitment

To assess whether the differential effect of the stromal cell lines on the haematopoietic activity was due to a differential effect on early mesoderm commitment, we used an ES cell line that had been engineered to express GFP driven by the *Brachyury* (*Bry*) promoter [27]. We did not observe a significant increase in the proportion of cells expression GFP in ES cells differentiated in co-culture with AM20-1B4 compared to ES cells co-cultured on gelatin or on the UG26-1B6 or EL08-1D2 stromal cell lines (Fig. 5). This suggests that the effect of the AM20-1B4 stromal cell line on the amount of haematopoietic activity likely occurs after the commitment of ES cells to a mesodermal cell fate.

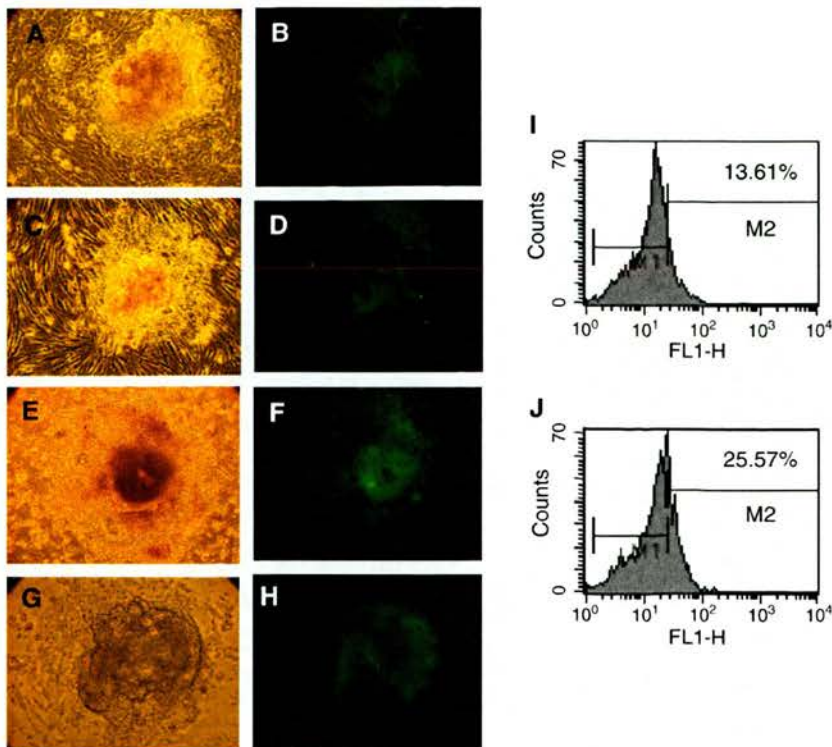
## Discussion

We have established a co-culture system that combines the requirements for primary AGM organ culture with the defined conditions for optimal haematopoietic differentiation of ES cells into three-dimensional EBs. We show that the primary AGM region environment increases haematopoietic progenitor numbers in differentiating ES cells. The increase in EB derived CFU-A activity is also observed when the AM20-1B4 stromal cell line derived from the aorta and surrounding mesenchyme of the AGM region is substituted in the co-culture system. In contrast to the primary AGM tissue, which results in a 19-fold increase in the number of EB derived CFU-A colonies, the maximum increase in haematopoietic progenitor activity achieved using this stromal cell line was only 3.5-fold.

**Table 1** – Expression of haematopoietic markers on differentiating ES cells cultured on gelatin or in co-culture with the AM20-1B4, UG26-1B6 and EL08-1D2 stromal cell lines

Co-culture	c-Kit (%)	Sca-1 (%)	CD45 (%)
Gelatin	8.14 ± 6.5	4.42 ± 4.8	3.51 ± 1.98
AM20.1B4	28.95 ± 18.8	13.33 ± 10.2	17.2 ± 13.92
UG26.1B6	7.24 ± 4.2	3.06 ± 2.4	1.69 ± 0.45
EL08.1D2	3.67 ± 2.0	2.12 ± 2.5	0.51 ± 0.61





**Fig. 5** – *Bry*-GFP expression after culture of ES cells on AGM region and foetal liver stromal cell lines. *Bry*-GFP expression in EBs after 4 days of differentiation on gelatin (A, B) or on the stromal cell lines AM20-1B4 (C, D), UG26-1B6 (D, F) or EL08-1D2 (G, H). Individual EBs were photographed using light (A, C, E, G) and UV fluorescence microscopy (B, D, F, H) to visualise GFP. Flow cytometry analysis of ES cells co-cultured on AM20-1B4 (I) and UG26-1B6 (J) stromal cells showing that the proportion of *Bry*-GFP expression cells was not significantly increased after co-culture on the AM20-1B4 cell line.

This could be explained by the fact that the single stromal cell line is only one component of a complex microenvironment but it is also possible that the immortalised cell line does not function as efficiently *in vitro* as analogous cells *in vivo*. A higher level of induction was observed after co-culture on the AM20-1B4 stromal cell line when haematopoietic activity was assayed using the CFU-Mix and CFU-GM assays that possibly represent more mature haematopoietic progenitors. This could suggest that the primary AGM provides a superior inductive and/or expansive environment for the more primitive haematopoietic precursors compared to the AM20-1B4 stromal cell line. Despite differences in the level of induction, the fact that haematopoietic amplification is observed with the AM20-1B4 cell line using a range of different progenitor assays is encouraging and, consequently, this cell line will be useful in the identification of factors involved in ES-derived haematopoietic induction and/or amplification. We also observed a significant increase in the number of ES-derived cells expressing a range of haematopoietic markers (including CD45, *c-Kit*, *Sca-1*) by flow cytometry after co-culture on the AM20-1B4 stromal cell line. This further supports our finding that haematopoietic differentiation is enhanced by this stromal cell line. Experiments are under way to directly compare the AGM-derived AM20-1B4 cell line to the OP9 stromal cell line that was derived from the CSF-1-deficient bone marrow stroma and also shown to induce haematopoietic activity in differentiating ES cells [4].

We observed that co-culture of ES cells on the AM20-1B4 stromal cell lines did not increase the proportion of cells that had committed to a mesodermal fate as assessed by the appearance of *Bry*-GFP<sup>+</sup> expressing cells during differentiation. These data suggest that the factor(s) produced by the AM20-1B4 cell line affecting the numbers of haematopoietic colonies produced were acting after the induction of ES cells into a mesodermal cell fate. We are currently investigating whether the cell lines promote differentiation of ES cells into mesodermal lineages other than haematopoietic cell types, and preliminary data suggest that the differentiation of ES cells into beating cardiomyocytes is differentially affected (A.K., unpublished).

Previous studies have shown that the AM20-1B4 cell line was less supportive than the UG26-1B6 and the EL08-1D2 cell lines in the maintenance and expansion of HSCs derived from adult bone marrow [22,23]. In our studies on the effects of these cells on ES cell differentiation, we found that AM20-1B4 can increase the numbers of ES-derived haematopoietic progenitors while the other two cell lines had no significant effect after 6 days of co-culture. The long-term maintenance of adult bone marrow-derived HSCs by the UG26-1B6 and the EL08-1D2 cell lines did not require contact, suggesting that secreted molecules are involved in this support [24], whereas we show using transwell cultures that the haematopoietic amplification observed in our ES cell differentiation system is dependent upon stromal cell contact. Taken

together, these data suggest, not surprisingly, that the signals required for the induction and/or amplification of haematopoietic progenitors from ES cells are different to that required for the maintenance of adult bone marrow-derived HSCs.

To date, there has been only limited success in obtaining engraftable long-term repopulating HSCs from ES cells directly in vitro [32–35]. This limitation of ES-derived cells to engraft in the adult haematopoietic system may be attributed to the sub-optimal microenvironment provided by existing in vitro protocols for the differentiation of mature HSCs or the inability of these cells to home to the appropriate niche. Recently, it was shown that c-Kit<sup>+</sup>CD45<sup>+</sup> cells derived from differentiated ES cells were capable of high levels of multilineage engraftment when injected directly into the femur cavity of the mice, providing strong evidence for a homing defect in ES-derived HSCs [36]. Experiments are underway to determine whether our novel co-culture system promotes the production of engraftable HSCs via the intra-femoral route.

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