Emergence and Expansion of Embryonic Definitive Haematopoietic Stem Cells

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References
I declare that the work present in this thesis is my own, except where otherwise stated

Samir Taoudi
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Abstract

_De novo_ generation and physiological expansion of long-term repopulating definitive haematopoietic stem cells (LTR-dHSCs) occurs exclusively during embryogenesis and is initiated within the dorsal aorta/para-aortic mesenchyme (Ao) of the E11.5 aorta-gonad-mesonephros (AGM) region. Elucidation of the _in vivo_ mechanisms involved in LTR-dHSC emergence and expansion is likely to be a prerequisite to the success of generating LTR-dHSCs from non-haematopoietic materials and facilitating an effective expansion of pre-existing LTR-dHSC _in vitro_.

Using flow cytometry and the _in vivo_ long-term haematopoietic reconstitution assay we herein describe the endothelial affiliation of early LTR-dHSCs as they emerge within the embryonic aorta-gonad-mesonephros (AGM) region and yolk sac, fundamentally defined by a VE-cadherin^+^CD45^+^ immunophenotype, and the successive restriction to a haematopoietic identity upon hepatic colonisation. Well defined haematopoietic colony forming unit-culture and endothelial network forming assays were used to reveal that the endothelio-haematopoietic plasticity observed in the early embryo is lost at mid-gestation in favour of exclusive lineage commitment. A functional benefit from haematopoietic-endothelial interaction is alluded to by experiments using a novel liquid suspension culture system in which successful multilineage haematopoietic differentiation of HSC/progenitor cells is dependent on the presence of endothelium.

To directly examine the hypothesis of an anatomical polarity of haematopoietic activity in the AGM region the dorsal aorta (Ao) from the E10.5-11.5 AGM region was bisected along the dorsoventral axis. The ventral (AoV) and dorsal
(AoD) aspects of the Ao were studied for \textit{in vitro} and \textit{in vivo} haematopoietic capacity. Herein, the first functional data to support the hypothesis of a ventral polarity of LTR-dHSC emergence and expansion is described. Investigation into the transcriptional activity of the E11.5 AoV and AoD highlight the differential expression of \textit{GATA-2, GATA-3, Hoxb4, Runx1, BMP4, Noggin} and \textit{Chordin}. Preliminary investigations into the effect of exogenous Noggin in explant cultures suggest a role for BMP4 in the regulation of progenitor cell expansion.

I have discovered a method to facilitate the rapid expansion of heterogeneous CFU-C, CFU-S and LTR-dHSC populations. Previous studies report that following whole organ explant culture of E11.5 AGM region a single LTR-dHSC can be expanded to a pool of 12. Here, using a novel reaggregation system the generation of 98-140 LTR-dHSCs per AGM region is described within 96 hours. Thus, in our hands we have a system with the potential to allow useful investigation into the origin of LTR-dHSCs and mechanisms of their induction and expansion.
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<tr>
<td>7-AAD</td>
<td>7-Amino-Actinomycin D</td>
</tr>
<tr>
<td>µ</td>
<td>Micro</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>Ac-LDL</td>
<td>Acetylated low-density lipoprotein</td>
</tr>
<tr>
<td>AGM</td>
<td>Aorta-Gonad-Mesonephros</td>
</tr>
<tr>
<td>Ao</td>
<td>Dorsal aorta</td>
</tr>
<tr>
<td>Aoc</td>
<td>Cultured dorsal aorta</td>
</tr>
<tr>
<td>AoD</td>
<td>Dorsal aorta - Dorsal</td>
</tr>
<tr>
<td>AoV</td>
<td>Dorsal aorta - Ventral</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>BFU-E</td>
<td>Burst Forming Unit - Erythroid</td>
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<td>BMP4</td>
<td>Bone Morphogenetic Protein 4</td>
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<td>Ca²⁺</td>
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<td>C/D</td>
<td>Collagenase Dispase</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CD45&lt;sup&gt;SP&lt;/sup&gt;</td>
<td>VE-cadherin&lt;sup&gt;CD45&lt;/sup&gt;</td>
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<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
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<td>CFU-GEMM</td>
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<td>dHSC</td>
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<td>Ethyelene diamine tetra-acetate</td>
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<td>e.e.</td>
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<td>Foetal Liver Kinase-1</td>
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<td>Flt3-ligand</td>
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<td>g</td>
<td>Grams</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
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<tr>
<td>HC</td>
<td>Hydrocortisone (sodium salt)</td>
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<td>HSC</td>
<td>Haematopoietic Stem Cell</td>
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<td>Haematopoietic stem cell equivalent</td>
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<td>IL-11</td>
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<td>IMDM</td>
<td>Iscove’s Modified Dulbecco’s Medium</td>
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<td>LTR-dHSC</td>
<td>Long-term repopulating definitive haematopoietic stem cell</td>
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</tr>
<tr>
<td>STR-dHSC</td>
<td>Short-term repopulating definitive haematopoietic stem cell</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>UGR</td>
<td>Urogenital ridge</td>
</tr>
<tr>
<td>VE-cadherin^{sp}</td>
<td>VE-cadherin^{*CD45}</td>
</tr>
<tr>
<td>YS</td>
<td>Yolk Sac</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 General introduction

In vitro generation of long-term repopulating definitive haematopoietic stem cells (LTR-dHSCs) has been a major challenge for researchers. In vivo, LTR-dHSC induction is hypothesised to occur exclusively during embryogenesis. Thus elucidating the in vivo mechanisms of LTR-dHSC development is likely to be essential for designing strategies for the in vitro generation of stem cells.

Unlike in the adult, embryonic haematopoiesis follows a complex developmental pattern that results in the successive appearance of oligopotent, bipotent and multipotent haematopoietic progenitors followed by the emergence of the LTR-dHSC (Johnson and Barker, 1985; Medvinsky and Dzierzak, 1996; Medvinsky et al., 1996; Medvinsky et al., 1993; Moore and Metcalf, 1970; Muller et al., 1994; Palis et al., 1999; Toles et al., 1989; Wong et al., 1986; Yoder and Hiatt, 1997). Elucidating the ontogeny of LTR-dHSCs is further complicated by the existence of two distinct haematopoietic hierarchies that are likely to involve ontogenetically distinct pathways: the primitive (embryonic) and definitive (adult) haematopoietic hierarchies.

Under physiological conditions adult bone marrow LTR-dHSCs exist in either a quiescent state or support the immediate haematopoietic requirements of the organism by entry into the cell cycle and the production of progeny poised to differentiate (Arai et al., 2004; Arai et al., 2005; Cheshier et al., 1999; Goodell et al., 1996; Yoder, 2004). In contrast, during embryogenesis the LTR-dHSC pool undergoes an acute phase of expansion resulting in the generation of thousands of
stem cells in a matter of days (Ema and Nakauchi, 2000; Kumaravelu et al., 2002; Morrison et al., 1995a). Additionally, de novo formation of LTR-dHSCs is hypothesised to only occur during embryogenesis. Therefore, despite possible complications, the study of embryonic LTR-dHSC biology is of great interest.

In this introduction issues regarding LTR-dHSC ontogeny, the co-existence of primitive and definitive haematopoietic hierarchies during embryogenesis and the ability of researchers to induce de novo formation and ex vivo expansion of LTR-dHSCs will be reviewed.
1.2 The definitive haematopoietic hierarchy

The definitive haematopoietic hierarchy is classically represented by the steady-state haematopoiesis that exists within the adult organism*. This process involves a small number of LTR-dHSCs, located in the bone marrow, that are able to support the production and maintenance of downstream progeny. The definitive system is hierarchically arranged according to the progressive loss of lineage plasticity and capacity for self-renewal (Figure 1.1).

1.2.1 The haematopoietic stem cell compartment

At the foundation of the definitive hierarchy is the definitive haematopoietic stem cell (dHSC) compartment (Abramson et al., 1977; Ford et al., 1956) which is capable of simultaneously providing progeny primed to proliferate and differentiate while preserving its existence through the processes of self-renewal and quiescence that are governed by instructive and permissive signals of the stem cell niche (Arai et al., 2004; Arai et al., 2005; Askenasy et al., 2002; Calvi et al., 2003; Nilsson et al., 2001; Orkin, 1995; Schofield, 1978; Wilson et al., 2004; Zhang et al., 2003a).

The dHSC compartment of the adult bone marrow is composed of two functionally distinct components: the long-term repopulating dHSC (LTR-dHSC) and the short-term repopulating dHSC (STR-dHSC)*. Both LTR-dHSCs and STR-HSCs are pluripotent† cells endowed with a high proliferative potential and the ability to home to and engraft upon adult haematopoietic organs. Unlike LTR-

* Unless stated otherwise, all discussions are presented in the context of murine organism
† Short-term repopulating cells are not capable of life-long self-renewal, a property considered to be the most fundamental for stem cells. This term is used for convenience.
‡ The term pluripotent is used in this context to describe the ability of a single cell to produce all lineages of the definitive haematopoietic hierarchy and not to allude to a capacity for organ plasticity
Figure 1.1: Classical representation of the definitive/adult haematopoietic hierarchy

The definitive haematopoietic hierarchy is organised according to the linear differentiation of the LTR-dHSC resulting in the formation of progeny with descending differentiation potential and self-renewal capacity. The founding stem cell compartment (A) is the most potent of the hierarchy, giving rise to CFU-S (B). Further downstream are the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) (C), these cells are restricted to the production of erythromyeloid or lymphoid CFU-Cs (D), respectively. Circular arrows represent self-renewal capacity.

Inset is a schematic semi-quantitative representation of the definitive hierarchy in which a small number of founding stem cells support the production of all downstream progeny and committed mature lineages.

CFU-S denotes colony forming unit-spleen; CFU-C, colony forming unit-culture
dHSCs, STR-dHSCs are restricted by an inability to undergo prolonged self-renewal and are thus only able to contribute to an acute period of haematopoietic reconstitution (Zhao et al., 2000; Zhong et al., 1996). The ability of the LTR-dHSC to sustain long-term self-renewal confers the capacity to robustly support haematopoiesis for the lifetime of the organism (Dick et al., 1985; Jordan and Lemischka, 1990; Keller et al., 1985; Lemischka et al., 1986; Smith et al., 1991). This quality provides the criteria for the gold-standard of LTR-dHSC detection and assessment in the form of the in vivo competitive long-term repopulation assay and limiting dilution analysis (Szilvassy et al., 1990).

LTR-dHSCs are present in the bone marrow of the (CBAxC57/BL6)F1 adult mouse at a frequency of 1 in 10,000 nucleated cells (Kumaravelu et al., 2002) but can be enriched as either a side-population of cells that effectively efflux the nuclear Hoechst 33342 dyes (Goodell et al., 1996) or by a complex immunophenotype based on the low/absent expression of plasma membrane proteins normally present on fully differentiated lineages (Lin). These include: Ter119, Mac-1, Gr-1, B220, CD3ε, CD4 and CD8, and the differential expression of Thy-1, Sca-1, c-Kit, and CD34 (Berman and Basch, 1985; Coffman and Weissman, 1981; Ikuta and Weissman, 1992; Osawa et al., 1996; Spangrude et al., 1988; Whitlock et al., 1987). This classical immunophenotype, as detected using a combination of fluorochrome conjugated monoclonal antibodies, allows LTR-dHSCs to be enriched to 1 in 5 Lin⁻c-Kit⁻Sca-1⁻CD34low/neg or Lin⁻c-Kit⁺Sca-1⁺Thy1.1low cells by flow cytometry assisted cell sorting (Osawa et al., 1996; Wagers et al., 2002). Recently two SLAM family receptors, CD48 and CD150, have been used to purify LTR-HSCs from adult bone
marrow to a frequency of 1 in 5 cells on the basis of a CD48\(^+\)CD150\(^+\) phenotype and to 1 in 2 CD48\(^+\)CD150\(^+\)CD41\(^-\) cells (Kiel et al., 2005).

1.2.2 Colony forming unit-spleen

Downstream of the CIHSC compartment resides the colony forming unit-spleen (CFU-S) which are able to home to the spleen and form macroscopic colonies (Till and McCulloch, 1961). In common with further downstream progenitors, CFU-S are restricted to myeloerythroid differentiation yet they are able to engraft upon adult haematopoietic territories following intravenous injection and undergo a limited period of self-renewal (Becker et al., 1963; Siminovitch et al., 1963; Till and McCulloch, 1961; Wu et al., 1967).

The CFU-S compartment is comprised of early and late developing components. For example, CFU-S\(_8\) are capable of rapidly generating colonies by 7-9 days post-transplantation which recede by day 10, and CFU-S\(_{11}\) produce colonies by day 9-12 that persist until day 14 (Magli et al., 1982). Early and late differentiating CFU-S are further distinguished by their respective capacities for self-renewal, a property restricted to late CFU-S (Magli et al., 1982; Siminovitch et al., 1963). Accordingly it was hypothesised that the CFU-S\(_8\) represented a committed progenitor and the CFU-S\(_{12}\) a more primitive cell type, possibly the LTR-dHSC.

Purified LTR-dHSCs have been reported to possess CFU-S\(_{12}\)-like activity (Spangrude et al., 1988). Evidence against late differentiating CFU-S representing pluripotent stem cells came following the segregation of long-term repopulating and CFU-S activity by counterflow centrifugal elutriation (Jones et al., 1990).
In addition to the clear functional differences between terminally differentiated haematopoietic lineages the myeloid and lymphoid compartments can be resolved according to the mutually exclusive and differential expression of plasma membrane proteins (immunophenotype), many of which can be effectively resolved using fluorochrome conjugated monoclonal antibodies and flow cytometry. The most commonly used lineage specific markers are summarised in Table 1.1.
<table>
<thead>
<tr>
<th>Haematopoietic lineage</th>
<th>Plasma membrane protein</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucocyte</td>
<td>CD45/Ly-5</td>
<td>Ledbetter and Herzenberg, 1979; Thomas, 1989; Lagasse et al, 2000</td>
</tr>
<tr>
<td>Erythroid</td>
<td>Ter119</td>
<td>Kina et al, 2000; Zhang et al, 2003</td>
</tr>
<tr>
<td></td>
<td>CD71</td>
<td>Kemp et al, 1987; Zhang et al, 2003</td>
</tr>
<tr>
<td>Lymphoid</td>
<td>B220</td>
<td>Hardy et al, 1991; Allman et al, 1992; Rolink et al, 1996</td>
</tr>
<tr>
<td></td>
<td>CD19</td>
<td>Krop et al, 1996; Tedder et al, 1994</td>
</tr>
<tr>
<td></td>
<td>CD43</td>
<td>Hardy et al, 1991</td>
</tr>
<tr>
<td>Lymphoid</td>
<td>CD3ε</td>
<td>Leo et al, 1987; Nakano et al, 1996</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>Pierres et al, 1984</td>
</tr>
<tr>
<td></td>
<td>CD8α</td>
<td>Ledbetter et al, 1980; van Ewijk et al, 1981</td>
</tr>
<tr>
<td>Natural killer</td>
<td>NK1.1</td>
<td>Koo and Peppard, 1984; Yokoyama and Seaman, 1993</td>
</tr>
<tr>
<td>Myeloid</td>
<td>CD34</td>
<td>Drew et al, 2002</td>
</tr>
<tr>
<td>Mast cell</td>
<td>c-Kit/CD117</td>
<td>Drew et al, 2002</td>
</tr>
<tr>
<td></td>
<td>Sca-1</td>
<td>Drew et al, 2002</td>
</tr>
<tr>
<td></td>
<td>FceRI</td>
<td>Ishizaka and Ishizaka, 1984; Dombrowicz et al, 1993; Turner et al, 1999</td>
</tr>
<tr>
<td></td>
<td>Gr-1</td>
<td>Hestdal et al, 1991; Fleming et al, 1993; Lagasse and Weissman, 1996</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>Mac-1/CD11b</td>
<td>Lagasse and Weissman, 1996</td>
</tr>
<tr>
<td></td>
<td>Gr-1</td>
<td>Hestdal et al, 1991; Fleming et al, 1993; Lagasse and Weissman, 1996</td>
</tr>
</tbody>
</table>

Table 1.1: Erythroid, lymphoid and myeloid lineage specific markers expressed on the plasma membrane following haematopoietic differentiation
1.3 Development of the definitive haematopoietic system

During embryogenesis four major organs are linked during the development of the LTR-dHSC pool by complex temporal and spatial patterns of stem cell emergence, migration and expansion which have taken nearly 100 years to elucidate. These organs are the aorta-gonad-mesonephros (AGM) region, the yolk sac (YS), the liver and the placenta (highlighted in Figure 1.2).

1.3.1 Extra-embryonic hypothesis of LTR-dHSC emergence

During murine ontogeny haematopoiesis is first initiated in the extra-embryonic YS between embryonic day (E) 7.0-8.0 and is characterised by the presence of primitive nucleated erythroid cells (Russell and Bernstein, 1966; Russell, 1979) and the detection of myeloid in vitro colony forming units (CFU-C) (Johnson and Barker, 1985; Moore and Metcalf, 1970; Palis et al., 1999; Wong et al., 1986). Based on haematopoietic interchange experiments between parabiosed female-male chicks (Moore and Owen, 1965; Moore and Owen, 1967) and the capacity of early YS explants to produce CFU-S and CFU-C (Moore and Metcalf, 1970) it was hypothesised that the LTR-dHSC, from which committed cells were presumed to derive, must have originated from an extra-embryonic source and migrated via the peripheral circulation to the intra-embryonic liver and subsequently to the bone marrow which would succeed as foci of haematopoietic activity (Moore and Metcalf, 1970).

The idea of an extra-embryonic origin of LTR-dHSCs persisted until quail-chick chimera experiments revealed that sustained haematopoietic contribution derived exclusively from an intra-embryonic source (Dieterlen-Lievre, 1975; Martin et al.,
Figure 1.2: Protagonists of LTR-dHSC development

Image of an E11.5 murine embryo highlighting the aorta-gonad-mesonephros (AGM) region, which is a transient intra-embryonic organ located between the forelimbs and hind limbs between E10.5-E12.5, the yolk sac, the placenta and the liver.
Furthermore, although the YS is capable of generating a transient wave of erythroid cells (Dieterlen-Lievre et al., 1976; Lassila et al., 1982) chick-chick chimera experiments showed that HSC generation was predominantly localised to the embryo proper, with the possibility of only minor contribution being attributable to the YS (Lassila et al., 1978; Lassila et al., 1982).

1.3.2 Establishing an intra-embryonic origin of murine definitive haematopoiesis

Cells with the capacity to contribute to definitive haematopoiesis and eventually contribute to the adult dHSC compartment have been identified in the E9.0-10.5 YS and para-aortic splanchnopleura (P-Sp)/AGM region following *in utero* injection of cells into E11.0-15.0 embryos or intra-hepatic injection into conditioned neonates (Toles et al., 1989; Yoder and Hiatt, 1997; Yoder et al., 1997a; Yoder et al., 1997b). Once integrated into the adult recipient dHSC compartment E9.0-10.5 derived cells are subsequently able to repopulate secondary adult recipients. Although it is clear that cells with the potential to become adult-type LTR-dHSCs reside within embryonic organs as early as E9.0 it remains an open question whether these cells contribute to definitive haematopoiesis under physiological conditions.§

Evidence in support of a intra-embryonic source of murine definitive haematopoiesis is provided by *ex vivo* culture experiments: P-Sp was discovered to possess the ability to produce both myeloid and lymphoid progenitors prior to the

§ In the murine embryo only one study reports the successful reconstitution of adult mice from whole E6.0 embryo Hollands, P. (1987). Differentiation and grafting of haemopoietic stem cells from early postimplantation mouse embryos. *Development* 99, 69-76. This observation has to date never been independently reproduced and is inconsistent with subsequent reports.
onset of circulation while the differentiation capacity of the YS was restricted to the myeloid lineage (Cumano et al., 1996). It was therefore argued that cells with definitive haematopoietic potential are likely to have an intra-embryonic origin. Furthermore, significant numbers of lymphoid progenitors are only identified within the YS in situ following the onset of circulation which suggests that an intra-embryonic source of lymphoid progenitors most likely seeds the YS (Godin et al., 1995).

The first adult engrafting cells have been shown to emerge within the AGM region, which precedes the liver in harbouring and expanding CFU-S (Medvinsky et al., 1996; Medvinsky et al., 1993). At E10.5 low-level reconstituting activity, restricted to 1-5% contribution to recipient peripheral blood leucocyte chimerism, has been reported in cells derived from the AGM region (Bertrand et al., 2005; Muller et al., 1994) and YS (Muller et al., 1994). Rare high-level reconstituting capacity at E10.5 is restricted to the AGM region (Muller et al., 1994). Because 112 E10.5 AGM regions were required to successfully repopulate 3 adult mice the appearance of LTR-dHSCs at this developmental stage is likely to be an infrequent event possibly associated with asynchronous development.

Robust detection of LTR-dHSCs during embryogenesis is not possible until E11.0-11.5 at which stage LTR-dHSCs are detected in the YS, the AGM region, the liver, and the vitelline and umbilical arteries (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Muller et al., 1994). These experiments, which involved the injection of freshly dissected organs into adult recipient mice, were unable to distinguish whether extra-embryonic and intra-embryonic organs were capable of
simultaneously and autonomously generating LTR-dHSCs or whether LTR-dHSCs of intra-embryonic origin were rapidly seeding the YS, or vice versa.

Resolving the anatomical origin of the first LTR-dHSC was achieved through the use of an in vitro organ explant system in which dissected embryonic organs could be independently cultured ex vivo. Following the explant and transplantation of E10.5 YS, AGM region and liver only the AGM region was capable of reconstituting irradiated adult recipient mice thereby confirming the AGM region as the first site of LTR-dHSC emergence (Medvinsky and Dzierzak, 1996).

1.3.3 Quantifying E11.5-12.5 LTR-dHSC number: evidence for a dual extra- and intra-embryonic contribution to the hepatic stem cell pool

Combining the above ex vivo culture system with limiting dilution analysis for accurate quantification of LTR-dHSC numbers a model of stem cell ontogeny has been described in which the foetal liver is seeded during two sequential waves of LTR-dHSC production: the first wave occurs in the AGM region between E11.5-12.5 and the second wave, between E12.5-13.5 from the YS (Kumaravelu et al., 2002). The detection of low numbers of mobilised LTR-dHSCs in the peripheral circulation from E11.5 onwards led to the hypothesis that both organs are able to produce LTR-dHSCs that immediately emigrate to the foetal liver via the peripheral circulation.

Importantly, the above studies reveal that although at any single point in time between E11.5 and E12.5 only 1-2 LTR-dHSCs can be detected in the AGM region or the YS both organs are capable of generating far greater numbers, a capacity that
can only be appreciated if the organs are cultured in the absence of potential for circulatory exchange.

These data suggest that the foetal liver pool of LTR-dHSCs derives from both extra-embryonic and intra-embryonic sources. However, in the absence of direct evidence, for example through the differential labelling of AGM region and YS derived cells, it is difficult to be sure that LTR-dHSCs from both the AGM region and the YS actually contribute to the accumulating pool of hepatic LTR-dHSCs.

Additionally, a marked quantitative disparity between the number of LTR-dHSCs within the entire conceptus between E11.5 and E12.5 was observed: quantification of LTR-dHSC number by limiting dilution analysis revealed that approximately 3 LTR-dHSCs are present in the E11.5 embryo which are equally distributed between the AGM region, the YS and the liver; by E12.5 the number of LTR-dHSCs increases to approximately 66 which are distributed between the AGM region, the YS, the liver, the peripheral circulation and the rest of the body. The dramatic increase in LTR-dHSC number observed during a 24 hour period cannot be convincingly explained by cell division alone and thus implicates the involvement of a synchronous induction of an existing pool of precursors followed by rapid hepatic colonisation (Kumaravelu et al., 2002; Medvinsky, 1993).

1.3.4 Embryonic cell lines: further evidence of the haematopoietic competence of the AGM region and the YS

The potent capacity of the AGM region and the YS to facilitate the activity of LTR-dHSCs is further illustrated by the ability of established cell lines to maintain
LTR-dHSCs from both embryonic and adult sources. Interestingly multiple cell types demonstrate supportive abilities.

Non-endothelial cell lines derived from the E11.5 urogenital ridge (UGR) (cell lines UG26-1B6 and UG26-3D4) are capable of sustaining AGM region and adult bone marrow derived LTR-dHSCs during co-culture or with the use of conditioned medium (Harvey and Dzierzak, 2004; Oostendorp et al., 2002a; Oostendorp et al., 2002b; Oostendorp et al., 2005). An established endothelial cell line has been derived from the E11.5 AGM region, the DAS104-4 cell line (Ohneda et al., 1998). As observed with the UG26-1B6 and UG26-3D4 cell lines, DAS104-4 has only been reported to maintain the input number of adult LTR-dHSCs.

The AGM-S3 cell line, derived from the E10.5 AGM region, is unique amongst the cell lines derived from the murine embryo. AGM-S3 has been reported to be capable of facilitating LTR-dHSC induction from E8.0-8.5 YS and P-Sp (Matsuoka et al., 2001; Xu et al., 1998). Despite the unprecedented potential of the AGM-S3 cell line no follow up papers have been published.

Although no successful maintenance of the LTR-dHSC activity has been described from established YS derived cell lines the use of primary endothelial cells from the E9.5 embryo has yielded a positive response: primary endothelial cells derived from the E9.5 YS and P-Sp are able to support the ex vivo maintenance of adult bone marrow LTR-dHSCs (Li et al., 2003).

It is unclear whether the UGR has a preferential capacity for LTR-dHSC maintenance and expansion compared with the region of the dorsal aorta/para-aortic mesenchyme (Ao). Of note, with the exception of the AGM-S3 cell line, none of the currently described embryo-derived cell lines have been reported to facilitate a de
novo induction or expansion of LTR-dHSCs (Harvey and Dzierzak, 2004; Oostendorp et al., 2002b).

1.3.5 Evidence for in vivo LTR-dHSC generation by the YS

A caveat to the dual origin hypothesis of hepatic LTR-dHSCs (section 1.3.3) is the ambiguous ability of the YS to form LTR-dHSCs in the absence of influence from the AGM region. Evidence in support of the ability of the YS to autonomously generate LTR-dHSC will therefore be considered.

As discussed above, co-culture of E8.0 YS cells or P-Sp with the AGM-S3 cell line was reported to generate adult repopulating cells (Matsuoka et al., 2001). These observations support the idea that both the YS and the P-Sp contain progenitors capable of generating LTR-dHSCs prior to the establishment of circulation**. This suggests that migration of LTR-dHSCs or precursors between the AGM region and the YS is not required to explain the presence of extra-embryonic LTR-dHSCs.

Given that in the study presented by Matsuoka et al (2001) LTR-dHSCs were only induced from pre-circulation YS after exposure to an AGM region-derived environment it still remained unclear whether or not the YS can autonomously initiate LTR-dHSC emergence. Using an organ explant system that does not require exogenous stromal support data has been presented to suggest that between E7.5-8.5 autonomous generation of HSCs occurs exclusively within the embryo proper (Cumano et al., 2001). A limitation to this study is that only low-level contribution

** which begins at E8.5 with the completion of the omphalomesenteric artery and is finalised at E10.5 with substantial haematopoietic circulation driven by the beating heart McGrath, K. E., Koniski, A. D., Malik, J. and Palis, J. (2003). Circulation is established in a stepwise pattern in the mammalian embryo. Blood 101, 1669-76.
to haematopoietic reconstitution could be achieved by cultured P-Sp. Although an uncomfortable logical leap is required to make an ontogenetic link between low-level repopulating cells and LTR-dHSCs these data illustrate that long-term adult engrafting cells first arise from an intra-embryonic organ.

A line of evidence in support for the ability of YS to independently generate LTR-dHSCs comes from the GATA-2 haploinsufficient mouse model: a single functional copy of GATA-2, a transcription factor essential for the development of the definitive haematopoiesis (see section 1.4.4.2.2), results in the failure of the E11.5-12.5 AGM region to facilitate LTR-dHSC expansion. In contrast, YS-derived GATA-2<sup>+</sup> stem cells expand in a manner comparable to wild-type LTR-dHSCs (Ling et al., 2004) suggesting the existence of distinct extra-embryonic and intra-embryonic pathways for LTR-dHSC induction/expansion or the ability of the YS environment to compensate for GATA-2 haploinsufficiency.

On balance, it remains unclear whether or not the wildtype YS is capable of autonomously generating LTR-dHSCs in a manner analogous to the AGM region.

1.3.6 Role of the placenta during LTR-dHSC development

It has long been recognised that the placenta is a rich source for haematopoietic progenitor cells (Dancis et al., 1977; Melchers, 1979), an appreciation that has recently been revived (Alvarez-Silva et al., 2003) culminating in two groups independently recognising the organ as a reservoir for a large pool of
LTR-dHSCs between E11.5-15.5 (Gekas et al., 2005; Ottersbach and Dzierzak, 2005) which at E12.5 equals that of the hepatic pool††.

Unlike the E10.5-11.5 AGM region and the E12.5 YS, the placenta is unable to independently initiate LTR-dHSC emergence or expansion under established organ explant conditions (Otterbach and Dzierak, 2005; H.Mikkola, personal communication). It has been proposed that the placenta acts as a stem cell safe house during the early stages of maturation and expansion prior to hepatic colonisation, as supported by the concomitant reduction of the placental LTR-dHSC pool as the hepatic pool expands (Gekas et al., 2005; Mikkola et al., 2005). Importantly, these data provide the basis for an alternative explanation for the quantitative disparity in LTR-dHSCs between E11.5 and E12.5 that favours an extra-embryonic pooling of stem cells that are subsequently liberated and undergo hepatic colonisation.

During the subsequent days of gestation the number of hepatic LTR-dHSCs continues to increase until colonisation of the long bones is initiated at E17.5 (Christensen et al., 2004; Ema and Nakauchi, 2000; Gekas et al., 2005; Morrison et al., 1995a).

The combination of the above data allows the formulation of a model for the development of the LTR-dHSC pool which involves an initial wave of activity at E10.5-12.5 during which the first LTR-dHSCs are produced by the AGM region and emigrate to either the placenta or the liver; a second wave is subsequently initiated by the YS at E12.5 which continues to supplement the continually expanding stem

cell pool until E13.5. Placental LTR-dHSCs are hypothesised to begin to relocate to
the liver between E12.5-15.5 where they remain until the early stages of neonatal life
(summarised in Figure 1.3).

1.3.7 De novo LTR-dHSC formation: an embryonic privilege

It has been speculated that all dHSCs present in the adult organism are
produced during embryogenesis and no de novo induction of LTR-dHSCs occurs
after birth (Dzierzak et al., 1998; Godin and Cumano, 2002; Keller et al., 1999).
Some indirect evidence exists to support this theory which includes the dispensable
role of the transcription factors SCL/Tal-1 and Runx1/AML1 in adult LTR-dHSCs,
which in the embryo are essential for the initiation and completion of definitive
haematopoiesis (Elefanty et al., 1997; Ichikawa et al., 2004; Mead et al., 1998;
Mikkola et al., 2003b; North et al., 1999; Okuda et al., 1996; Porcher et al., 1996;
Robb et al., 1996; Robb et al., 1995; Shivdasani et al., 1995; Wang et al., 1996).

An attempt to directly address this question has been undertaken in which a
compound transgenic mouse line bearing a silent reporter protein that could be
activated in cells expressing the SCL gene (Gothert et al., 2005). Following the
activation of the reporter gene at E10.5-11.5 not all hepatic LTR-dHSCs were
labelled. However, Gothert et al (2005) observed that the same proportion of
irreversibly marked cells contributed to haematopoiesis in the foetal liver and the
adult bone marrow. From this the authors deduced that no de novo stem cell
formation is likely to occur in the adult.
Figure 1.3: Composite model for the development of the LTR-dHSC pool
The first wave of LTR-dHSCs emerge from the AGM region at E11.5; AGM derived stem cells proceed to colonise the foetal liver (A), the placenta (B) and possibly the yolk sac (C). A second wave of LTR-dHSC expansion is initiated in the yolk sac at E12.5; yolk sac derived stem cells continue the colonisation of the liver (D) and placenta (F). Once the yolk has seized LTR-dHSC production the hepatic pool of LTR-dHSCs are supplemented by the placental pool (F). The liver remains the focus of LTR-dHSC maintenance until the neonatal bone marrow is colonised (G).

LTR-dHSC, Long-term repopulating definitive haematopoietic stem cell
AGM, Aorta-gonad-mesonephros region
1.3.8 Dorsoventral distribution of haematopoietic activity during embryogenesis

Transplantation of freshly isolated Ao and UGR from the E11.5 AGM region has localised the first LTR-dHSCs exclusively to the Ao (de Bruijn et al., 2000). However, if a period of explant culture is introduced prior to transplantation the capacity of the E11.5 UGR to generate LTR-dHSC becomes apparent (de Bruijn et al., 2000). To date no direct functional data in support of a polarity in haematopoietic activity within the murine Ao has been presented. Circumstantial evidence in support of an enhanced ventral haematopoietic capacity includes:

1. Identification of intra-aortic haematopoietic clusters (IAHCs) restricted to the ventral luminal wall of the 5-week old human Ao that express CD34, PECAM-1 and CD45 (Figure 1.4A) (Tavian et al., 1996; Tavian et al., 1999) and in the murine E10.5-11.5 Ao that express the LTR-dHSC/low-level repopulating cell associated markers CD45, CD34, PECAM-1, AA4.1 and Runx1 (Figure 1.4B) (Baumann et al., 2004; Bertrand et al., 2005; Garcia-Porrero et al., 1995; Garcia-Porrero et al., 1998; Godin et al., 1999; Jordan et al., 1990; Manaia et al., 2000; North et al., 2002; Sanchez et al., 1996).


3. A ventral enrichment of sub-aortic patches (SAPs) (Figure 1.4C) which are regions of cells defined by the high-level expression of GATA-2,
Figure 1.4: Ventral localisation of intra-aortic haematopoietic clusters and sub-aortic patches

(A) Transverse section through the dorsal aorta (da) of a day 32 human embryo. Note the presence of the intra-aortic haematopoietic cluster (IAHC) on the ventral surface of the dorsal aorta (arrow heads). CD34 expression is seen in brown. da denotes dorsal aorta. Scale bar represents 100μm. Image taken from Tavian et al (1999).

(B) Transverse section through a murine E11.5 AGM region demonstrating the presence of a CD34 expression IAHC on the ventral surface of the dorsal aorta (arrow heads). Scale bar represents 57μm. Image taken from Garcia-Porrero et al (1998).

(C) In this example of a murine E10.5 AGM region sub-aortic patches are distinguished by the expression of GATA-3 (blue staining); pink staining represents alkaline phosphatase positive primordial germ cells. GR denotes genital ridge; SV, subcardinal vein; WD, Wolfian duct. Scale bar represents 100μm. Image taken from Manaia et al (2000).
$GATA-3$ and $LMO2$ transcripts (see section 1.4.4). It has been theorised that SAPs generate HSCs that subsequently migrate to the ventral luminal side of the aortic wall to form IAHCs (Bertrand et al., 2005; Godin and Cumano, 2002; Godin et al., 1999; Manaia et al., 2000).

Despite reports of the appearance of IAHCs and SAPs coinciding with the induction of intra-embryonic progenitor activity (Bertrand et al., 2005; Godin and Cumano, 2002; Godin et al., 1999) to date no groups have been able to directly attribute functional haematopoietic stem/progenitor activity to these structures.

1.3.9 Ancestry of haematopoiesis and the LTR-dHSC

Early investigations into the endothelio-haematopoietic composition of extra-embryonic blood islands and the perceived intra-embryonic phenomenon of the luminal budding of haematopoietic cells from aortic endothelium led to the proposal of haematogenic endothelium (Jordan, 1917) and formulation of the haemangioblast theory (Sabin, 1920): a bipotent cell restricted to endothelio-haematopoietic differentiation. Since its inception many studies have focused on proving the existence of such progenitors during embryonic development.

1.3.9.1 Endothelial contribution to embryonic haematopoiesis

To successfully demonstrate the endothelial heritage of haematopoietic cells it is necessary to either specifically label the in vivo endothelial compartment and trace their progeny or purify putative haematogenic populations and investigate their
capacity in vitro. Plasma membrane proteins preferentially expressed in the endothelial lineage are summarised in Table 1.2.

Vascular endothelial cadherin (VE-cadherin; also known as CD144 and cadherin-5) is an indispensable adhesion molecule specifically expressed at the adherens junctions between endothelial cells where homotypic interactions preserve the integrity of vascular structure (Breier et al., 1996; Carmeliet et al., 1999; Crosby et al., 2005; Gory et al., 1999; Gotsch et al., 1997). To date, the expression of VE-cadherin is the most effective single marker to resolve the endothelial and haematopoietic lineages by immunophenotype: PECAM-1, Tie-2 and Flk-1 are commonly associated with the endothelial compartment however they are also present on mesoderm, components of the embryonic and adult haematopoietic lineages, and foetal and adult LTR-dHSCs (Arai et al., 2004; Baumann et al., 2004; DeLisser et al., 1994; Hamaguchi et al., 1999; Hirashima et al., 1999; Nishikawa et al., 1998a; North et al., 2002; Takakura et al., 1998; Vecchi et al., 1994; Yamashita et al., 2000; Yano et al., 1997). In contrast, the expression of VE-cadherin is restricted to endothelium (Breier et al., 1996; Carmeliet et al., 1999; Gory et al., 1999; Hisatsune et al., 2005).

Reports of non-endothelial VE-cadherin expression are restricted to the first LTR-dHSCs that emerge within the E11.5 AGM region, which are themselves hypothesised to be immediate progeny of the endothelial compartment (North et al., 2002). Apart from this exception, no functionally defined endothelial populations have been reported to express CD45. Thus the most effective basic endothelial immunophenotype appears to be VE-cadherin^CD45^, with haematopoietic cells
<table>
<thead>
<tr>
<th>Plasma membrane protein</th>
<th>Function/comments</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Ac-LDL receptor</td>
<td>Binds Ac-LDL</td>
<td>Voyta et al, 1984; Brown et al, 1979; Brown et al, 1980</td>
</tr>
<tr>
<td></td>
<td>Expressed on macrophages</td>
<td></td>
</tr>
<tr>
<td>Flk-1</td>
<td>VEGF receptor expressed by endothelium and mesoderm</td>
<td>Millauer et al, 1993; Quinn et al, 1993; Nishikawa et al, 1998</td>
</tr>
<tr>
<td></td>
<td>Expressed on foetal liver and quiescent adult bone marrow LTR-dHSCs</td>
<td></td>
</tr>
<tr>
<td>PECAM-1/CD31</td>
<td>Involved in inter-endothelial homotypic interactions</td>
<td>Vecchi et al, 1994; Delisser et al, 1994; Suri et al, 1996; Baumann et al, 2004; North et al, 2002</td>
</tr>
<tr>
<td></td>
<td>Expressed on some adult haematopoietic cells and all HSCs during ontogeny and adulthood</td>
<td></td>
</tr>
<tr>
<td>VE-cadherin/CD144</td>
<td>Essential for angiogenesis and preserving vascular Integrity at endothelial adherens junctions</td>
<td>Gotsch et al, 1997; Lampugnani et al, 1992; Breier et al, 1996; Carmeliet et al, 1999; Crosby et al, 2005; Gory et al, 1999</td>
</tr>
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</table>

VE-cadherin, vascular endothelial cadherin; Flk-1, foetal liver kinase-1, PECAM-1, platelet endothelial cell adhesion molecule-1
Ac-LDL, acetylated low density lipoprotein; VEGF, vascular endothelial growth factor; HSC, haematopoietic stem cell; LTR-dHSC, long-term repopulating definitive haematopoietic stem cell

Table 1.2: Endothelial associated plasma membrane proteins
being most robustly defined by the VE-cadherin^CD45^ phenotype (Fraser et al., 2003; Nishikawa et al., 1998b).

**1.3.9.1.1 Haematogenic endothelium**

**1.3.9.1.1 In vivo tracing**

Preliminary functional experiments alluding to the haematopoietic capacity of endothelium involved tagging cells within the circulation of early avian embryo using fluorochrome conjugated acetylated low-density lipoprotein and non-replicating retroviral vectors (Jaifredo et al., 2000; Jaifredo et al., 1998). Both studies relied on the validity of two assumptions: 1. only cells lining the vascular lumen would be labelled; 2. only endothelial cells would be successfully marked. Based on these experiments Jaifredo et al (1998; 2000) described the budding and ingression of haematopoietic cells from the chick aorta. However, the absence of any mutually exclusive criteria by which endothelium can be differentially labelled from the haematopoietic compartment has made interpretation of these observations problematic.

**1.3.9.1.1.2 The embryonic stem cell differentiation model**

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of pre-implantation embryos which can be maintained as undifferentiated cell lines when co-cultured with fibroblast feeder cells (Evans and Kaufman, 1981; Martin, 1981) or in the presence of leukaemia inhibitory factor and BMP4 (Smith et al., 1988; Williams et al., 1988; Ying et al., 2003). ES cells retain the capacity for *in vitro* and *in vivo* differentiation along the endoderm, the ectoderm and the mesoderm.
pathways (Beddington and Robertson, 1989; Bradley et al., 1984), including cells of the haematopoietic lineage.

The first direct functional evidence of haematogenic endothelium was achieved using an *in vitro* ES cell differentiation model in which cells with a VE-cadherin$^+CD45^{-}\text{Ter119}^-$ immunophenotype gave rise to cells of the erythroid and myeloid lineages (Nishikawa et al., 1998a). An analogous *in vivo* haematogenic population bearing a VE-cadherin$^+CD45\alpha_4$-integrin$^+$ immunophenotype has been isolated from the E8.5-10.5 YS and embryo proper (Fraser et al., 2003; Nishikawa et al., 1998b; Ogawa et al., 1999). Furthermore, endothelial cells purified from both extra-embryonic and intra-embryonic sources are capable of generating lymphoid and myeloid progeny (Nishikawa et al., 1998b). This raises the possibility that either haematogenic endothelium is capable of generating LTR-dHSCs which can later differentiate into lymphocytes or that haematogenic endothelium is able to undergo lymphoid differentiation thus represent part of the primitive haematopoietic hierarchy.

1.3.9.1.2 The haemangioblast

Using the *in vitro* ES cell differentiation model a blast colony-forming cell (BL-CFC) has been identified which is proposed to represent the *in vitro* equivalent of the hypothesised *in vivo* haemangioblast (Choi et al., 1998; Kennedy et al., 1997). The BL-CFC transiently exists within three-dimensional embryoid bodies between 2.5-4.0 days of differentiation; the emergence of the BL-CFC precedes the onset of haematopoiesis and is capable of forming colonies composed of at least endothelial and haematopoietic cells upon transfer to secondary culture. The fact that the BL-
CFC arises before the onset of haematopoietic and endothelial development supports the idea that this progenitor could represent an early haemangioblast. However, it is also possible that the cells responsive to secondary culture are a population of uncommitted mesoderm, as implied by the Flk-1⁺Brachyury⁺ phenotype (Fehling et al., 2003).

Attempts to prove that the BL-CFC can fulfil the criteria required to confirm its haemangioblast function, the ability of a single cell to differentiate exclusively along haematopoietic and endothelial pathways, have been restricted to eliminating the possibility that colonies arise from the migration and association of cells of varying differentiation potential through cell mixing experiments using genetic markers (Choi et al., 1998). The ability of a single purified Flk-1⁺Brachyury⁺ cell to produce a colony composed exclusively of haematopoietic and endothelial cells has to date not been formally proven.

An analogous in vivo Flk-1⁺Brachyury⁺ population has been identified in the posterior region of the primitive streak as a transient population in the E7.0-7.5 murine embryo (Huber et al., 2004). These cells are capable of producing ex vivo colonies with a similar lineage composition to those derived from the in vitro BL-CFC (Huber et al., 2004). The study presented by Huber et al (2004) suffered from the same failure to demonstrate the clonal origin of in vitro colonies. However, the major flaw in the study which prevents the colony forming cell(s) being classified as a haemangioblast is the clear presence of smooth muscle cells in the differentiated colony. These progenitors should be more accurately considered as a sub-fraction of mesoderm capable of giving rise to haematopoietic, endothelial and smooth muscle lineages.
1.3.9.2 Endothelial origin of LTR-dHSCs

Because of the difficulties associated with studying the rare first LTR-dHSCs far less information has been collected regarding their provenance. Evidence for the capacity of the embryonic endothelial compartment to produce adult repopulating cells was first provided by the discovery that the neonatal repopulating cells from the E9.5 YS express the endothelial specific protein VE-cadherin and lack the expression of the pan-haematopoietic marker CD45 (Fraser et al., 2002), suggesting that pre-LTR-dHSC function is first detected within an endothelial population. Low-level adult engrafting cells of the E10.5 AGM express the endothelial-associated AA4.1 and low levels of CD45 (Bertrand et al., 2005; Petrenko et al., 1999). Again, suggesting an affiliation of HSCs to the endothelial compartment.

The recurrent association of multipotent engrafting cells with endothelium continues with the first E11.5 AGM LTR-dHSC. High-level repopulating capacity is isolated exclusively within the VE-cadherin and CD45 co-expressing fraction (North et al., 2002), suggesting that these cells had recently diverged from the endothelial compartment.

Thus adult repopulating HSCs between E9.5-11.5 share in common the expression of traditionally endothelial associated proteins. This observation provides the foundation for two interpretations: 1. that these cells represent an ontogenetic continuity completed with the acquisition of CD45 expression and high-level adult repopulating potential, or 2. that putative pre-LTR-dHSCs, low-level adult engrafting cells and LTR-dHSCs derive independently from endothelial precursors.
1.3.9.2.1 Insights from gene inactivation studies

1.3.9.2.1.1 VE-cadherin knockout

VE-cadherin is an essential component of vascular integrity (see section 1.3.9.1); ablation of physiological expression results in extensive abnormalities in vascular development including incomplete Ao formation, the absence of YS vascular organisation and possibly the failure to establish circulation between extra-embryonic and intra-embryonic compartments (Carmeliet et al., 1999; Gory-Faure et al., 1999; Rampon and Huber, 2003). Null embryos die between E9.5-11.5 which is thought to arise from inadequate vascular development caused by increased endothelial apoptosis (Carmeliet et al., 1999; Gory-Faure et al., 1999).

The YS of VE-cadherin\textsuperscript{−/−} embryos are able to develop blood islands and between E8.5-10.5 demonstrates no quantitative or qualitative disparity in CFU-C content (Carmeliet et al., 1999; Gory-Faure et al., 1999; Rampon and Huber, 2003). To date, no functional studies on VE-cadherin\textsuperscript{−/−} intra-embryonic haematopoiesis have been reported, however, the embryo proper suffers from developmental retardation and the failure to produce any obvious signs of ongoing erythropoiesis, as evidenced by pallor and the absence of detectable embryonic β-globin (βH1) transcripts (Gory-Faure et al., 1999).

Absence of investigations into the presence of adult engrafting cells, such as CFU-S or pre-LTR-dHSCs, means that it is difficult to appreciate the level to which normal vascular development is required for extra-embryonic haematopoietic progress. Furthermore, the lack of formal reports of the status of intra-embryonic haematopoiesis and the development of definitive haematopoiesis in the absence of
functional VE-cadherin expression means that the necessity for VE-cadherin during LTR-dHSC development is unclear.

1.3.9.2.1.2 Flk-1 knockout

The expression of Foetal Liver Kinase-1 (Flk-1), an endothelial and mesodermal associated Vascular Endothelial Growth Factor (VEGF) receptor (Hirashima et al., 1999; Millauer et al., 1993; Nishikawa et al., 1998a; Quinn et al., 1993; Yamashita et al., 2000) is vital for haematopoietic development. Although the in vitro differentiation of Flk-1 ES cells along a haematopoietic pathway is possible (Schuh et al., 1999; Shalaby et al., 1997) the Flk-T" embryo suffers from a combined failure of early endothelial precursors to form extra- and intra-embryonic blood vessels. Furthermore, the induction of both primitive and definitive haematopoietic hierarchies is blocked as evidenced by the absence of E7.5 YS blood islands or circulating erythroid cells, and the inability of null ES cells to contribute to the definitive haematopoietic hierarchy in chimeric adult mice created by the injection in Flk-I" ES cells into wildtype blastocysts (Shalaby et al., 1997; Shalaby et al., 1995). The cell autonomous defect caused by the absence of functional Flk-1 is hypothesised to arise from the failure of mesoderm or early endothelio-haematopoietic progenitors to migrate from the primitive streak to extra- and intra-embryonic niches where they would contribute to the formation of LTR-dHSCs.

These data are consistent with the hypothesis of a haemangioblast origin of primitive and definitive haematopoiesis that is disrupted in the absence of functional Flk-1. However, it is also possible that the endothelial and haematopoietic
compartments have an independent ontogeny, both of which could be affected by the failure of mesodermal migration or appropriate response to VEGF.

1.3.9.2.1.3 Tie-2 knockout

Tie-2 is a receptor tyrosine kinase expressed by all endothelium within the embryo and adult that interacts with angiopoietin-1 and angiopoietin-2 to orchestrate the growth and modelling of vascular development (Davis et al., 1996; Maisonpierre et al., 1997; Takakura et al., 2000).

Deletion of Tie-2 results in embryonic lethality at E10.5 and is characterised by the failure of vascular network formation (Sato et al., 1995). With regard to the haematopoietic system, sequestering available ligand inhibits ex vivo CFU-C expansion from the E9.5 P-Sp and ablation of Tie-2 results in the absence of in vitro multilineage haematopoietic differentiation (Takakura et al., 1998). To date no studies regarding the ability of Tie-2 null cells to contribute to definitive haematopoiesis have been published.

Analysis of Tie-1/Tie-2 compound null embryos reveals that CFU-Cs are produced in the E8.5 YS (Puri and Bernstein, 2003). Investigation into the contribution of Tie-1/Tie-2Δ cells to definitive haematopoiesis during foetal development and adult life was achieved through morula-morula aggregation of wildtype and null cells: null cells were found to contribute to E13.5 liver CFU-Cs but not to haematopoiesis in the adult bone marrow (Puri and Bernstein, 2003). These findings have been interpreted as demonstrating the differential requirement of embryonic and adult HSCs for Tie mediated signalling (Arai et al., 2005; Puri and Bernstein, 2003), however, given the absence of any in vivo long-term repopulation
studies from either embryonic or adult haematopoietic organs it is not clear whether LTR-dHSCs are ever formed in the absence of functional Tie-1 or Tie-2.

1.3.9.3 Primordial germ cells

When considering non-haematopoietic sources of LTR-dHSCs the haematogenic potential of primordial germ cells (PGCs) can be considered. It has been postulated that PGCs, which seed the UGR by E8.5 (Ginsburg et al., 1990), retain a totipotent capacity (Matsui et al., 1992) and possibly undergo *in vitro* haematopoietic differentiation (Rich, 1995); it is therefore possible that PGC might possess the ability to contribute to LTR-dHSC generation.

1.3.9.4 Pre-definitive stem cells as a source of LTR-dHSCs

Proving the existence of a pool of pre-LTR-dHSCs would eliminate the necessity of a physical emergence of LTR-dHSCs in the E11.5 AGM region and would suggest that a population of pluripotent haematopoietic cells exists in the embryo that completes maturation between E10.5-11.5, thus representing a functional emergence.

As previously argued (Medvinsky and Dzierzak, 1999), the distinction between LTR-dHSCs and putative pre-LTR-dHSCs (Yoder and Hiatt, 1997; Yoder et al., 1997a; Yoder et al., 1997b), which require further processing within the neonatal liver to achieve functional maturation, is of vital importance to preserve the integrity of this valid scientific question. For example, while a linear developmental relationship between neonatal repopulating, low-level adult repopulating (Bertrand et al., 2005; Muller et al., 1994) and high-level adult repopulating HSCs is commonly
assumed this is yet to be proven. It remains possible that each type of stem cell derives from ontogenetically distinct precursors each being inherently restricted in their capacity for self renewal, proliferation or effective niche occupation. Thus the oversimplification of the HSC definition might lead to important mechanisms in the induction and expansion of LTR-dHSCs being overlooked.

There are three lines of evidence in support of the existence of immediate pre-cursors to LTR-dHSCs:

1. The capacity of CD34⁺c-Kit⁺ cells from E9.0-10 YS and P-Sp to engraft conditioned neonates and contribute to the definitive stem cell compartment during the lifetime of the recipient (Yoder and Hiatt, 1997; Yoder et al., 1997a; Yoder et al., 1997b).

2. The ability to induce LTR-dHSC activity within E8.25 YS cells, prior to the onset of circulation, by overexpression of the Hoxb4 gene (Kyba et al., 2002). However, recipients of serial transplants from chimeric bone marrow demonstrated a restricted ability to undergo lymphoid differentiation and possibly an impeded capacity for secondary haematopoietic reconstitution.

3. The ability of E8.0 YS and P-Sp to generate LTR-dHSCs following co-culture with the AGM-S3 cell line (see section 1.3.4.).

These data suggest that cells with the potential to acquire LTR-dHSC properties are present in major haematopoietic organs prior to the emergence of the first detectable E11.5 AGM region LTR-dHSC. However, it remains to be clarified whether or not CD34⁺c-Kit⁺ cells from the E9.0-9.5 YS and P-Sp mature into the
LTR-dHSCs present in the E11.5-12.5 embryo. Although it can be argued that upon engraftment these cells are capable of developing into LTR-dHSCs, as demonstrated by the ability to reconstitute irradiated adult recipients upon serial transplantation, it is unclear whether these observations represent an in vivo recapitulation or an experimental artefact created following the bypass of foetal development. Circumstantial evidence in support of the existence of a pre-LTR-dHSC pool is provided by the recognition that a common CD34\(^+\)c-Kit\(^+\) immunophenotype is shared by E9.0 YS and P-Sp putative pre-LTR-dHSCs and LTR-dHSCs from the E11.5 AGM region, E12.5 liver and the E12.5 placenta (Gekas et al., 2005; Sanchez et al., 1996).

Whether the *Hoxb4* responsive cells from the E8.25-5 YS are the same pre-LTR-dHSCs described in the E9.5 YS is an unresolved question. An important distinction to be made when considering the elucidation of the development of LTR-dHSCs is between those factors that can induce stem cell formation from those factors which do influence the induction and expansion of LTR-dHSCs.
1.4 Defining primitive and definitive haematopoiesis

Distinguishing between primitive and definitive haematopoiesis and the cells from which these processes occur is a continuing debate in the field of developmental haematopoiesis. Resolution of this issue is essential as it likely to provide useful information regarding the induction of LTR-dHSCs from non-haematopoietic sources such as ES cells.

Following the intricate elucidation of the stages of adult bone marrow LTR-dHSC differentiation to CFU-S, CLP and CMP (Adolfsson et al., 2005; Akashi et al., 2000; Kondo et al., 1997; Till and McCulloch, 1961) it has been proposed that the sequence of emergence of lineage restricted progeny during embryogenesis recapitulates the definitive hierarchy and thus is supportive of the existence and activity of HSCs in the murine embryo prior to E10.5-11.5 (Jaffredo et al., 2005).

This argument challenges the hypothesis of the independent mid-gestation emergence of LTR-dHSCs by proposing that HSCs are present within extra-embryonic and intra-embryonic tissues during the early embryonic stages of haematopoietic development and that the pluripotent potential of HSCs is restricted by the limits of their environment. For example, HSCs in the E7.5-11.0 YS are limited by immature signals and are thus restricted to the production of cells of the erythroid and macrophage lineages; as embryonic tissues become more conducive cells of increasing potential can be detected culminating in the ‘appearance’ of the LTR-dHSC.

The counter argument is that during embryogenesis haematopoiesis occurs in two independent waves: the first wave is initiated at E7.5 in the YS and is supported by cells with limited differentiation potential meeting the immediate needs of the
developing organism (primitive haematopoiesis); the second wave is initiated in the AGM region between E10.5-11.5 with the emergence of the LTR-dHSC (Dzierzak and Medvinsky, 1995; Medvinsky and Dzierzak, 1999). From E11.5 it is possible that haematopoietic differentiation occurs in an adult-like manner, this is to say that lineage restricted progeny derive directly from a pluripotent LTR-dHSC compartment.

1.4.1 Evidence for ontogenetically distinct primitive and definitive haematopoietic hierarchies

1.4.1.1 ES cell differentiation model

ES cells provide a valuable resource for the investigation of haematopoietic differentiation which has been used successfully to study the induction of haematopoietic differentiation following mesoderm formation (Fehling et al., 2003; Kabrun et al., 1997; Keller et al., 1993; Kennedy et al., 1997; Robertson et al., 2000). In addition to providing an abundant source of cellular material the ES cell model is particularly intriguing because in vitro differentiation recapitulates some of the stages of extra-embryonic yolk sac haematopoiesis (Burkert et al., 1991; Keller et al., 1993; Nakano et al., 1994; Potocnik et al., 1994; Wiles and Keller, 1991).

Attempts to use in vitro ES cell differentiation to resolve the processes of primitive and definitive haematopoiesis during development are convoluted, this is in part a result of the inability to accurately resolve primitive and definitive haematopoiesis beyond the erythroid lineage (Nakano et al., 1996). This an inherent problem associated with the failure to generate, or preserve, LTR-dHSCs during in vitro ES cell differentiation and the failure to unambiguously prove the existence of a
common clonal origin of primitive and definitive erythroid cells using a wildtype model (Kennedy et al., 1997; Perlingeiro et al., 2001) (see section 1.8.2).

### 1.4.1.2 Xenopus model of haematopoietic development

The clearest *in vivo* dissection of primitive and definitive haematopoiesis comes from research using amphibian models. Grafting and transplantation experiments using *Xenopus laevis* have revealed that both primitive and definitive haematopoiesis develops from cells derived from distinct locations of the posterior region of the embryo. The primitive supply of haematopoietic cells to the early embryo derives from the ventral blood island (VBI), which is functionally analogous to the mammalian YS, while the cells that constitute the adult (late larval-adult stage) haematopoietic hierarchy derive exclusively from the dorsolateral plate (DLP), which is functionally analogous to the mammalian P-Sp/AGM region (Bechtold et al., 1992; Kau and Turpen, 1983; Maeno et al., 1985a; Maeno et al., 1985b; Turpen and Knudson, 1982).

A major advantage of using amphibian organisms, such as *Xenopus laevis*, is the ability to trace the fate of early embryonic tissues through direct physical labelling of target cells. Initial fate tracing experiments in which transplantation of differentially labelled tissue from stage 10 *Xenopus* ventral marginal zone (VMZ) gave rise to both primitive and definitive haematopoietic cells within the VBI and DLP, and thus provided support for the hypothesis of a single origin of both primitive and definitive haematopoiesis (Ciau-Uitz et al., 2000; Turpen et al., 1997). However, improved resolution of individual VMZ and DMZ fated blastomeres revealed that cells of the primitive and the definitive haematopoietic lineages cells
derive from spatially distinct tissues. It was observed that although VMZ blastomeres are capable of primitive and definitive haematopoiesis, no single VMZ blastomere is capable of contributing to both hierarchies (Ciau-Uitz et al., 2000), thus providing empirical \textit{in vivo} proof-of-principle evidence for an independent origin of primitive and definitive haematopoiesis.

1.4.2 CD41 expression as a marker of definitive haematopoiesis

CD41 interacts with CD61 to form the platelet glycoprotein receptor IIb/IIIa which facilitates the binding of platelets to adhesive proteins such as fibronectin, fibrinogen and von Willebrand factor (Phillips et al., 1988). It was observed that during \textit{in vitro} ES cell differentiation CD41 was the first haematopoietic protein to be expressed at the plasma membrane (Mikkola et al., 2003a). Additionally, in \textit{SCL}^{-} ES cells, which are unable to initiate haematopoietic commitment from mesoderm, CD41 expression was undetectable (Mikkola et al., 2003a). Further supported by the expression of CD41 on E10.5 IAHCs (Corbel and Salaun, 2002) it was hypothesised that during embryogenesis CD41 may provide an early marker for the initiation of definitive haematopoiesis (Mikkola et al., 2003a).

Some empirical validation of this hypothesis was achieved following the purification of CFU-Cs within the CD41 expressing populations from the E9.5 YS and from ES cell derivatives (Ferkowicz et al., 2003; Mikkola et al., 2003a; Mitjavila-Garcia et al., 2002). Moreover, haematogenic capacity of CD45-negative YS cells could be enriched on the basis of CD41 expression (Mikkola et al., 2003a). However, this hypothesis does not hold true for progenitor cells from the E9.5 P-Sp and the E12.5-13.5 liver which are found in both CD41-positive and CD41-negative
populations (Corbel and Salaun, 2002; Ferkowicz et al., 2003; Mitjavila-Garcia et al., 2002). Concluding that CD41 marks definitive progenitor cells from these data is problematic because even if it is conceded that dHSCs exist in the conceptus at E9.5 it is not currently possible to determine which CFU-Cs derive from primitive or definitive pathways.

Although all E9.5 YS neonatal repopulating pre-dHSCs express CD41 all E12.5 hepatic LTR-dHSCs are CD41-negative (Ferkowicz et al., 2003). This may reflect a temporal loss of CD41 expression following haematopoietic commitment resulting in developmentally nascent pre-LTR-dHSCs expressing CD41 which becomes lost from developmentally older LTR-dHSCs. An alternative interpretation to this circular argument is that the differential expression of CD41 on pre-LTR-dHSCs and LTR-dHSCs reflects an ontogenetic difference.

On balance, no conclusive evidence has been provided to support the hypothesis that CD41 expression specifically marks founder cells of the definitive haematopoietic hierarchy. The significance of CD41 expression during embryogenesis may be restricted to a pre-CD45 marker of haematopoietic commitment.

1.4.3 Molecular regulation of primitive and definitive haematopoiesis

Targeted deletion of indispensable haematopoietic transcription factors have provided some of the clearest functional resolution of murine primitive and definitive haematopoiesis by illustrating similarities and differences in essential molecular mechanisms.
1.4.3.1 GATA transcription factors

The GATA family is composed of highly conserved transcription factors that bind to a common consensus DNA sequence, (A/T)GATA(A/G), (Cantor and Orkin, 2005; Orkin, 1992). In vertebrates, the GATA family comprises six members (GATA-1-6) which can be divided into two subgroups based on the pattern of gene expression: the first includes GATA-4, GATA-5 and GATA-6 which are predominantly expressed within cardiac cells and lineages of endodermal heritage (Arceci et al., 1993; Gove et al., 1997; Heikinheimo et al., 1994; Jiang and Evans, 1996; Kelley et al., 1993; Laverriere et al., 1994) and the second includes GATA-1, GATA-2 and GATA-3 which are predominantly expressed within the central nervous system, the haematopoietic lineages and non-neural ectoderm (George et al., 1994; Nardelli et al., 1999; Pandolfi et al., 1995; Pevny et al., 1995; Pevny et al., 1991; Read et al., 1998; Tsai et al., 1994). Although GATA-1-3 are expressed in many tissues these transcription factors play particularly intriguing roles during haematopoietic development.

1.4.3.1.1 GATA-1

GATA-1 is involved in the differentiation of the erythroid, megakaryocyte, mast cell and eosinophil lineages (Fujiwara et al., 1996; Migliaccio et al., 2003; Shivdasani et al., 1997; Yu et al., 2002). Ablation of functional GATA-1 results in the failure to initiate definitive erythropoiesis but causes only a mild perturbation of primitive erythropoiesis characterised by incomplete erythroid maturation (Pevny et al., 1991; Weiss et al., 1994). Interestingly, the compound ablation of both GATA-1
and GATA-2 expression results in a complete block in both primitive and definitive erythropoiesis (Fujiwara et al., 2004). This suggests that a functional redundancy exists between GATA-1 and GATA-2 that is unable to rescue the development of definitive haematopoiesis but is sufficient to preserve the emergence of primitive erythropoiesis.

1.4.3.1.2 GATA-2

GATA-2 is expressed within, but is not specific to, many of the haematopoietic lineages including the adult bone marrow LTR-dHSC compartment (Jippo et al., 1996; Nagai et al., 1994; Orlic et al., 1995; Tsai et al., 1994; Walmsley et al., 1994) and endothelial cells (Lee et al., 1991). Although primitive haematopoiesis proceeds normally in the absence of GATA-2 expression, as revealed in GATA-2\textsuperscript{-/-}/wildtype chimeras, nulls cells are not able to contribute to definitive haematopoiesis in either embryonic or adult haematopoietic organs (Tsai et al., 1994). This implicates a vital role for GATA-2 in the emergence, expansion or survival of the LTR-dHSC pool.

Utilising in situ hybridisation and gene reporter mouse lines expression of GATA-2 has been reported in Ao endothelium of the E9.5 P-Sp (Robert-Moreno et al., 2005), the non-haematopoietic and non-endothelial cells of the E10.5 AGM region (Minegishi et al., 1999) and within the E11.5 UGR (Zhou et al., 1998). Although it is clear that GATA-2 plays a vital role in the progression of definitive haematopoiesis, and presumably in the emergence and expansion of LTR-dHSCs as suggested by reports from GATA-2\textsuperscript{+/-} embryos (Ling et al., 2004), it remains unclear which processes are regulated by GATA-2.
1.4.3.1.3 GATA-3

Ablation of *GATA-3* expression results in normal morphological development until E10.5 when haematopoietic and neurological abnormalities become apparent. Embryo lethality occurs between E11.75-12.5 (Pandolfi et al., 1995). Data presented regarding the haematopoietic phenotype of *GATA-3*<sup>−/−</sup> embryos are restricted to the quantitative analysis of YS-derived CFU-Cs which appears normal yet hepatic CFU-C content is markedly decreased. From these observations it was concluded that GATA-3 is indispensable for definitive haematopoiesis but unnecessary for primitive extra-embryonic haematopoiesis (Pandolfi et al., 1995; Ting et al., 1996). To date, no investigation for possible signs of normal intra-embryonic haematopoietic development has been presented, which is possible given that embryos are viable during the crucial developmental stages of E9.5-11.5.

These concerns are validated by reports from chimera experiments in which *GATA-3*<sup>−/−</sup> null ES cells were injected into *RAG-2*<sup>−/−</sup> blastocysts. These experiments reveal that all null cells are capable of differentiating along B-lymphoid and myeloid lineages, which presumably derive from *GATA-3*<sup>−/−</sup> LTR-dHSCs, but are not capable of completing T-lymphocyte differentiation (Ting et al., 1996).

Intriguingly, *GATA-3* expression has been reported in sub-aortic patches (see section 1.3.8) of the E10.5-11.5 AGM, which are hypothesised to be involved with the emergence of the first LTR-dHSCs, and perhaps weakly by aortic endothelium (Bertrand et al., 2005; Godin and Cumano, 2002; Manaia et al., 2000).
The unknown function of SAPs and the unresolved requirement of embryonic and foetal LTR-dHSCs on functional GATA-3 make the significance of GATA-3 expression during LTR-dHSC emergence, expansion and self-renewal unclear.

1.4.3.2 SCL

The basic-helix-loop-helix transcription factor SCL is expressed within embryonic and adult haematopoietic organs (Begley et al., 1989; Mellentin et al., 1989) and is essential for the initiation of haematopoietic commitment in vivo and in vitro, as evidenced by the inability of mesoderm to initiate haematopoietic differentiation (Elefanty et al., 1997; Mead et al., 1998). In vivo, ablation of SCL expression results in the failure to initiate primitive haematopoiesis or the emergence of LTR-dHSCs as evidenced by yolk sac pallor, the absence of extra-embryonic blood islands, the lack of CFU-Cs and the inability of null ES cells to contribute to adult haematopoiesis following chimera generation (Porcher et al., 1996; Robb et al., 1996; Robb et al., 1995; Shivdasani et al., 1995). Conditional gene inactivation studies reveal that SCL expression is not required for the maintenance of LTR-dHSCs in the bone marrow nor for the preservation of the cardinal characteristics of engraftment and long-term maintenance upon serial transplantation (Mikkola et al., 2003b).

1.4.3.3 LMO2

Lmo2IRbtn2 encodes a LIM-domain protein which during murine development is expressed in the brain, spleen, endothelium and erythroid cells of the liver and peripheral circulation (Foroni et al., 1992; Warren et al., 1994; Yamada et
As in the case of SCL, Lmo2 is also involved in the development of T cell acute leukaemias (Fisch et al., 1993; Rabbitts, 1994). Under physiological conditions Lmo2 functions as a bridging protein in SCL- and GATA-containing complexes (Ono et al., 1998; Osada et al., 1995; Osada et al., 1997; Valge-Archer et al., 1994; Wadman et al., 1994; Wadman et al., 1997). The anatomical pattern of LMO2 expression, which is detected in E8.0-11.0 aortic endothelium and IAHCs (Manaia et al., 2000), provides circumstantial evidence of a role for LMO2 during LTR-dHSC emergence.

In the Lmo2<sup>−/−</sup> embryo a complete failure of haematopoietic cells to undergo erythroid differentiation <em>in vivo</em> and <em>in vitro</em> is observed (Warren et al., 1994). As observed with the GATA-1<sup>−/−</sup> embryo, Lmo2 null YS cells are able to undergo myeloid differentiation which suggests that a complete failure of primitive haematopoiesis had not occurred. Analysis of adult chimeric mice created following the injection of Lmo2<sup>−/−</sup> ES cells into wild-type blastocysts revealed that the knockout genotype imposes an inability to contribute to definitive haematopoiesis (Yamada et al., 2000).

### 1.4.3.4 Runx1

Runx1 (also known as AML1, PEBPα2 and CBFA2) is involved in many oncogenic chromosomal translocations (Lutterbach and Hiebert, 2000; Tenen et al., 1997). Although fidelity of Runx1 expression is dispensable for primitive erythroid development in the YS, expression is essential for the initiation of myelopoiesis, intra-embryonic haematopoiesis, haematopoietic commitment of haematogenic endothelium, IAHC formation and the induction of LTR-dHSC emergence (Cai et al.,
Overexpression of functional \textit{RUNX1} cDNA in cells derived from \textit{Runx1}^{+/+} E9.5 P-Sp revealed that CFU-C formation/haematopoietic differentiation could be rescued (Goyama et al., 2004; Mukouyama et al., 2000). Transfection of \textit{Runx1}^{+/+} E11.5 AGM with rescue cDNA did not yield a positive haematopoietic response (Mukouyama et al., 2000). These findings suggest that during early \textit{Runx1} embryogenesis haematogenic cells develop which either do not survive until mid-gestation or commit to non-haematogenic pathways.

Altering the level of \textit{Runx1} expression reveals that the spatial and temporal development of the LTR-dHSC pool results from a flexible regulatory process rather than an intrinsic haematopoietic potential of extra- and intra-embryonic organs. In the E10.5 wildtype embryo LTR-dHSCs are infrequently detected in the AGM region (Medvinsky and Dzierzak, 1996; Muller et al., 1994). However, in the presence of a single functional copy of \textit{Runx1} LTR-dHSCs become readily detected in both the AGM region and the YS at E10.5 (Cai et al., 2000).

Marked deviation in the LTR-dHSC phenotype becomes apparent in the \textit{Runx1} haploinsufficient embryo: at E10.5 \textit{Runx1}^{+/+} LTR-dHSCs are exclusively found in the CD45^- AGM fraction. By E11.5 LTR-dHSCs are detectable in both the CD45^+ (haematopoietic) and CD45^- (non-haematopoietic) populations (North et al., 2002). It may be interpreted that the dysregulation of \textit{Runx1} expression results in the
induction, or preservation, of LTR-dHSC properties in non-haematopoietic populations. Further support for this hypothesis is provided by studies of the expression of VE-cadherin, which in the wildtype embryo is co-expressed with CD45 on all LTR-dHSCs (North et al., 2002): in the Runx1+/c model haematopoietic reconstitution of adult mice is possible from both VE-cadherin+ (endothelial) and VE-cadherin− (non-endothelial) fractions (North et al., 2002). This observation may shed light on previously unrecognised pathways of LTR-dHSC development.

1.4.3.5 Notch

Notch proteins are a highly conserved family of transmembrane receptors which are involved in cell fate determination of multiple systems including haematopoiesis (Allman et al., 2002; Artavanis-Tsakonas et al., 1999). Four members of the Notch family are currently recognised of which Notch1 and Notch2 have been identified within human and murine haematopoietic cells (Bigas et al., 1998; Wrier et al., 1994).

No early embryonic haematopoietic phenotype has been reported following the inactivation of Notch-2 (Kumano et al., 2003). In the absence of Notch-1 expression extra-embryonic primitive haematopoiesis progresses relatively unperturbed however intra-embryonic haematopoietic development and the emergence of both extra- and intra-embryonic pre-LTR-dHSCs is ablated (Hadland et al., 2004; Kumano et al., 2003). Furthermore, Notch-1+/− ES cells do not contribute to definitive haematopoiesis in null/wildtype chimeric adults (Hadland et al., 2004).

The disruption of in vitro haematopoietic differentiation from the E9.5 P-Sp and haematogenic endothelium from the E10.5 AGM region (Kumano et al., 2003)
and the restricted requirement of LTR-dHSCs on Notch1 during embryogenesis was revealed by the dispensable role of Notch1 in adult bone marrow dHSCs using conditional gene inactivation (Radtke et al., 1999), has led to the hypothesis that defective definitive haematopoiesis in the Notch-1<sup>−/−</sup> embryo arises from the failure of haematogenic endothelium, or the haemangioblast, to engage haematopoietic commitment (Kumano et al., 2003). However, it must be stated that until an endothelial/haemangioblast origin of LTR-dHSCs is proven this hypothesis is largely speculative.

Ablating the expression of GATA-2, SCL, LMO2, Runx1 or Notch-1 results in failure to initiate definitive haematopoiesis but not primitive haematopoiesis suggesting the involvement of distinct molecular and possibly cellular regulatory mechanisms.

A limitation of the studies discussed above is the difficulty in resolving the gene products involved in LTR-dHSC emergence, expansion and maintenance from those involved in haematopoietic differentiation. Investigating the failure of LTR-dHSC formation by the absence of contribution to haematopoiesis in an adult organism involves the use of a test system with a compromised read out, thus even if 'stem cells' were present they would be incapable of initiating and/or completing haematopoietic differentiation programs. Precedents for such concerns are supported by gene knockout studies of c-Myc and Bmi-1 which result in the failure of LTR-dHSCs to initiate differentiation (Lessard and Sauvageau, 2003; Wilson et al., 2004).
1.5 Homeobox (Hox) genes

1.5.1 Haematopoietic influence of Hox gene expression

Mammalian Homeobox (Hox) genes are arranged in four clusters designated A, B, C and D (Scott, 1992). In human adult bone marrow and peripheral blood genes from the HOXA and HOXB gene clusters are differentially expressed between the most primitive stem/progenitor cells, lineage committed progenitors and terminally differentiated cells (Sauvageau et al., 1994). Overexpression studies have revealed marked differences in the physiological and pathological capacities of HOX genes within the haematopoietic system.

The overexpression of multiple Hox genes in the haematopoietic compartment contributes to leukaemic transformation. To date the overexpression of Hoxa7, Hoxa9, HOXA10, and Hoxb8 have been shown to collaborate with factors such as Meis1, NUP98 and interleukin-3 in the formation of myeloid leukaemias (Iwasaki et al., 2005; Kroon et al., 1998; Lessard and Sauvageau, 2003; Nakamura et al., 1996a; Nakamura et al., 1996b; Perkins et al., 1990; Thorsteinsdottir et al., 2002; Thorsteinsdottir et al., 1997).

Enforced expression of HOXA10 within adult bone marrow cells confers the capacity for ex vivo CFU-S survival and expansion, however, this occurs at the expense of successful multipotent myeloid and B lymphoid terminal differentiation and the preservation of long-term physiological steady-state haematopoiesis (Thorsteinsdottir et al., 1997). The overexpression of HOXB3 does not result in leukaemic transformation but causes disruption of in vivo lymphoid differentiation in favour of enhanced proliferation of myeloid restricted progenitors (Sauvageau et al., 1997).
The effect of the enforced expression of *HOXB4* is unique amongst *HOX* genes. As reported with *HOXA10*, overexpression of *HOXB4* within the adult bone marrow resulted in the *ex vivo* survival and expansion of CFU-S. More significantly, overexpression resulted in the *ex vivo* expansion of LTR-dHSCs (Sauvageau et al., 1995). Overexpression of *Hoxa9* also results in an enhanced capacity for LTR-dHSC expansion; however, this is accompanied by diminished *in vivo* B-cell lymphopoiesis and latent myeloid leukemogenesis (Kroon et al., 1998; Thorsteinsdottir et al., 2002). Under similar conditions *HOXB4* is able to facilitate the expansion of LTR-dHSCs for at least 14 days *ex vivo* and instruct enhanced *in vivo* expansion without incurring a differentiation bias or pathological consequence (Antonchuk et al., 2002; Kroon et al., 1998; Sauvageau et al., 1995; Thorsteinsdottir et al., 1997).

Uniquely, the overexpression of *HOXB4* not only achieves a qualitative restoration of LTR-dHSC activity but facilitates a quantitative reconstitution of the LTR-dHSC compartment. Following transplantation of wildtype adult LTR-dHSCs into an irradiated adult recipient approximately 25% of the original femoral bone marrow LTR-dHSC pool is reconstituted; under similar circumstances *HOXB4* overexpressing cells are able to reconstitute 100% of LTR-dHSCs (Antonchuk et al., 2001; Antonchuk et al., 2002; Sauvageau et al., 1995).

### 1.5.2 Limits to the clinical application of Hoxb4/HOXB4

In contrast to the studies performed in the murine organism, *HOXB4* overexpression in human cord blood HSCs results in a concurrent proliferative growth advantage and differentiation deficiency (Schiedlmeier et al., 2003) illustrating the need for a temporally restricted *HOXB4* expression/delivery system in
which LTR-dHSC induction, expansion and maintenance can be achieved *in vitro* while not compromising the ability of stem cells to contribute to all haematopoietic compartments *in vivo*.

Two groups have pioneered such solutions by alternative means: utilising a recombinant HOXB4 protein fused with the protein transduction domain of the Human Immunodeficiency Virus transactivating protein (TAT-HOXB4), (Krosl et al., 2003) have reported a four to six fold induction of HSC expansion following a 96 hour culture period. The application of this strategy is restricted by the short half-life of the TAT-HOXB4 protein which demands regular supplementation. (Amsellem et al., 2003) were able to induce a 2.5-fold expansion of human cord blood-derived HSCs following co-culture with a bone marrow stromal cell line overexpressing *HOXB4*.

1.5.3 Physiological role for Hoxb4 during LTR-dHSC development

Given the powerful ability of *HOXB4* overexpression to facilitate adult bone marrow LTR-dHSC expansion it might be reasonably expected that the expression of this gene would have a physiological role. However, deletion of *Hoxb4* results in only a mild perturbation in the cellularity of foetal and adult haematopoietic organs and the proliferative capacity of *in vitro* progenitors (Bijl et al., 2005; Bjornsson et al., 2003; Brun et al., 2004). The impact of ablating *Hoxb4* expression on the adult LTR-dHSC compartment is restricted to the speed of haematopoietic reconstitution; no marked deficiency from *Hoxb4*+ LTR-dHSCs from the E14.5 liver. Thus, the homeostatic mechanisms of expansion and self-renewal are not entirely dependent on the expression of *Hoxb4*, if at all.
Functional redundancy and collaboration between paralogous and neighbouring \(Hox\) genes has been proposed as a mode of compensation for the deletion of a single gene (Chen and Capecchi, 1997; Greer et al., 2000; Horan et al., 1995; Rancourt et al., 1995). No evidence of an upregulation in \(Hoxa4\), \(Hoxc4\) or \(Hoxd4\) expression in response to \(Hoxb4\) ablation has been reported (Biji et al., 2005; Brun et al., 2004). Although \(Hoxb6-9\) and \(Hoxb13\) are upregulated in the \(Hoxb4^{\text{E14.5}}\) liver deletion of all \(HoxB\) genes expressed in LTR-dHSC-enriched E14.5 liver (\(Hoxb2\), \(Hoxb3\), \(Hoxb4\), \(Hoxb5\) and \(Hoxb8\)) has little impact on the functional properties of the developing LTR-dHSC pool (Biji et al., 2005).

On balance, it appears that the expression of \(HoxB\) genes is dispensable for LTR-dHSC expansion and self-renewal. To date no studies into the mid-gestation development of LTR-dHSCs have been reported using the \(Hoxb4^{\text{E14.5}}\) mouse model thus it remains unclear whether disruption of \(Hoxb4\) expression affects the normal mechanisms of LTR-dHSC emergence/induction.
1.6 Role of Polycomb group genes in dHSC development and homeostasis

Polycomb group (PcG) gene products are organised into two multimeric complexes: complex 1 comprises Rae28, M33, Bmi-1, Mel18, Ring1A and Ring1B (Alkema et al., 1997; Gunster et al., 1997; Hemenway et al., 1998; Satijn et al., 1997; Satijn and Otte, 1999; Schoorlemmer et al., 1997); complex 2 comprises Eed, Enx1 and EnX2 (Denisenko et al., 1998; Sewalt et al., 1998; van Lohuizen et al., 1998). Members of the PcG are primarily involved in regulating the spatial initiation and temporal maintenance of Hox gene expression during axial patterning (Akasaka et al., 2001; Gould et al., 1997; Schumacher and Magnuson, 1997). Loss of function studies from Bmi-1, Eed, M33, Mel18 and Rae28 gene knockout models have demonstrated the involvement of the PcG during haematopoiesis (Core et al., 1997; Iwama et al., 2004; Jacobs et al., 1999; Lessard et al., 1999; Ohta et al., 2002; van der Lugt et al., 1996). Gain-of-function, loss-of-function and rescue studies demonstrate that the development and preservation of LTR-dHSC activity is most markedly affected by Rae28 and Bmi-1 expression.

1.6.1 Rae28

Expression of Rae28 is detected in the LTR-dHSC enriched fractions of the E14.5 liver and adult bone marrow (Ohta et al., 2002). Mice deficient in both alleles of the Rae28 gene demonstrate multiple developmental defects and die at the perinatal period (Takahara et al., 1997). Rae28<sup>−/−</sup> foetuses show signs of haematopoiesis at late stages of gestation (Takahara et al., 1997) and neonates do not markedly differ in the number of mature haematopoietic cells present in the
peripheral blood, however, in the foetus significantly fewer CFU-Cs, CFU-S<sub>12</sub> and LTR-dHSCs are detected (Ohta et al., 2002). Furthermore, \textit{in vivo} studies into the ability of \textit{Rae28<sup>+</sup>} contribution into the stem cell compartment of primary transplant recipients revealed the deficiency of knockout cells to efficiently induce or sustain LTR-dHSC expansion or robust haematopoietic reconstitution (Kim et al., 2004; Ohta et al., 2002).

It is unclear which aspect of LTR-dHSC biology is affected by the ablation of \textit{Rae28}. It is possible that LTR-dHSC induction/emergence is unaffected, although to date such investigations have not been reported. The plausibility of such a hypothesis is supported by the progressive loss of CFU-C and CFU-S activity in \textit{Rae28<sup>+</sup>} knockout foetuses while analogous wildtype populations are expanding and further by the detection of low numbers of HSCs with restricted potential for \textit{in vivo} expansion.

1.6.2 \textit{Bmi-1}

\textit{Bmi-1} is differentially expressed in human haematopoietic stem/progenitor cells and mature populations, with greater levels of expression observed in the most potent compartments (Iwama et al., 2004; Lessard et al., 1998; Lessard et al., 1999; Park et al., 2002; Park et al., 2003; Sauvageau et al., 1994). Studies of the \textit{Bmi-1<sup>−/−</sup>} murine model provide some interesting insights into the development of LTR-dHSCs and definitive haematopoiesis. Initial observations revealed that deletion of \textit{Bmi-1} results in viable mice that survive until 2-20 weeks of age (van der Lugt et al., 1996). In addition to suffering from ataxia and skeletal defects, null mice demonstrate diminished cellularity of haematopoietic organs with a marked quantitative and
qualitative disparity in CFU-Cs from the E14.5 liver, neonatal bone marrow and adult bone marrow (Iwama et al., 2004; Lessard and Sauvageau, 2003; Lessard et al., 1999; van der Lugt et al., 1996).

Although studies into the mid-gestation emergence of LTR-dHSCs are lacking it is interesting that despite the presumably successful foetal haematopoietic progress which continues into adulthood no cells capable of long-term haematopoietic reconstitution are functionally detectable in either the Bmi-1 \(^{+}\) E14.5 liver or the adult bone marrow (Iwama et al., 2004; Lessard and Sauvageau, 2003; Park et al., 2003).

Transfection of Bmi-1 \(^{-}\) E14.5 liver cells with a rescue construct ubiquitously expressing functional Bmi-1 cDNA results in the detection of long-term low-level repopulating HSCs and a quantitative restoration of in vitro progenitors (Lessard and Sauvageau, 2003). These findings infer that haematopoietic stem and progenitor cells are present in the Bmi-1 \(^{-}\) liver but are restricted by either a proliferative or differentiation defect. Assuming that over-expression of Bmi-1 does not facilitate a de novo induction of dHSCs, these observations suggest that Bmi-1 is dispensable for the emergence and the initial expansion of dHSCs. It remains unclear whether the effect of Bmi-1 deficiency on HSC behaviour is cell autonomous or is secondary to an environmental defect.

Analogous rescue experiments performed using purified Bmi-1 \(^{-}\) Lin^-Kit^+Sca-1^+CD34^- LTR-dHSC-enriched adult bone marrow cells reveals that a partial quantitative and complete qualitative rescue of in vitro proliferation/differentiation is possible and the function of high-level long-term competitive repopulation can be restored (Iwama et al., 2004). This again infers the presence of ‘stem cells’ prior to
rescue. Interestingly, overexpression of Hoxb4 in Bmi-1−/− cells did not result in the rescue of LTR-dHSC function suggesting that either Hoxb4 plays an dispensable role in bone marrow LTR-dHSCs or that Bmi-1 expression is a prerequisite to prime cells to a Hoxb4 mediated response.

There are currently two interpretations of the role of Bmi-1 in the adult LTR-dHSC compartment: the first is the regulation of self-renewal (Park et al., 2003); the second is the regulation of proliferation, as supported by the dependence of leukaemic clones on Bmi-1 expression for long-term engraftment upon transfer to a secondary recipient (Lessard and Sauvageau, 2003).

Gain-of-function experiments, in which Bmi-1 was overexpressed in purified wildtype LTR-dHSCs, reveal that long-term repopulating activity can be preserved for greater periods of time ex vivo in the presence of elevated levels of Bmi-1 (Iwama et al., 2004). Furthermore, under controlled conditions allowing the clonal analysis of paired daughter cells following a single cellular division, Bmi-1 overexpression facilitates an increased likelihood of a functional symmetric division (Iwama et al., 2004; Takano et al., 2004).

1.6.3 Other PcG members

Transplantation studies of murine E14.5 liver from M33−/− reveals no obvious LTR-dHSC phenotype (Iwama et al., 2004). Transplantation of Mel18−/− E14.5 liver results in a statistically significant decrease in the percentage of peripheral blood leucocyte chimerism established upon engraftment of adult recipients (Iwama et al., 2004). It is unclear whether this observation results from a qualitative or quantitative perturbation of the LTR-dHSC pool.
1.7 Deriving LTR-dHSCs from ES cells

1.7.1 Limitations of tissue culture

ES cells could provide a useful source of cellular material for the molecular reduction of LTR-HSC expansion and maintenance. Furthermore, the use of ES cells could circumvent the current limitations associated with the transplantation of primary HSCs that include the limited numbers of HSCs available from cord blood and the graft-versus-host disease associated with bone marrow HSCs (Kollman et al., 2001; Lane, 2005; Sudo et al., 2000). However, before any use can be made of this resource protocols for the induction of LTR-dHSCs from ES cells must be developed.

When grown in vitro as either embryoid bodies or methylcellulose based medium ES cell derivatives are capable of generating CFU-Cs and undergoing terminal haematopoietic differentiation (Burkert et al., 1991; Wiles and Keller, 1991). However, ES cells or ES cell-derivatives are unable to produce cells with the ability to robustly engraft adult haematopoietic organs following injection into the peripheral circulation at either the level of LTR-dHSC or CFU-S (Muller and Dzierzak, 1993; Potocnik et al., 1997). Furthermore, cells that do successfully engraft are only able to contribute to low-levels (0.1-4.4%) of lymphoid restricted peripheral blood leucocyte chimerism upon injection into neonatal recipients (Muller and Dzierzak, 1993) or to the transient rescue of lymphopoiesis in adult mice (Potocnik et al., 1997).

The production of adult repopulating cells following co-culture with an adult bone marrow cell line in the presence of foetal liver cell line conditioned medium has been reported (Palacios et al., 1995). The data presented in the study was limited but describes the successful induction of LTR-dHSCs from ES cells following 25 days of
differentiation as assessed by the successful multi-lineage reconstitution of primary SCID recipient mice and secondary mice following serial transplantation. No data independently verifying these observations has been reported.

Induction of LTR-dHSCs from ES cells has been reported following a 4 day culture period as embryoid bodies (Hole et al., 1996). In this study it was argued that LTR-dHSC could only be preserved for a short period of time. A major limitation of the presented study is the failure of the investigators to confirm the haematopoietic identity of the engrafting cells. Taking into account reports that injection of embryoid bodies at time points earlier than 6 days of culture can result in the engraftment and circulation of non-haematopoietic cells (Kyba et al., 2002; Muller and Dzierzak, 1993) the limited analysis provided by Hole et al (1996) makes interpretation of the presented data problematic.

1.7.2 The use of enforced gene expression

1.7.2.1 Overexpression of Bcr/Ab1

The raw ability of ES cell derivatives to engraft upon adult haematopoietic territories was alluded to following transduction of wildtype cells with the Bcr/Ab1 oncogene which resulted in the successful contribution of injected cells to the lymphoid and myeloid compartments of irradiated adult mice (Perlingeiro et al., 2001; Peters et al., 2001). However, because grafts deriving from cells that had undergone leukaemic transformation transplanted mice were unable to survive beyond 5-9 weeks post-injection and so the presence of a transformed LTR-dHSC-like cells could not be confirmed.
1.7.2.2 Overexpression of *Hoxb4*

The overexpression of *Hoxb4*, shown to induce the efficient expansion of adult bone marrow LTR-dHSC (see above), in murine ES cells not only enhances the formation of CFU-Cs (Helgason et al., 1996) but facilitates the induction of high-level repopulating cell without inducing leukaemic transformation (Kyba et al., 2002). Although these data provide a significant advance in realising the practical capacity of ES cells to produce HSCs the nature of the stem cells produced *in vitro* with respect to complete LTR-dHSC function is yet to be effectively characterised. For example, the ability of ES cell-derived HSCs to sustain life-long repopulation and effectively expand *in vivo* appears to be compromised in the wild-type host and is likely to be dependent on the level of *Hoxb4* expression (Brun et al., 2004; Schiedlmeier et al., 2003). As a testament to the power of this strategy genetically and functionally repaired LTR-dHSCs have been induced from ES cells that were derived following somatic cell nuclear transfer from *Rag2*−/− adult cells into wildtype enucleated oocytes (Rideout et al., 2002). Clinical translation for the use of *HOXB4* overexpression may be limited because a similar response from human ES cells has not been observed (Wang et al., 2005) suggesting that different pathways may be involved in the induction and expansion of LTR-dHSCs derived from humans and mice.
1.8 Aims and objectives

The focus of this thesis is to further investigate the emergence and expansion of definitive haematopoietic stem cells within embryonic organs. The first aim is to investigate a possible common ontogenetic pathway for the emergence and development of extra-embryonic and intra-embryonic LTR-dHSCs. To this end key organs during the development of the LTR-dHSC pool: the E11.5 AGM, the E12.5 YS, the E12.5 PB and the E13.5 liver will be harvested and cell populations purified by flow cytometry on the basis of the differential expression of endothelial-specific and pan-haematopoietic proteins VE-cadherin and CD45, respectively. Collected cells will then be subjected to functional assessment for LTR-dHSC, CFU-C and endothelial activity.

Secondly, I aim to empirically examine the long-standing hypothesis of a ventral restriction of LTR-dHSC activity in the AGM region. This will be achieved through the bisection of the dorsal aorta from the E11.5 AGM along the dorsoventral axis and the functional assessment of their respective abilities to initiate LTR-dHSC emergence, provide suitable niches for LTR-dHSC maintenance and facilitate LTR-dHSC expansion.

Finally, methods to efficiently harness the environment of the E11.5 AGM will be investigated with the ultimate aim of developing a system that can be used to study the cellular and molecular basis of LTR-dHSC induction and expansion. Such a system could be applied to the in vitro generation of LTR-dHSCs from non-haematopoietic sources and allow further understanding of the in vivo events that facilitate the emergence of definitive haematopoiesis.
Chapter 2: Materials and methods

2.1 General solutions

Solution 1: room temperature Dulbecco’s PBS (with Mg\(^{2+}\) and Ca\(^{2+}\); Sigma) containing 7% FCS (Gibco); 50 units/ml Penicillin and Streptomycin (P/S; Gibco).

Solution 2: room temperature Dulbecco’s PBS (with Mg\(^{2+}\) and Ca\(^{2+}\)) containing 7% FCS; 50 units/ml P/S; 0.1% collagenase/dispsase (Boehringer).

Solution 3: ice-cold Dulbecco’s PBS (without Mg\(^{2+}\) and Ca\(^{2+}\); Sigma) containing 7% FCS; 50 units/ml P/S.

2.2 Animals

2.2.1 Animal husbandry

C57BL6 and CBA mice were housed and bred within the University of Edinburgh according to the provisions of the Animals (Scientific Procedures) Act (UK) 1986. Mice were housed in a stabilised environment with a 14 hours light/10 hours dark cycle (midpoint 12 O’clock, midnight). Mice were provided with a constant supply of water and chow food. As standard practice, litters from matings were left with parents until 3 weeks of age when they were weaned by separating the offspring from their parents. At weaning animals were sexed and if required tail tips were taken for genotyping. From 6 weeks of age mice could be used for mating.

2.2.2 Timed matings and isolation of embryonic organs

All animals were culled by the schedule 1 method of cervical dislocation. For collection of tissues from embryos at specific stages of gestation, matings were
set up overnight and the females examined for the presence of a vaginal plug the following morning. The day of discovery of the vaginal plug was designated as embryonic day (E) 0.5. Pregnant females at the correct stage of gestation were sacrificed and the uterus dissected into solution 1. Subsequently the embryos were dissected from the uterus and the extra-embryonic tissues removed with the exception of the YS and where appropriate the placenta. Embryos were scored according to Theiler criteria [for example, E11.0-E11.5 (41-47 somite pairs) is equal to stages 18 and 19] (http://genex.hgu.mrc.ac.uk/intro.html). Embryonic organs were dissected out into solution 1 using sharpened tungsten with the aid of a dissecting microscope (LEICA MZ8 microscope). As required, embryonic peripheral blood was placed immediately on ice.

For the isolation of ventral and dorsal aspects of the dorsal aorta/para-aortic mesenchyme (Ao) the Ao from the E11.5 AGM region was dissected free from the embryo-proper. Using the remnants of the mesentery and the remnants of the somites and the notochord as anatomical landmarks the ventral (AoV) and dorsal (AoD) aspects could be clearly identified, respectively. AoV and AoD were then separated along the mid-line of the Ao and immediately placed in separate dishes.

### 2.2.3 Isolation of adult organs

Adult spleen and thymus were dissected free of connective tissues and fat, and placed in a container of solution 1. Bone marrow was isolated by aspirating femurs with 26 gauge syringe needles (BD Microlance) and placed directly on ice. Adult peripheral blood was obtained by bleeding from the lateral tail vein and collected into 500µl EDTA/PBS (200µg/ml).
2.3 Preparation of haematopoietic organs

2.3.1 Cellular suspensions

Tissues were isolated from appropriately staged embryos or adult mice as described. To obtain single cell suspensions embryonic organs and reaggregates were digested in solution 2 for 45 minutes at 37°C with gentle shaking. Placentas were prepared as described previously (Gekas et al, 2005): E11.5-12.5 placentas were dissected free of maternal tissues in solution 1 and dissociated mechanically through a 16 gauge (G) syringe needles and then incubated in solution 2 for 1.5 hours at 37°C with gentle shaking. Samples were then centrifuged at 1,500rpm (MSE Mistral 2000 centrifuge) for 5 minutes and washed twice in solution 1. Cellular suspensions were resuspended in solution 3, filtered through a 40µm cell strainer (BD Falcon) and placed on ice.

Cell suspensions from adult spleen and thymus were obtained by pressing the organs with a 1.0ml syringe plunger (BD Plastipak) in the presence of solution 1 in a round-bottom 96 well plate (Bibby Sterilin). Cells from adult bone marrow were disaggregated by pipetting.

2.3.2 Erythrocyte depletion

2.3.2.1 Embryonic organs

E12.5 peripheral blood and E13.5 liver was depleted of erythrocytes by passing sample that had been incubated with an anti-Ter119 monoclonal antibody conjugated with magnetic beads (Miltenyi Biotec) through a VarioMACS magnetic separator (Miltenyi Biotec) according to the manufacturer’s instructions.
2.3.2.2 Adult organs

To cell suspension centrifuged at 2,000rpm (Biofuge pico from Heraeus Instruments) for 2 minutes at room temperature 1.0ml of PharM Lyse solution (BD Bioscience) was added; preparations were processed according to the manufacturers' instructions. Following a 15 minute period of incubation cells were centrifuged at 1,500rpm (Labofuge 400R from Heraeus Instruments) for 5 minutes at 4°C. Supernatant was removed and cells were resuspended in 1.0ml of solution 3 and centrifuged at 1,500rpm for 5 minutes at 4°C. Cells were generally resuspended in solution 3 to a 1x10^7 cells/ml concentration.

2.4 In vivo haematopoietic assays

2.4.1 Competitive long term repopulation assay

Cells were isolated directly from dissected organs or after purification by flow cytometry (see section 2.5). Organs for transplantation were obtained from (CBAxC57BL/6)F1 embryo or adult bone marrow bearing the Ly-5.2/5.2 genotype. Cells were resuspended in ice-cold 1% FCS/PBS at appropriate doses and injected with 2x10^4 nucleated adult bone marrow competitor cells (Ly-5.1/5.2) into the lateral tail vein of adult recipient mice (Ly-5.1/5.1). Recipient mice were irradiated at 9.5 Gr split into two doses separated by a 3 hour interval in the Cs source at a rate of 21.6rad/min. The volume of cell suspension received did not exceed 300μl per recipient. Resolution of contribution to haematopoietic reconstitution derived from donor, competitor or endogenous recipient cells was performed by flow cytometry.
according to the differential expression of Ly-5.1 and Ly-5.2 proteins as detected following incubation with appropriate antibodies.

2.4.2 CFU-S assay

Cellular suspensions from E11.5 AGM region reaggregates after 96 hours in culture were injected into irradiated adult mice without any bone marrow carrier cells. 11 days after injection mice were sacrificed and spleens removed. Splenic colonies were scored under a dissecting microscope (LEICA MZ8 microscope). To assess the origin of CFU-S reaggregates were generated from the AGM regions embryos ubiquitously expressing GFP (Gilchrist et al., 2003). The donor origin of colonies was confirmed by observing the presence of GFP under a dissecting microscope enabled to detect fluorescence (LEICA MZFLIII).

2.5 Flow cytometry

2.5.1 Staining and analysis of adult cells

Cell suspensions were obtained as described above. Cells were counted in a haemocytometer and between 1x10^5-10^6 cells in 100μl of solution 3 were placed in 5.0ml polystyrene tubes (BD Falcon). 100μl of antibody solution at the appropriate concentration (Tables 2.1 and 2.2) was added to each tube. Cells were incubated on ice in the dark for 20-45 minutes; 1ml of solution 3 was added and cells were centrifuged at 1,500rpm (Heraeus Instruments Lubofuge 400R centrifuge) for 5 minutes at 4°C. Supernatants were removed and 100μl solution 3 added.

For secondary staining with streptavidin conjugated fluorochromes 100μl of secondary reagent diluted to the appropriate concentration (Table 2.2) was added.
Cells were incubated on ice in the dark for 10 minutes. 0.5ml solution 3 was added to each tube and cells were centrifuged at 1,500rpm for 3 minutes at 4°C. Finally, supernatant was removed and cells were resuspended in 200μl of 0.25-0.5μg/ml 7-AAD.

Cells were analysed using a dual laser FACScalibur. FITC, PE and APC labelled cells were identified in the fluorescence level (FL)-1, FL-2 and FL-4 channels, respectively, and viable cells were selected according to the low uptake of 7-AAD as detected in FL-3. Cell size and granularity were assessed according to forward scatter and side scatter profile.

2.5.2 Staining and analysis of embryonic cells

Cells either harvested directly from embryonic organs or following ex vivo differentiation were processed as described above with the exception that a maximum of 5x10⁶ cells was added to each tube.

2.5.3 Cell sorting

Cell sorting was performed using either FACStar (Becton Dickinson) or MoFlo (DakoCytomation) flow cytometers. Cells were stained as described above with the exception that cells were resuspended at a concentration of 1x10⁶ cells/ml in 0.25μg/ml 7-AAD. To minimise cell loss during simultaneous two-way sorting 50ml Polypropylene centrifuge tubes (Corning) containing 25ml solution 3 were used as collection vessels; during simultaneous four-way sorting 5ml Polystyrene tubes (BD Falcon) containing 2.5ml solution 3 were used. Throughout the sorting process cells were maintained at 4°C. When possible, a sample of cells was analysed to check the.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Isotype</th>
<th>Working concentration</th>
<th>Conjugate</th>
<th>Supplier</th>
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Table 2.1: Primary antibodies used for confocal microscopy, flow cytometry and immunohistochemistry

APC denotes Allophycocyanin; Bio, biotin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; None, non-conjugated.
<table>
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<th>Reagent</th>
<th>Clone</th>
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<td>Streptavidin</td>
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<td>Pharmingen</td>
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</tbody>
</table>

Table 2.2: Secondary reagents and isotype control antibodies for flow cytometry
7-AAD denotes 7-amino-actinomycin D; Ac-LDL, acetylated low density lipoprotein; APC, Allophycocyanin; PE, phycoerythrin; n/a, not applicable
purity of the sorted population. Sorting was performed by either Andrew Sanderson or Jan Vrana.

2.6 Tissue culture

All tissue culture procedures were performed in class 2 laminar flow sterile hoods using a strict sterile technique which included wiping the hood down and spraying all items entering the hood with 70% industrial methylated spirits. All cells were incubated in 5.0% CO₂ at 37°C in a humidified incubator (Heraeus Instruments). Cells were routinely examined using an inverted microscope (Olympus CK2). All solutions were pre-warmed to 37°C prior to use. Unless otherwise stated, all growth factors were supplied by PeproTech. All media and supplementary reagents were supplied by Gibco. All supplementary reagents were prepared by the in-house tissue culture service and were stored at -20°C.

2.6.1 OP-9 cell line

OP-9 cells (Courtesy of Dr. Lesley Forrester) were routinely maintained in αMEM cell culture medium containing 20% FCS; 4mM glutamine; 0.1mM 2-mercaptoethanol; 50 units/ml P/S. Cells were maintained in 75cm³ tissue culture flasks (Iwaki).

2.6.1.1 Freezing of OP-9 cells

Approximately 1x10⁶ cells were suspended in 500µl of the above maintenance medium containing 10% DMSO and transferred to a 1.0ml cyrotubes
Cells were kept overnight at -80°C then transferred to liquid nitrogen for long-term storage.

### 2.6.1.2 Thawing of OP-9 cells

Cells were rapidly thawed by placing a vial of frozen cells in a 37°C water bath. Once thawed, cells were transferred to a universal tube containing 9.5ml of pre-warmed maintenance medium in order to dilute out the DMSO. Cells were centrifuged at 1,200rpm (MSE Mistral 2000 centrifuge) for 5 minutes at room temperature. Following the removal of the supernatant the cell pellet was resuspended in 10ml of pre-warmed maintenance medium and then transferred to a 75cm³ tissue culture flask.

### 2.6.1.3 Passaging of OP-9 cells

Cells were allowed to grow until 80% confluent at which point the medium was removed and replaced by two changes of 10ml pre-warmed PBS. 2.0ml trypsin solution (PBS containing 0.025% trypsin (Gibco); 0.1% chicken serum (Flow Labs); 1.3mM EDTA (Sigma)) was added to the cells and incubated at 37°C for 2-5 minutes until a single-cell suspension was obtained. 8.0ml pre-warmed maintenance medium was added to neutralise the trypsin and the cell suspension centrifuged at 1,200rpm for 3 minutes at room temperature. Supernatant were removed, cells resuspended in 10ml of pre-warmed maintenance medium and then transferred to a fresh 75cm³ flask at a dilution of 1:4.

### 2.6.2 Endothelial tubule/network forming assay
Endothelial tubule and network formation was assessed using OP-9 stromal cells in the presence of αMEM medium containing 10% FCS; 4mM glutamine; 0.1mM 2-mercaptoethanol; 50units/ml P/S; 50ng/ml VEGF as described previously (Fraser et al., 2003; Nishikawa et al., 1998). Cells purified from the E11.5 AGM region were added to a confluent layer of OP-9 cells in a 96 well plate. Following 4 days of co-culture the number of PECAM-1+ tubules was counted. A two-step anti-PECAM-1 staining protocol was followed to identify endothelial development: following a primary anti-PECAM-1 antibody incubation (BD Bioscience) endothelial structures were visualised using a secondary antibody conjugated with alkaline phosphatase (Southern Biotechnology Associates) and a Vector Blue Alkaline Phosphatase Substrate Kit III (Vector) according to the manufacturer’s instructions. Phase contrast images were taken with an inverted Olympus IX50 microscope using ViewFinder software. Images were prepared using Adobe Photoshop.

2.6.3 Methylcellulose based differentiation

Cell suspensions were added to growth factor supplemented methylcellulose-based MethoCult medium containing erythropoietin, IL-3, IL-6 and SCF (M3434; Stem Cell Technologies) according to the manufacturer’s instructions. Colonies were counted and scored after 7-12 days of differentiation. The criteria for colony scoring are described in Appendix 3. Cells were harvested by diluting 1.0ml medium in 2.0ml solution 3 followed by centrifugation at 1,500rpm for 5 minutes at room temperature. Images were taken with an inverted Olympus IX50 microscope using ViewFinder software. Images were prepared using Adobe Photoshop.
2.6.4 Organ explant culture

AGM region and yolk sac explant cultures were carried out as described previously (Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996). AGM region and YS were dissected from E11.5-12.5 embryos into solution 1 and grown in organ cultures as described. Durapore 0.65μm membrane filters (Millipore) were placed at the gas-liquid interface in 6 well tissue culture plates containing 5.0ml of Myelo-cult medium (M5300; Stem Cell Technologies) supplemented with 1x10^-6 M Hydrocortisone 21-hemisuccinate sodium salt (H2270; Sigma) and 50 units/ml P/S. In some experiments recombinant Noggin (R&D Systems) or BMP4 (R&D Systems) was added to the explant medium. A maximum of four AGM regions or two yolk sacs were placed on each filter and incubated for 72 hours. Scalpel blades were used to collect cultured organs.

2.6.5 Liquid suspension culture (LSC)

Unless otherwise stated, all growth factors were present at a concentration of 100ng/ml. After 7-21 days of culture phase contrast images of colonies were taken with an inverted Olympus IX50 microscope using ViewFinder software. Images were prepared using Adobe Photoshop.

2.6.5.1 Unfractionated E11.5 AGM region

1.0e.e. of cell suspension from whole E11.5 AGM region was added to 300μl of IMDM medium (containing 20% FCS; 4mM glutamine; 0.1mM 2-mercaptoethanol; 50 units/ml P/S) supplemented with various combinations of growth factors, these included: SCF, IL-3, IL-6, IL-7, IL-11, Flt3L and 0.01%
leukaemia inhibitory factor (LIF) conditioned medium (in-house preparation). Cells were transferred to wells from a 96 U-bottom well non-tissue culture treated plate (Bibby Sterlin); to eliminate possible effects from the evaporation of medium outer wells contained IMDM medium only. Plates were centrifuged at 1,300rpm for 5 minutes at room temperature and then transferred to an incubator. At the end of the culture period cells were harvested by pipetting. To remove remnants of growth factor enriched medium cells were centrifuged at 1,500rpm for 3 minutes at room temperature, supernatant was removed, cells resuspended in 1.0ml solution 3, passed through a 40μm cell strainer and centrifuged at 1,500rpm for 5 minutes. Supernatant was removed and cells were resuspended in 100μl solution 3.

2.6.5.2 Purified populations from the E11.5 AGM region

Various doses of cells purified from the E11.5 AGM region by flow cytometry was added to 300μl of IMDM medium (containing 20% FCS; 4mM glutamine; 0.1mM 2-mercaptoethanol; 50 units/ml P/S) supplemented with stem cell factor (SCF), interleukin (IL)-3, IL-6, IL-7, IL-11 and Flt3-ligand (Flt3L) and transferred to a well from a 96 U-bottom well non-tissue culture treated plate (Bibby Sterlin). To eliminate possible effects from the evaporation of medium outer wells contained IMDM medium only. Plates were centrifuged at 1,300rpm for 5 minutes at room temperature and then transferred to an incubator. At the end of the culture period cells were harvested by pipetting. To remove remnants of growth factor enriched medium cells were centrifuged at 1,500rpm for 3 minutes at room temperature, supernatant was removed, cells resuspended in 1.0ml solution 3, passed
through a 40μm cell strainer and centrifuged at 1,500rpm for 5 minutes. Supernatant was removed and cells were resuspended in 100μl solution 3.

2.6.6 E11.5 AGM region reaggregation

Reaggregates were produced by modifying a method developed by J.Sheridan (personal communication). E11.5 AGM region was prepared as described above. Cells from pooled organs were resuspended in appropriate culture medium at a concentration of 1.0e.e./15μl. 15μl of AGM suspension was drawn into a 200μl pipette tip and the tip orifice blocked using Parafilm (VWR International). Tips were subsequently placed in 50ml centrifuge tubes (Corning) and centrifuged at 1,200rpm (GS-6R centrifuge from Beckman) for 5 minutes at room temperature. Cell pellets were expelled onto Durapore 0.65μm membrane filters (Millipore) that had been placed at the gas-liquid interface in 6 well tissue culture plates containing 5.0ml of either organ explant medium (see above) or IMDM+ medium (containing 20% FCS; 4mM glutamine; 0.1mM 2-mercaptoethanol; 50 units/ml P/S; SCF; IL-3; Flt3L; in some cases 1x10⁻⁶M Hydrocortisone 21-hemisuccinate sodium salt was included). A maximum of four cell pellets were placed on each filter and incubated for 24-96 hours. To harvest reaggregates filters were submersed into the culture medium and adherent organs were dislodged following gentle pipetting. Cell suspensions were produced as described above.

Bright field and fluorescent images of reaggregates were taken using a LEICA MZFLIII dissecting microscope and a Digital Sight DS-L camera (Nikon). Images were prepared using Adobe Photoshop.
2.7 Confocal microscopy

2.7.1 Frozen sections

2.7.1.1 Embedding and sectioning

Fresh or reaggregated E11.5 AGM region was snap-frozen in O.C.T compound (BDH Gurr) on dry ice. 10µm thick transverse sections were produced using a LEICA CM1900 cryostat (Leica). Sections were transferred to polysine coated slides and air-dried for 1 minute then stored at -20°C. Frozen sections were allowed to equilibrate to room temperature then fixed in 100% acetone (-20°C) for 2.5 minutes and then air-dried for a further 2.5 minutes.

2.7.1.2 Immunofluorescence staining

Individual acetone fixed transverse sections were encircled using a PAP pen and then rehydrated in PBS for 2 minutes. Blocking serum (PBS containing 5% FCS) was added to sections for 15 minutes at room temperature. Sections were then washed with 1 change of PBS. Fluorochrome conjugated antibodies diluted in PBS was added to sections and incubated for 30 minutes in the dark. Staining solution was removed and replaced by 3 changes of PBS, each wash lasted 5 minutes. Nuclei were stained by incubating sections in adding PBS containing 0.2% 4-6-Diamidino-2-phenylindole (DAPI) (Molecular probes) for 10 minutes in the dark. Sections were washed in 3 changes of PBS then mounted in a drop of VECTORSHIELD hard set medium (Vector Laboratories). Number 1.5 glass cover slips (BDH) were used. Mounted slides were allowed to harden according to the manufacturer’s instructions.

Sections were studied and images were taken using an inverted confocal microscope (Leica DM IRE2). Images were prepared using Adobe Photoshop.
2.7.2 Whole-mount preparation and imaging

All stages of preparation was performed in 4 well plates (Nunc).

Organs were fixed in PBS containing 2.0% paraformaldehyde (pH7.4) for 20 minutes at room temperature. Following 3 changes of PBS organs were permeabilised using PBS containing 0.6% Triton-X (Sigma) for 30 minutes at room temperature. Following a further 3 changes of PBS fluorochrome conjugated antibodies diluted in 0.3% Triton-X/PBS were added; organs were incubated overnight in the fridge.

On the second day the antibody solution was replaced with 4 changes of PBS over a period of 2 hours at room temperature in the dark. The final change of PBS was replaced with 0.2% DAPI/PBS for 1 hour at room temperature in the dark. Following 3 changes of PBS over 30 minutes organs were ready for analysis.

Three-dimensional Z-stacks were compiled from 1.0μm optical sections taken using an inverted confocal microscope (Leica DM IRE2) and constructed using Velocity software (Improvisation). Images were prepared using Adobe Photoshop.

2.8 Cytological examination

2.8.1 Cytospin preparation

Cells suspended in 100μl of PBS were loaded into a cytospin chamber which includes a polysine coated slide (VWR International) and a filter card (Thermo). Preparations were centrifuged at 1,000rpm (Cytospin 3; Shandon) for 5 minutes at room temperature. Slides were removed from the chamber and allowed to air-dry for 2 minutes and then fixed in 100% methanol (room temperature) for 2.5 minutes.
Following fixation slides were left to air-dry for 2 minutes. Prior to staining the area around the cells was marked using a PAP pen (Daido Sangyo).

2.8.2 May-Grunwald-Giemsa staining

May-Grunwald stain (BDH Gurr) was prepared as a 50% solution in PBS (Sigma); Giemsa stain (BDH Gurr), as a 10% solution in PBS (Sigma). May-Grunwald and Giemsa stains were always freshly prepared. Stains were filtered immediately before using number 1 Whatman filter paper (Whatman International Ltd).

Methanol fixed cells were incubated in May-Grunwald stain in the dark for 14 minutes; the primary stain is discarded and replaced with Giemsa stain (1:9 dilution in PBS/filtered through Whatman No.1) for 12 minutes. Slides were washed 3 times with running distilled water and then incubated in clean water for 3-5 minutes. Finally, slides were air-dried for approximately 5 minutes.

Images were taken using an upright Vanox AHBT3 microscope (Olympus) and Openlab software (Improvision). Images were prepared using Adobe Photoshop.

2.9 Reverse transcription polymerase chain reaction (RT-PCR)

2.9.1 RNA extraction

Total RNA from the ventral (AoV) and dorsal (AoD) aspects of the E11.5 dorsal aorta was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. AoV and AoD RNA was extracted from 10-15 embryos. Samples were stored at -80°C.
2.9.2 cDNA synthesis

cDNA was synthesised from extracted RNA using SuperScript First-strand Synthesis System (Invitrogen) according to the manufacturer's instructions.

2.9.3 Polymerase Chain Reaction (PCR)

PCR reactions were carried out using a T3 Thermocycler (Biometra). dNTPS was supplied by Boehringer and *Thermus aquaticus* DNA polymerase (Taq), by Qiagen. To 1µg of cDNA was added 10x PARR PCR buffer; 1.5mM MgCl$_2$ (Cambio); 0.2µg sense primer; 0.2µg antisense primer; 0.2µM dNTPs; 0.4µl Taq (10 units/µl) and the volume adjusted to 50µl with PCR grade H$_2$O (Sigma). The PCR conditions were as follows: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds; 62°C for 30 seconds; 72°C for 60 seconds; followed by 72°C for 5 minutes. PCR reactions with products of cDNA synthesis in the absence of a reverse transcriptase step were performed using *Noggin* primers.

PCR products were analysed by electrophoresis using 5% agarose (Cambrex) gels cast and run in 1xTAE buffer (0.04M Tris-Acetate; 0.001M EDTA) at an appropriate concentration containing 0.5µg/ml ethidium bromide. 0.5µg of 100bp DNA ladder (New England Biolabs) was loaded on each gel as a size standard.

2.9.4 PCR primers

Unless stated otherwise, oligonucleotide sequences were designed using Primer3 software (http://cbrmain.cbr.nrc.ca:8080/cbr/jsp/ServicePage_e.jsp?id=81) and were synthesised by Sigma.
2.10. Statistical analysis

The statistical significance of differences in the efficacy of haematopoietic reconstitution within the \textit{in vivo} LTR assay was calculated using 2x2 contingency tables and the Fisher exact test. The statistical significance in all other contexts was calculated using the Students t-test; variances between experimental groups were calculated using the F-test. Statistical significance was calculated at the 5% significance level.

Calculation of limiting dilution was performed using L-Calc software (Stem Cell Technologies).
<table>
<thead>
<tr>
<th>Transcript</th>
<th>Oligonucleotide sequence</th>
<th>Expected PCR product (bp)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Sense (5'-3')/Antisense (3'-5')</td>
<td>cDNA template</td>
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<tr>
<td><strong>BMP-4</strong></td>
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<tr>
<td></td>
<td>GGGACGTAAGTCCCTCCACCA</td>
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</tbody>
</table>


**Table 2.3: Primers used for PCR**

Note the difference in the expected PCR product from cDNA and gDNA templates for the majority of primer pairs. Distinction between cDNA and gDNA templates providing the basis for a product in the presence of Noggin primers was achieved by performing the PCR reaction with products of cDNA synthesis in the absence of a reverse transcription step.
Chapter 3: Results 1

Endothelial affiliation of the emerging LTR-dHSC pool†‡

3.1 Introduction

The cascade of LTR-dHSC development follows a complex temporal and spatial pattern with the sequential involvement of multiple organs beginning with the emergence of the first LTR-dHSCs from the E10.5-11.5 AGM region (see section 1.3.). There are currently three models proposed to explain the origin of LTR-dHSCs: 1. divergence from haematogenic endothelium lining the floor of the dorsal aorta; 2. production from sub-aortic patches and; 3. commitment of haemangioblasts residing beneath the aortic endothelium (summarised in Figure 3.1).

Each of the models integrates the formation of intra-aortic haematopoietic clusters (IAHC) as a means by which LTR-dHSCs enter the peripheral circulation thus universally implicating an endothelial stage of LTR-dHSC development. We therefore reasoned that if functionally competent LTR-dHSCs emerge from the endothelial compartment they might bear hallmarks of both the endothelial and haematopoietic lineages. Using a combination of flow cytometry, *in vivo* transplantation, organ explant culture and *in vitro* differentiation the involvement of the embryonic endothelial compartment during LTR-dHSC development was investigated.

†‡ This study was performed in collaboration with Dr Aline Morrison.
Based on histological and transcription evidence there are currently three models used to explain the emergence of LTR-dHSCs in the AGM region:

(A) Divergence of LTR-dHSCs from haematogenic endothelial cells lining the dorsal aorta, as evidenced by the identification of intra-aortic haematopoietic clusters during the period of intra-embryonic LTR-dHSC emergence.

(B) LTR-dHSCs are formed in sub-aortic patches and subsequently migrate to the dorsal aorta where they traverse the endothelial lining and bud into the vascular lumen.

(C) Intra-aortic haematopoietic clusters contain LTR-dHSCs derived from haemangioblasts that underlie the endothelial lining of the dorsal aorta.

(Schematic representation after Godin and Cumano, 2002; Bertrand et al, 2005)
3.2 Results

3.2.1 Rare cells in the E11.5 AGM region co-express VE-cadherin and CD45

For an initial analysis of a potential relationship between endothelial and haematopoietic cells the expression of two basic markers was used: vascular endothelial cadherin (VE-cadherin) and the pan-leucocyte CD45. Viable cells from the E11.5 AGM region, the YS and the liver were analysed. The obtained results confirmed that in all organs tested expression is predominantly mutually exclusive (Table 3.1). Intriguingly, co-expression of VE-cadherin and CD45 is observed in a rare population of cells (Table 3.1 and Figure 3.2).

At E11.5 LTR-dHSCs can be detected in the AGM region, the YS and the liver, however, only the AGM region is capable of expanding these cells ex vivo (sections 1.3.2-1.3.3). Based on flow cytometric analysis the E11.5 AGM region is composed of 2.5±0.4% VE-cadherin\(^+\)CD45\(^-\) (VE-cadherin single-positive/VE-cadherin\(^{\text{SP}}\)) cells, 2.6±0.39% VE-cadherin\(^+\)CD45\(^+\) (CD45 single-positive/CD45\(^{\text{SP}}\)) cells, 95±0.76% VE-cadherin-CD45- (double-negative/DN) cells and 0.05±0.01% VE-cadherin\(^+\)CD45\(^+\) (double-positive; DP) cells (Table 3.1 and Figure 3.2A).

3.2.2 Defining AGM region lineages according to the co-expression of multiple lineage-specific proteins

Given that this rare population of DP cells can be consistently identified within the E11.5 AGM region during the time when it is hypothesised that LTR-dHSCs diverge from the embryonic endothelial compartment (section 1.3.9.2.) the expression of multiple endothelial and haematopoietic lineage associated adhesion,
<table>
<thead>
<tr>
<th>Embryo age</th>
<th>Organ</th>
<th>n</th>
<th>Phenotype frequency (%) ± SEM (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>VE-cadherin&lt;sup&gt;+&lt;/sup&gt;CD45&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>E11.5</td>
<td>AGM region</td>
<td>7</td>
<td>2.5±0.40 (1.3-4.0)</td>
</tr>
<tr>
<td></td>
<td>Yolk sac</td>
<td>4</td>
<td>6.9±1.3 (3.5-9.6)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>6</td>
<td>10.7±0.71 (8.9-12.3)</td>
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Table 3.1: Frequency of haematopoietic (VE-cadherin<sup>+</sup>CD45<sup>-</sup>), endothelial (VE-cadherin<sup>-</sup>CD45<sup>+</sup>) and double-positive (VE-cadherin<sup>-</sup>CD45<sup>-</sup>) cells in major E11.5 haematopoietic organs
Figure 3.2: Analysis of the E11.5 AGM region by flow cytometry

(A) Flow cytometric analysis of E11.5 AGM region demonstrating endothelial (VE-cadherin^+CD45^-), haematopoietic (VE-cadherin^-CD45^+) and double-positive (DP) (VE-cadherin^+CD45^+) populations. The contour plot shown is a representative example compiled from approximately 10e.e. of E11.5 AGM (1.5x10^6 viable cells). The percentage of cells in each quadrant is indicated; quadrants are based on appropriate isotype control staining (Figure A4.1).

(B) Detailed immunophenotyping of E11.5 AGM region endothelial, haematopoietic and DP populations. The endothelial affiliation of the double-positive population extends beyond the expression of VE-cadherin as evidenced by endothelial-like levels of Tie-2, Flk-1, PECAM-1, Ac-LDL, CD34 and Sca-1 expression; the DP demonstrates greater affiliation with the endothelial population than with the haematopoietic fraction as evidenced by the common enrichment of endothelial and pan-HSC markers. Isotype control (dark line) and specific antibody staining (filled curve) are presented. Values indicate the percentage of positive cells. Histograms of the analysis of DP, endothelial and haematopoietic populations are composed from approximately 200-700, 15,000-20,000, and 9,000-30,000 viable cells, respectively.

Data are representative of 2-4 independent experiments for each marker analysed.
A  
E11.5 AGM region

B  
VE-cadherin^CD45^-

VE-cadherin^CD45^+

VE-cadherin^CD45^+

Tie-2    Flk-1    PECAM-1    Ac-LDL    α4-integrin    CD41    Mac-1    CD34    c-Kit    Sca-1    AA4.1
<table>
<thead>
<tr>
<th>Population</th>
<th>Tie-2 ±</th>
<th>Flk-1 ±</th>
<th>PECAM-1 ±</th>
<th>Ac-LDLr ±</th>
<th>CD41 ±</th>
<th>α4-integrin ±</th>
<th>Mac-1 ±</th>
<th>Sca-1 ±</th>
<th>c-Kit ±</th>
<th>AA4.1 ±</th>
<th>CD34 ±</th>
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<tr>
<td>VE-cadherin^CD45^+</td>
<td>2.2±1.4</td>
<td>2.0±1.0</td>
<td>92±3.0</td>
<td>80±15</td>
<td>2.0±1.1</td>
<td>63±13</td>
<td>98±0.5</td>
<td>1.4±0.3</td>
<td>16±1.1</td>
<td>1.5±0.5</td>
<td>0±0</td>
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<tr>
<td>VE-cadherin^CD45^-</td>
<td>96±1.4</td>
<td>96±1.0</td>
<td>98±0.9</td>
<td>99±0.5</td>
<td>4.9±2.1</td>
<td>2.4±0.7</td>
<td>0±0</td>
<td>90±4.3</td>
<td>67±7.5</td>
<td>81±6.5</td>
<td>78±11</td>
</tr>
<tr>
<td>VE-cadherin^CD45^-</td>
<td>69±2.3</td>
<td>85±1.5</td>
<td>98±0.5</td>
<td>95±4.0</td>
<td>15±6.5</td>
<td>86±5.1</td>
<td>90±5.4</td>
<td>81±2.4</td>
<td>81±4.1</td>
<td>38±11</td>
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<tr>
<td>VE-cadherin^CD45^-</td>
<td>0.6±0.1</td>
<td>0.18±0.13</td>
<td>2.0±0.4</td>
<td>89±1.3</td>
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<td>6.3±3.3</td>
<td>0±0</td>
<td>4.9±3.3</td>
<td>18±6.5</td>
<td>4.9±3.3</td>
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</table>

Table 3.2: Co-expression of endothelial and haematopoietic lineages markers in the E11.5 AGM region
signalling and homing molecules was studied with the aim of investigating the extent of the endothelio-haematopoietic phenotype of the DP population.

3.2.2.1 Expression of endothelial markers

Expression of Tie-2, Flk-1 and PECAM-1 proteins and the presence of Ac-LDL receptors (Ac-LDLr) are characteristics of the endothelial lineage (section 1.3.9.1.). Within the E11.5 AGM region the VE-cadherinSP population is highly enriched for these endothelial markers: 98±1.4% of cells express Tie-2, 96±1.0% express Flk-1, 98±0.9% express PECAM-1 and 99±0.5% express Ac-LDLr (Table 3.2 and Figure 3.2B).

Rare cells from the CD45SP population expressed Tie-2 and Flk-1, 2.2±1.4% and 2.0±1.0%, respectively. Some qualitative similarity to the VE-cadherinSP population was observed as evidenced by the expression of PECAM-1 on 92±3.0% of cells and Ac-LDLr on 80±15% of cells (Table 3.2 and Figure 3.2B).

The DP population demonstrates a greater qualitative affiliation to the endothelial (VE-cadherinSP) compartment than the haematopoietic (CD45SP) compartment: 69±2.3% of DP cells express Tie-2, 85±1.5% express Flk-1, 98±0.5% express PECAM-1 and 95±4.0% took up Ac-LDL (Table 3.2 and Figure 3.2B).

Tie-2, Flk-1 and PECAM-1 expression was largely absent from the DN population: 0.6±0.1%, 0.18±0.13% and 2.0±0.4%, respectively. Interestingly, 89±1.3% of DN cells were able to take up Ac-LDL (Table 3.2). Thus, contrary to the supposition that within the embryo Ac-LDL incorporation is unique to the endothelium (section 1.3.9.1.1.1) Ac-LDL receptors are broadly expressed.
Endothelial associated proteins Tie-2 and Flk-1 are abundantly expressed within both the VE-cadherin<sup>SP</sup> and the DP populations with some expression present within CD45<sup>SP</sup> and DN cells. PECAM-1 expression is abundant in the VE-cadherin<sup>SP</sup>, CD45<sup>SP</sup> and DP populations but absent from DN cells. CD45<sup>SP</sup> cells are characterised by a PECAM-1<sub>low</sub> phenotype and VE-cadherin<sup>SP</sup> cells by a PECAM-1<sub>high</sub> phenotype (Figure 3.2B). The DP population exhibits characteristics of both the VE-cadherin<sup>SP</sup> and CD45<sup>SP</sup> populations, seen as a bimodal distribution of PECAM-1<sub>low</sub> and PECAM-1<sub>high</sub> cohorts (Figure 3.2B). The ratio between these low-level and high-level expressing fractions is variable, favouring a trend towards PECAM-1<sub>high</sub> cells.

With respect to Ac-LDL uptake a similar distinction between the VE-cadherin<sup>SP</sup>, CD45<sup>SP</sup> and DP populations can be made, with the greatest levels of expression being found within VE-cadherin<sup>SP</sup> and DP populations (Figure 3.2B).

### 3.2.2.2 Expression of haematopoietic markers

The plasma membrane expression of haematopoietic lineage-associated proteins demonstrates that in keeping with the expression of CD45 the DP population bears a haematopoietic profile. In the E11.5 AGM region the CD45<sup>SP</sup> population is characterised by 63±13% of cells expressing α4-integrin, 98±0.5% expressing Mac-1 and 2.0±1.1% expressing CD41 (Table 3.2 and Figure 3.2B). In line with this pattern 86±5.1% of DP cells express α4-integrin, 90±5.4% expresses Mac-1 and 15±6.5% expresses CD41. Highlighting the value of these markers in segregating the endothelial and haematopoietic compartments α4-integrin was only identified on
2.4±0.7% of VE-cadherin<sup>SP</sup> cells, Mac-1 was completely absent and CD41 was found to be expressed on 2.0±1.1% of cells (Table 3.2 and Figure 3.2B).

### 3.2.2.3 Expression of HSC markers

c-Kit and CD34 have been identified on the surface of pre-LTR-dHSCs, the first embryonic LTR-dHSCs to emerge from the E11.5 AGM region, and the LTR-dHSC pools of the E12.5-14.5 liver and the E12.5 placenta (Gekas et al., 2005; Sanchez et al., 1996; Yoder et al., 1997; Zeigler et al., 1994). In the E11.5 AGM region the CD45<sup>SP</sup> population contains 16±1.1% c-Kit<sup>+</sup> cells but no CD34 expressing cells. In contrast, c-Kit and CD34 expression is highly enriched within the VE-cadherin<sup>SP</sup> and DP populations: c-Kit was expressed by 67±7.5% of VE-cadherin<sup>SP</sup> cells and in 81±4.1% of DP cells; CD34 was expressed in 78±11% of VE-cadherin<sup>SP</sup> cells and in 86±7.9% of DP cells (Table 3.2 and Figure 3.2B).

Sca-1 is readily detected on LTR-dHSCs in the adult bone marrow and the foetal liver (Morrison et al., 1995; Spangrude et al., 1988). Following conventional antibody staining LTR-dHSCs are detected in both the Sca-1<sup>+</sup> and the Sca-1<sup>-</sup> fractions, however, using a transgenic reporter line, that is proposed to improve the resolution of Sca-1 detection, all LTR-dHSCs are found within the Sca-1<sup>+</sup> population (de Bruijn et al., 2002). These observations suggest that the level of expression is likely to be low in the LTR-dHSC compartment. Interestingly, Sca-1 is most abundantly expressed in the VE-cadherin expressing component of the E11.5 AGM region: Sca-1 is expressed in 90±4.3% of VE-cadherin<sup>SP</sup> cells, 81±2.4% of DP cells, 1.4±0.3% of CD45<sup>SP</sup> cells and 4.9±3.3% of DN cells (Table 3.2). As predicted, the level of Sca-1 expression in the E11.5 AGM region is low (Figure 3.2B).
AA4.1 labels LTR-dHSCs within the foetal liver (Harrison et al., 1997; Jordan et al., 1995) and low-level adult repopulating cells within the E10.5 AGM region (Bertrand et al., 2005). This HSC marker is also enriched within the VE-cadherin\textsuperscript{SP} and DP populations: 81±6.5% and 38±11% of cells expressed AA4.1, respectively. AA4.1 was detected on 1.5±0.5% of CD45\textsuperscript{SP} cells and on 4.9±3.3% of DN cells (Table 3.2 and Figure 3.2B).

The results of this investigation demonstrate that with the exception of Mac-1 the plasma membrane proteins associated with the HSC compartment during embryogenesis are particularly enriched within the VE-cadherin\textsuperscript{SP} population. Of note, the CD45\textsuperscript{SP} population lacks marked expression of HSC-associated markers, particularly CD34. Importantly, rare VE-cadherin\textsuperscript{+}CD45\textsuperscript{+} cells bear the qualitative and quantitative phenotypic hallmarks of both the endothelial and haematopoietic lineages, and express markers associated with HSCs.

3.2.3 Detection of VE-cadherin\textsuperscript{+}CD45\textsuperscript{+} cells in E12.5-13.5 haematopoietic organs

Investigation of major E12.5-13.5 haematopoietic organs revealed that populations of DP cells are present at many sites during key stages of LTR-dHSC development (Table 3.3 and Figure 3.3). Of note, DP cells continue to reside in the AGM region at E12.5.

In addition to the AGM region, DP populations are present in 0.03±0.008% of the E12.5 YS and 0.005±0.0013% of the E12.5 peripheral circulation (Table 3.3). The presence of these rare cells within the YS and the peripheral blood at E12.5 is intriguing because at this developmental stage two distinct processes are being
<table>
<thead>
<tr>
<th>Embryo age</th>
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<th>VE-cadherin$^*$CD45$^+$</th>
<th>VE-cadherin$^+$CD45$^+$</th>
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<td>0.035±0.005 (0.017-0.045)</td>
<td>0.005±0.0013 (0.001-0.008)</td>
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<td>5.5±0.32 (4.5-6.5)</td>
<td>1.3±0.15 (0.78-1.7)</td>
<td>0.31±0.036 (0.22-0.42)</td>
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</tr>
<tr>
<td>E13.5</td>
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<td>1.2±0.4 (0.48-2.8)</td>
<td>0.10±0.038 (0.022-0.22)</td>
<td>95±0.13 (95-96)</td>
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</table>

Table 3.3: Frequency of haematopoietic (VE-cadherin$^*$CD45$^-$), endothelial (VE-cadherin$^+$CD45$^+$) and double-positive (VE-cadherin$^+$CD45$^+$) cells in major E12.5 haematopoietic organs and the E13.5 liver
Figure 3.3: Expression of VE-cadherin and CD45 in the E12.5 yolk sac, the E12.5 peripheral circulation and the E13.5 liver
VE-cadherin$^+$CD45$^+$ cells are detected during the second wave of LTR-dHSC generation in the E12.5 yolk sac (A), during the initiation of LTR-dHSC mobilisation in the E12.5 peripheral blood (B) and during the hepatic phase of LTR-dHSC expansion in the E13.5 liver (C).

Data are representative examples from six experiments.
Each contour plot is composed from $1 \times 10^6$ viable yolk sac cells, $1 \times 10^5$ Ter119$^+$ viable peripheral blood cells and $3 \times 10^6$ viable foetal liver cells.
The percentage of cells in each quadrant is indicated; quadrants are based on appropriate isotype control staining (Figure A4.2).
undertaken: the first is the hypothesised extra-embryonic succession of LTR-dHSC generation; the second is the migration of LTR-dHSCs to secondary organs via the peripheral circulation (see section 1.3.3.).

Between E11.5-13.5 the number of LTR-dHSCs in the liver increases from approximately 1 to 260 (Kumaravelu et al., 2002). Interestingly, the liver harbours the greatest frequency and number of DP cells found between E11.5-13.5: the number of DP cells increases from 2,460±430 on E11.5 to 6,360±2,400 on E13.5 but the frequency decreases from 1.2±0.2% to 0.1±0.03% (Tables 3.3 and 3.4) suggesting that the hepatic DP population does expand during developmental progress but is diluted out by the accumulation of other lineages.

3.2.4 Expression of VE-cadherin as a means to trace LTR-dHSC ontogeny

The above observations provide the basis for a working hypothesis: if LTR-dHSCs emerge from the embryonic endothelial compartment then they may bear endothelial associated plasma membrane proteins which are subsequently lost upon continuation of haematopoietic development. In such a case all de novo LTR-dHSCs may bear the same dual endothelio-haematopoietic phenotype. In keeping with this hypothesis the distribution of LTR-dHSCs from the E11.5 AGM region, the E12.5 YS, the E12.5 PB and the E13.5 liver was investigated using the in vivo competitive long-term repopulating assay following purification of organs into VE-cadherin-expressing and CD45-expressing fractions (summarised in Figure 3.4).

3.2.4.1 E11.5 AGM region
<table>
<thead>
<tr>
<th>Embryo age</th>
<th>Organ</th>
<th>Viable cells (1x10^5) ± SEM</th>
<th>Number of VE-cadherin^+CD45^+ cells ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.5</td>
<td>AGM region</td>
<td>1.4±0.12</td>
<td>70±6</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>2.0±0.1</td>
<td>2,460±430</td>
</tr>
<tr>
<td>12.5</td>
<td>AGM region</td>
<td>1.0±0.15</td>
<td>20±3</td>
</tr>
<tr>
<td></td>
<td>Yolk sac</td>
<td>2.1±0.2</td>
<td>63±6</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>28.7±2.9</td>
<td>14±2</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>35.9±3.5</td>
<td>11,100±1,300</td>
</tr>
<tr>
<td>13.5</td>
<td>Liver</td>
<td>63.6±2.5</td>
<td>6,360±2,400</td>
</tr>
</tbody>
</table>

Table 3.4: Absolute numbers of VE-cadherin^+CD45^+ cells in major E11.5-13.5 haematopoietic organs
Figure 3.4: *In vivo* long-term repopulation assay

Experimental scheme for the analysis of VE-cadherin expression on LTR-dHSCs: candidate populations from E11.5-13.5 embryonic organs (*Ly-5.2/Ly-5.2* genotype) were purified by flow cytometry and injected into irradiated adult recipients (*Ly-5.1/Ly-5.1* genotype). High-level long-term multi-lineage contribution to haematopoiesis, ≥5% peripheral blood leucocyte chimerism, was determined after twelve weeks. Path *A* represents the transplantation of freshly dissected organs; path *B* indicates the additional 72 hour explant culture step prior to transplantation.
Cell suspensions produced from E11.5 AGM regions was stained using anti-VE-cadherin and anti-CD45 antibodies and purified into VE-cadherin\(^{SP}\), CD45\(^{SP}\), DP and DN populations. Sorted populations were transplanted into irradiated adult recipient mice. As previously described high-level contribution to the reconstitution of recipient haematopoiesis was restricted to the DP population (North et al., 2002). In this study 4 out of 12 mice were successfully reconstituted using 2.6e.e. of sorted cells, with a 36% mean peripheral blood leucocyte chimerism (PBLC) (range of 5-60%) (Table 3.5). No successful reconstitution was achieved following the injection of VE-cadherin\(^{SP}\), CD45\(^{SP}\) or DN cells into adult recipient mice even though in some cases twice the equivalent was injected (Table 3.5). Consistent with previously described findings, we find that as LTR-dHSCs first emerge they are defined by the co-expression of the largely mutually exclusive lineage-specific markers VE-cadherin and CD45.

3.2.4.2 E12.5 yolk sac

At E12.5 the production of LTR-dHSCs is superseded by the YS. To test if extra-embryonic LTR-dHSC emergence shares an affiliation with the endothelial compartment cell suspensions from E12.5 YS were separated according to the expression of either VE-cadherin or CD45.

7 out of 10 adult mice injected with the VE-cadherin\(^{+}\) fraction were successfully repopulated with a 33% mean PBLC (range 5-87%) (Table 3.5). None of the 10 mice injected with purified VE-cadherin\(^{-}\) cells were repopulated. Transplantation of YS cells on the basis of CD45 expression revealed that LTR-dHSCs are mostly CD45\(^{+}\): 3 out of 3 mice were repopulated with the CD45\(^{+}\) fraction
<table>
<thead>
<tr>
<th>Donor organ</th>
<th>Population transplanted</th>
<th>Embryo equivalent (e.e.)/HSC equivalent (HSC.e.) transplanted</th>
<th>Reconstituted/transplanted</th>
<th>Mean % chimerism (range)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E11.5 AGM</td>
<td>VE-cadherin⁺CD45⁺</td>
<td>2.6.e.e.</td>
<td>4/12</td>
<td>36 (5-60)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁺CD45⁺</td>
<td>4.0-5.0.e.e.</td>
<td>0/15</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁺CD45⁺</td>
<td>4.0-5.0.e.e.</td>
<td>0/10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁺CD45⁺</td>
<td>0.5-2.5.e.e.</td>
<td>0/10</td>
<td>-</td>
</tr>
<tr>
<td>E12.5 Yolk sac</td>
<td>CD45⁺</td>
<td>1.0.e.e.</td>
<td>3/3</td>
<td>74 (61-87)</td>
</tr>
<tr>
<td></td>
<td>CD45⁻</td>
<td>1.25.e.e.</td>
<td>1/4</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁺</td>
<td>0.4-0.8.e.e.</td>
<td>7/10</td>
<td>33 (5-87)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁻</td>
<td>0.7-1.0.e.e.</td>
<td>0/10</td>
<td>-</td>
</tr>
<tr>
<td>E12.5 Peripheral blood</td>
<td>VE-cadherin⁺</td>
<td>1.5-3.0.e.e.</td>
<td>8/12</td>
<td>54 (10-90)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁻</td>
<td>1.5-3.0.e.e.</td>
<td>1/10</td>
<td>84</td>
</tr>
<tr>
<td>E13.5 Foetal liver</td>
<td>VE-cadherin⁺CD45⁺</td>
<td>0.02.e.e.</td>
<td>4/5</td>
<td>30 (6-42)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁺CD45⁺</td>
<td>0.02.e.e.</td>
<td>5/5</td>
<td>70 (61-76)</td>
</tr>
<tr>
<td>Adult bone marrow</td>
<td>VE-cadherin⁺</td>
<td>2-10 HSC.e.</td>
<td>0/13</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁻</td>
<td>2-10 HSC.e.</td>
<td>7/10</td>
<td>≥ 10⁺</td>
</tr>
</tbody>
</table>

*Chimerism observed in peripheral blood leucocytes by flow cytometry. Only recipients demonstrating at least 5% chimerism after at least 12 weeks were regarded as reconstituted.

†Either male donor cells were transplanted into female recipient mice or Ly-5.1/5.2 cells were transplanted into Ly-5.2/5.2 recipient mice.

‡Chimerism determined in peripheral blood leucocytes by either Y-chromosome PCR or flow cytometry.

Table 3.5: VE-cadherin expression on LTR-dHSCs within primary haematopoietic organs, peripheral circulation, the liver and the adult bone marrow.
with a 74% mean PBLC (range 61-87%) and 1 out of 4 mice was repopulated with the CD45 fraction (40% PBLC). These data demonstrate that the vast majority of LTR-dHSCs bear the hallmarks of both the endothelial and haematopoietic lineages during the extra-embryonic phase of LTR-dHSC generation.

3.2.4.3 E13.5 liver

The working hypothesis of our laboratory is that the liver acts as a secondary haematopoietic organ which is seeded by LTR-dHSCs that emerge from the E11.5 AGM region and E12.5 YS during sequential waves of LTR-dHSC expansion. Having determined that as LTR-dHSCs emerge and expand within primary intra-embryonic and extra-embryonic organs they co-express VE-cadherin and CD45 we investigated if this phenotype is maintained in the liver at E13.5, at which time this organ is the only site of significant LTR-dHSC expansion (see section 1.3).

Cell suspensions from E13.5 liver was purified by flow cytometry into VE-cadherin\(^+\)CD45\(^+\) and VE-cadherin\(^-\)CD45\(^+\) populations and injected into irradiated adult mice at a dose of 0.02e.e. (approximately 5 LTR-dHSCs). 4 out of 5 mice injected with DP cells were successfully repopulated with a 30% mean PBLC (range 6-42%) (Table 3.5). All 5 mice that received the CD45\(^{SP}\) fraction were also successfully repopulated and demonstrated a 70% mean PBLC (range 61-76%).

Due to the limited numbers of experimental mice in our experiments it is not possible to accurately determine the numbers of LTR-dHSCs in either the DP or the CD45\(^{SP}\) fractions. However, given that all 5 mice injected with the CD45\(^{SP}\) fraction were repopulated and that the mean PBLC was two-fold higher than that observed in the cohort transplanted with the DP population our data supports the prediction that a
higher frequency of LTR-dHSCs are present in the CD45<sup>SP</sup> population. Presuming that the competitive pressure from host and carrier cells in both experimental groups was similar we predict that the total number of LTR-dHSCs in the CD45<sup>SP</sup> fraction was greater than in the DP fraction.

3.2.4.4 Preservation of VE-cadherin expression during LTR-dHSC migration

We next investigated if downregulation of VE-cadherin expression within the LTR-dHSC pool occurs during migration from the site of origin or upon colonisation of the liver. To this end we investigated the phenotype of LTR-dHSC in the peripheral circulation at E12.5, the time when circulatory LTR-dHSCs become readily detectable.

Ter<sub>119</sub>-depleted peripheral blood was separated into VE-cadherin<sup>+</sup> and VE-cadherin<sup>−</sup> fractions by flow cytometry. 1.5-3.0e.e. of each fraction was injected into adult recipients. 8 out of 12 mice that received VE-cadherin<sup>+</sup> cells were successfully repopulated with a 54% mean PBLC (range 10-90%) (Table 3.5). Only 1 out of 10 mice was repopulated with VE-cadherin<sup>−</sup> cells (84% PBLC). Thus, during embryogenesis significant loss of VE-cadherin expression occurs once LTR-dHSCs seed the liver.

3.2.4.5 VE-cadherin expression is lost in adult bone marrow LTR-dHSCs

Dr A. Morrison purified VE-cadherin<sup>+</sup> and VE-cadherin<sup>−</sup> cells from the adult bone marrow by flow cytometry and 2-10 HSC equivalents were injected into
irradiated adult mice. 0 out of 13 mice that received VE-cadherin\(^+\) cells demonstrated PBLC and 7 out of 10 mice transplanted with VE-cadherin\(^-\) cells were successfully reconstituted (Table 3.5). These findings indicate that although the expression of VE-cadherin is preserved during the hepatic phase of foetal LTR-dHSC development expression of this endothelial marker is completely lost during adulthood.

### 3.2.4.6 VE-cadherin expression in the E12.5 placental LTR-dHSC pool

The placenta has recently been discovered to be a niche for haematopoietic stem cells during embryonic and foetal development (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). A marked downregulation of VE-cadherin expression is observed within the CD34\(^-\)c-Kit\(^+\) placental LTR-dHSC fraction between E11.5 and E12.5 (Figure 3.5): at E11.5 95±0.6% and 60±0.3% the CD34\(^+\)c-Kit\(^+\) express CD45 and VE-cadherin, respectively. By E12.5 the expression of CD45 remains consistent (96±0.7%) while the expression of VE-cadherin is reduced to 30±3.0% (Figure 3.5). These data are consistent with our observations of the downregulation of VE-cadherin in the hepatic LTR-dHSC pool.

The above data allow us to formulate a working model of the development of LTR-dHSCs from the embryonic endothelial compartment: as LTR-dHSCs emerge from either the AGM region at E11.5 or the yolk sac at E12.5 they co-express the endothelial specific VE-cadherin and the pan-haematopoietic CD45, alluding to a common endothelial origin of LTR-dHSCs from intra- and extra-embryonic sources. As these nascent LTR-dHSCs migrate via the peripheral circulation they largely
Figure 3.5: Expression of VE-cadherin and CD45 on placental CD34<sup>+</sup>c-Kit<sup>+</sup> cells

(A) CD34 and c-Kit expression profile of E11.5 placenta

(B) CD34<sup>+</sup>c-Kit<sup>+</sup> cells from the E11.5 placenta were analysed for the expression of VE-cadherin and CD45. All CD34<sup>+</sup>c-Kit<sup>+</sup> cells co-express VE-cadherin and CD45.

(C) CD34 and c-Kit expression profile of E12.5 placenta.

(D) At E12.5 the placental CD34<sup>+</sup>c-Kit<sup>+</sup> cells continue to express CD45 but have markedly downregulated VE-cadherin expression

Contour plots are composed from approximately 1x10<sup>5</sup> viable cells. CD34<sup>+</sup>c-Kit<sup>+</sup> gates are based on previously described criteria (Gekas et al. 2005) and appropriate isotype control staining (Figure A4.3). Histograms are composed from approximately 2,000-4,000 gated cells.

All data are representative of three independent experiments.

Isotype control (dark line) and specific antibody staining (filled curve) is presented. Values represent the percentage of positive cells ± standard error of the mean.
Figure 3.6: Loss of VE-cadherin expression during LTR-dHSC development

As LTR-dHSCs emerge from intra-embryonic and extra-embryonic sites they co-express VE-cadherin and CD45. The expression of VE-cadherin is preserved during the migration via the peripheral circulation but is loss during the hepatic phase of LTR-dHSC expansion.
retain an endothelial identity which is progressively downregulated upon hepatic colonisation and is completely absent in the adult LTR-dHSC compartment (summarised in Figure 3.6).

3.2.5 VE-cadherin is downregulated during expansion of the LTR-dHSC pool

A limitation to the functional data described above is the inability to resolve whether downregulation of VE-cadherin expression within the LTR-dHSC pool during embryogenesis is dependent on direct interaction of LTR-dHSCs with the liver from the possibility that downregulation of expression would occur in any environment conducive to LTR-dHSC maintenance/expansion. To address this issue we employed the organ explant system which our laboratory has previously demonstrated to be conducive to the ex vivo expansion of LTR-dHSCs from the AGM region and the YS (Kumaravelu et al., 2002).

3.2.5.1 Ex vivo loss of VE-cadherin expression

3.2.5.1.1 E11.5 AGM region

The development of LTR-dHSCs in the AGM region was investigated by subjecting E11.5 AGM to explant culture at the gas-liquid interface for 72 hours. The cultured organ was subsequently purified into VE-cadherin⁺ and VE-cadherin⁻ fractions by flow cytometry and injected into irradiated adult mice. 7 out of 11 mice were repopulated with VE-cadherin⁺ cells with a 56% mean PBLC (range 5-85%), and 7 out of 9 mice were successfully repopulated with VE-cadherin⁻ cells with a 45% mean PBLC (range 5-82%) (Table 3.6). Thus, LTR-dHSCs of intra-embryonic
<table>
<thead>
<tr>
<th>Donor organ</th>
<th>Population transplanted</th>
<th>Embryo equivalent (e.e.) transplanted</th>
<th>Reconstituted/ transplanted</th>
<th>Mean % chimerism (range)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured E11.5 AGM region</td>
<td>VE-cadherin⁺</td>
<td>0.3.e.e.</td>
<td>7/11</td>
<td>56 (5-85)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁻</td>
<td>0.3-0.4.e.e.</td>
<td>7/9</td>
<td>45 (5-82)</td>
</tr>
<tr>
<td>Cultured E12.5 YS</td>
<td>VE-cadherin⁺</td>
<td>0.3-0.5.e.e.</td>
<td>7/10</td>
<td>40 (5-85)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁻</td>
<td>0.3-0.5.e.e.</td>
<td>10/10</td>
<td>55 (31-86)</td>
</tr>
<tr>
<td>E12.5 AGM region</td>
<td>CD45⁺</td>
<td>1.0-2.0.e.e.</td>
<td>6/11</td>
<td>69 (8-90)</td>
</tr>
<tr>
<td></td>
<td>CD45⁻</td>
<td>1.0-2.0.e.e.</td>
<td>0/10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁺</td>
<td>1.0-3.0.e.e.</td>
<td>8/17</td>
<td>61 (5-85)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin⁻</td>
<td>1.0-2.0.e.e.</td>
<td>6/15</td>
<td>61 (16-89)</td>
</tr>
</tbody>
</table>

*Chimerism observed in peripheral blood leucocytes by flow cytometry. Only recipients demonstrating at least 5% chimerism after at least 12 weeks were regarded as reconstituted.

Table 3.6: Downregulation of VE-cadherin occurs in LTR-dHSCs during ex vivo culture in the E11.5 AGM region and the E12.5 yolk sac, and in the E12.5 AGM region in situ.
origin were capable of downregulating VE-cadherin expression in the absence of the liver.

3.2.5.1.2 E12.5 yolk sac

We progressed to investigate the ability of the YS to downregulate VE-cadherin during explant culture. E12.5 YS was dissected free from the embryo proper and cultured under similar conditions for 72 hours. Cultured organs were then harvested and purified by flow cytometry into VE-cadherin\(^+\) and VE-cadherin\(^-\) fractions. VE-cadherin\(^+\) cells were capable of repopulating 7 out of 10 recipients with a 40% mean PBLC (range 5-85%) (Table 3.6). VE-cadherin\(^-\) cells were also capable of haematopoietic reconstitution with 10 out of 10 mice successfully repopulated, demonstrating a 55% mean PBLC (range 31-86%) (Table 3.6). As observed with the E11.5 AGM region, as LTR-dHSCs expand within the YS VE-cadherin expression is downregulated in the absence of hepatic influence.

3.2.5.3. In vivo loss of VE-cadherin expression

These ex vivo data are validated by the in vivo observation that the LTR-dHSCs that reside within the E12.5 AGM region are distributed between CD45\(^{SP}\) and DP populations. Following injection of purified E12.5 AGM region CD45\(^{+}\) cells into irradiated adults 6 out of 11 mice were successfully repopulated, demonstrating a 69% mean PBLC (range 8-90%) (Table 3.6). 0 out of 10 mice that received CD45\(^-\) cells were repopulated. Upon injection of VE-cadherin\(^+\) cells 8 out of 17 mice were successfully reconstituted with a 61% mean PBLC (range 5-85%) (Table 3.6). In contrast to the E11.5 AGM region, transplantation of the VE-cadherin\(^-\) fraction also
resulted in the successful repopulation of 6 out of 15 mice with a mean PBLC of 61% (range 16-89%). Thus by E12.5 the LTR-dHSC pool within the AGM region has developed from being exclusively within the DP fraction to being distributed between DP and CD45SP cells.

From these data we concluded that although in vivo VE-cadherin downregulation appears to occur as a consequence of LTR-dHSCs colonising secondary organs, such as the liver, this process can be successfully recapitulated in isolation from any external influence and is thus likely to be a consequence of developmental time. Therefore, following LTR-dHSC divergence from the endothelial compartment in the E11.5 AGM region and the E12.5 YS VE-cadherin expression may be downregulated following commitment to the haematopoietic lineage.

3.2.6 In vivo multilineage differentiation and proliferation of embryonic LTR-dHSCs

VE-cadherin expressing pre-LTR-dHSCs from the E9.5 YS have a restricted capacity for myeloid differentiation (Fraser et al., 2002). Therefore the extent of haematopoietic contribution derived from VE-cadherin expressing LTR-dHSCs from the E11.5 AGM, the E12.5 peripheral circulation and the E13.5 liver was assessed.

Detailed flow cytometric analysis for the presence of donor-derived CD43+B220+ pre/pro-B-lymphocytes, short-lived Mac-1+Gr-1+ granulocytes and CD4+CD8α+ thymic T-lymphocytes demonstrates the continued contribution of all VE-cadherin+ LTR-dHSCs to multilineage steady-state haematopoiesis of primary recipients 4-6 months post-transplantation (Figure 3.7A). Contribution of donor cells
Figure 3.7: Assessment of haematopoietic reconstitution by VE-cadherin expressing LTR-dHSCs

(A) High-level leucocyte chimerism could be detected in all major adult haematopoietic organs following the transplantation of VE-cadherin expressing LTR-dHSCs from the E11.5 AGM region, the E12.5 peripheral circulation, and the E13.5 liver. Note the contribution to short-lived granulocytes/monocytes (Mac-1^+Gr-1^+) and pro/pre-B-cells (B220^+CD43^+) within the bone marrow, and immature CD4^+CD8^+ thymic T-cells 12 weeks after transplantation.

(B) Successful haematopoietic reconstitution of secondary recipients demonstrates the long-term maintenance of transplanted stem cells.

Data presented are representative examples of one analysed mouse from each experimental group.
Each contour plot is composed from approximately 1x10^4 viable cells.
The percentage of cells in each gate/quadrant is indicated; gates and quadrants are based on appropriate isotype control staining (Figure A4.4).
A

Peripheral blood

Bone marrow

Spleen

Thymus

E11.5 AGM region
VE-cadherin'CD45'

0.7 42

50 23

19 1.5

11 84

E12.5 peripheral blood
VE-cadherin'

0.9 54

71 11

28 2.7

10 83

E13.5 liver
VE-cadherin'CD45'

0.3 34

47 25

14 1.3

4 70

Donor derived

Peripheral blood

B

Ly-5.1

CD43

Ly-5.2

Mae-1

B220

CD8α

Ly-5.1

B220

CD4

Ly-5.2
into the recipient LTR-dHSC compartment was confirmed by the successful haematopoietic reconstitution of secondary recipients following serial transplantation of primary recipient bone marrow (Figure 3.7B).

3.2.7 Divergence of haematopoietic populations from the endothelial compartment during circulatory migration and hepatic colonisation

Given the progressive loss of VE-cadherin expression from the LTR-dHSC compartment during developmental progress it was hypothesised that an accompanying loss of expression of endothelial proteins may also occur. As described for the E11.5 AGM region, the E12.5 peripheral circulation and the E13.5 liver was investigated for the co-localisation of endothelial, haematopoietic and HSC-associated proteins on VE-cadherin\textsuperscript{SP}, CD45\textsuperscript{SP}, DP and DN populations.

3.2.7.1 E12.5 peripheral circulation

3.2.7.1.1 Expression of endothelial markers

The expression of markers that distinguished the endothelial lineage from the haematopoietic lineages in the E11.5 AGM region provided less effective résolution in the E12.5 peripheral circulation, particularly with regard to the expression of PECAM-1, Flk-1 and Ac-LDL receptors.

\[ 66\pm9.0\% \text{ of the VE-cadherin}^{\text{SP}} \text{ population express Tie-2, a marked reduction in expression compared to that of immobilised endothelium.} \]

\[ 0.6\pm0.2\% \text{ of the CD45}^{\text{SP}} \text{ population and} \]

\[ 15\pm5.3\% \text{ of the DP population expressed Tie-2 (Table 3.7 and Figure 3.8A).} \]

\[ 9.7\pm4.9\% \text{ of the DN fraction were Tie-2}^+ \text{. Flk-1 expression was conspicuously absent from all of the fractions tested. Throughout circulation} \]
<table>
<thead>
<tr>
<th>Organ</th>
<th>Population</th>
<th>% antigen expression ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tie-2</td>
<td>Flk-1</td>
</tr>
<tr>
<td>E12.5 PB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VE-cadherin'CD45+</td>
<td>0.6±0.2</td>
<td>0±0</td>
</tr>
<tr>
<td>VE-cadherin'CD45-</td>
<td>66±9.0</td>
<td>0±0</td>
</tr>
<tr>
<td>VE-cadherin'CD45+</td>
<td>15±5.3</td>
<td>0±0</td>
</tr>
<tr>
<td>VE-cadherin'CD45-</td>
<td>9.7±4.9</td>
<td>0±0</td>
</tr>
<tr>
<td>E13.5 liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VE-cadherin'CD45+</td>
<td>14±1.2</td>
<td>1.0±1.0</td>
</tr>
<tr>
<td>VE-cadherin'CD45-</td>
<td>90±1.5</td>
<td>80±3.0</td>
</tr>
<tr>
<td>VE-cadherin'CD45+</td>
<td>69±2.0</td>
<td>75±2.5</td>
</tr>
<tr>
<td>VE-cadherin'CD45-</td>
<td>1.1±0.6</td>
<td>0.9±0.2</td>
</tr>
</tbody>
</table>

Table 3.7: Expression of endothelial and haematopoietic lineage proteins on mobilised and hepatic haematopoietic (VE-cadherin' CD45+), endothelial (VE-cadherin'CD45') and double-positive (VE-cadherin'CD45') populations
Figure 3.8: Co-expression of haematopoietic and endothelial lineage markers on endothelial, haematopoietic and double-positive cells in the peripheral circulation and the liver

(A) E12.5 peripheral circulation: circulating double-positive cells of E12.5 peripheral blood show attenuated expression of endothelial markers. Each histogram is composed from 80-110 DP, 1,000-4,000 endothelial or 8,000-13,000 haematopoietic cells.

(B) E13.5 liver: within the foetal liver some LTR-dHSCs remain associated with the DP fraction; the majority of HSCs reside within the haematopoietic fraction. This is reflected in the upregulation of essential HSC markers (Tie-2, c-Kit, Sca-1 and Mac-1) in the haematopoietic population.

Each histogram is composed from 1,000-4,000 DP, 8,000-20,000 endothelial or 30,000-70,000 haematopoietic cells.

All data are representative of 2-4 experiments for each marker analysed.

Isotype control (dark line) and specific antibody staining (filled curve) are presented. Values represent the percentage of positive cells.
PECAM-1 expression was most prevalent in the CD45-expressing populations: 27±16% of the VE-cadherin\(^{SP}\), 23±6.4% of the CD45\(^{SP}\), 76±3.1% of the DP and 5.3±2.7% of the DN populations expressed PECAM-1 (Table 3.7 and Figure 3.8A). Unlike in the E11.5 AGM region, the expression of PECAM-1 in the peripheral circulation was found at low intensity thus no distinction between the endothelial and haematopoietic lineages on the basis of quantitative differences was possible. Ac-LDLr were detected within all populations: 93±4.0% of VE-cadherin\(^{SP}\) cells, 89±8.5% of CD45\(^{SP}\) cells, 94±2.0% of DP cells and 64±6.5% of DN cells were positive for Ac-LDL uptake. As observed with the expression of PECAM-1, no quantitative distinction between the haematopoietic and endothelial lineages was possible on the basis of Ac-LDL uptake (Table 3.7 and Figure 3.8A).

3.2.7.1.2 Expression of haematopoietic markers

As observed in the E11.5 AGM region the expression of \(\alpha4\)-integrin was predominantly restricted to the CD45-expressing populations, however, CD41 expressing cohorts were detected in all populations. \(\alpha4\)-integrin was expressed on 0.5±0.5% of VE-cadherin\(^{SP}\) cells, 71±5.0% of CD45\(^{SP}\) cells, 75±3.5% of DP cells and 1.5±0.5% of DN cells (Table 3.7 and Figure 3.8A). CD41 is expressed on 20±6.5% of VE-cadherin\(^{SP}\) cells, 6.5±1.5% of CD45\(^{SP}\) cells, 14±6.5% of DP cells and 9.0±2.0% of DN cells. Although this pattern of expression is in agreement with previously observed reports of upregulation of CD41 expression during haematopoietic differentiation (see section 1.4.3) CD41 was also detected in the endothelial compartment.
3.2.7.1.3 Expression of HSC markers

Consistent with the AGM region, the expression of the myeloid/embryonic LTR-dHSC marker Mac-1 was restricted to the CD45-expressing populations and the pan-HSC markers c-Kit and Sca-1 were abundantly expressed on both the CD45 and the VE-cadherin expressing fractions. Mac-1 was expressed on 0.6±0.6% of VE-cadherin$^{SP}$ cells, 38±3.8% of CD45$^{SP}$ cells, 84±7.2% of DP cells and 0.1±0.1% of DN cells (Table 3.7 and Figure 3.8A). c-Kit is expressed on 78±9.0% of VE-cadherin$^{SP}$ cells, 52±6.5% of CD45$^{SP}$ cells, 48±15% of DP cells and 41±11% of DN cells. Sca-1 was expressed on 43±17% of VE-cadherin$^{SP}$, 17±1.8% of CD45$^{SP}$, 70±5.2% of DP cells and 5.2±4.4% of DN cells (Table 3.7 and Figure 3.8A).

In summary, during mobilisation through the peripheral circulation the expression of proteins that form the foundation of an endothelial identity is lost resulting in the endothelial compartment being most effectively resolved from the CD45-expressing populations according to the expression of high levels of Tie-2 and undetectable levels of α4-integrin and Mac-1 expression. During mobilisation the DP population appears closer in phenotype to the haematopoietic compartment than the endothelial lineage as accentuated by the minor expression of Tie-2 and abundant expression of α4-integrin and Mac-1. As observed in the AGM region the pan-HSC markers c-Kit and Sca-1 are highly enriched in the endothelial (VE-cadherin$^{SP}$) and the DP populations, however, an upregulation of c-Kit and Sca-1 expression becomes apparent within the CD45$^{SP}$ population.

3.2.7.2 E13.5 liver
3.2.7.2.1 Expression of endothelial markers

The VE-cadherin$^{SP}$ population of the E13.5 liver is phenotypically similar to that of the E11.5 AGM region. This similarity is characterised by a high frequency of Tie-2$^+$ cells, ubiquitous Flk-1 and PECAM-1 expression, and the ability to take up Ac-LDL. Distinct from both the AGM region and the peripheral circulation, expression of Ac-LDLr was predominantly restricted to the VE-cadherin$^{SP}$ and DP populations. Only minor Ac-LDL uptake was observed from the CD45$^{SP}$ population.

Tie-2 is expressed by 90±1.5% of VE-cadherin$^{SP}$ cells, 14±1.2% of CD45$^{SP}$ cells, 69±2.0% of DP cells and 1.1±0.6% of DN cells. Flk-1 expression was found to be largely restricted to the VE-cadherin$^{SP}$ and DP populations: 80±3.0% of VE-cadherin$^{SP}$ cells, 1.0±1.0% of CD45$^{SP}$, 75±2.5% of DP cells and 0.9±0.2% of DN cells express Flk-1 (Table 3.7 and Figure 3.813).

In contrast to the peripheral circulation, in the liver PECAM-1 was again detected within the VE-cadherin$^{SP}$, CD45$^{SP}$ and DP populations. PECAM-1 expression was found within 97±0.6% of VE-cadherin$^{SP}$ cells, 83±1.0% of CD45$^{SP}$ cells, 93±3.2% of DP cells and 1.0±0% of DN cells (Table 3.7 and Figure 3.813). As observed in the AGM region, the DP population expressed a bimodal distribution of PECAM-1 expression, however, in the liver the expression was biased towards a haematopoietic-like PECAM-1$^{low}$ profile.

We found that in the liver the boundaries between endothelial and haematopoietic lineage identity becomes more clearly defined as characterised by the restriction of Ac-LDLr expression to VE-cadherin expressing populations: 92±2.8% of VE-cadherin$^{SP}$ cells, 5.0±1.0% of CD45$^{SP}$ cells, 59±9.1% of DP cells (predominately Ac-LDLr$^{low}$) and 1.9±0.2% of DN cells were capable of taking up
Ac-LDL (Table 3.7 and Figure 3.8B). The pattern of Ac-LDL uptake indicates that by E13.5 the DP population has developed characteristics more consistent with the haematopoietic compartment.

3.2.7.2.2 Expression of haematopoietic markers

α4-integrin is expressed on the majority of cells within the E13.5 foetal liver: 37±6.0% of VE-cadherin$^{SP}$ cells, 98±1% of CD45$^{SP}$ cells, 96±2.5% of DP cells and 87±3.0% of DN cells (Table 3.7 and Figure 3.8B). With regard to the expression of α4-integrin, all the cells of the endothelial lineage exhibited a slight positive shift but cells from the CD45$^{SP}$ and DP population expressed α4-integrin at high level.

At E13.5 CD41 expression is readily detected in the endothelial and the haematopoietic lineages: 30±6.5% of VE-cadherin$^{SP}$ cells, 13±4.0% of CD45$^{SP}$ cells, 57±4.0% of DP cells and 0.9±0.2% of DN cells were found to express CD41 (Table 3.7 and Figure 3.8B).

Consistent with observations in the AGM region and the peripheral circulation the expression of Mac-1 is restricted to CD45 expressing populations and is particularly enriched within the VE-cadherin$^{+}$CD45$^{+}$ population: 0.1±0.1% of VE-cadherin$^{SP}$ cells, 80±4.5% of CD45$^{SP}$ cells, 93±2.0% of DP cells and 0±0% of DN cells express Mac-1 (Table 3.7 and Figure 3.8B).

3.2.7.2.3 Expression of HSC markers

In common with the E11.5 AGM region and the E12.5 peripheral circulation, the expression of the pan-HSC markers Sca-1 and c-Kit was strongly represented in the endothelial, haematopoietic and DP populations. Sca-1 is expressed in 59±3.0%
of VE-cadherin\textsuperscript{SP} cells, 25±6.0\% of CD45\textsuperscript{SP} cells, 57±3.0\% of DP cells and 0.3±0.3\% of DN cells (Table 3.7 and Figure 3.8B). c-Kit is detected in 33±6.0\% of VE-cadherin\textsuperscript{SP} cells, 23±5.5\% of CD45\textsuperscript{SP} cells, 57±8.0\% of DP cells and 5.5±2.5\% of DN cells. As would be predicted by data from the above \textit{in vivo} repopulation studies (that both DP and CD45\textsuperscript{SP} populations contain LTR-dHSCs) the DP population is highly enriched for markers known to be expressed in the hepatic pool of LTR-dHSCs notably high levels of Tie-2, PECAM-1, Mac-1, Sca-1 and c-Kit (Baumann et al., 2004; de Bruijn et al., 2002; Morrison et al., 1995; North et al., 2002; Sanchez et al., 1996; Yano et al., 1997). Also consistent with our \textit{in vivo} data is the expression of these LTR-dHSC markers on CD45\textsuperscript{SP} cells.

From these observations it is clear that within all organs DP cells retain a promiscuous endothelio-haematopoietic immunophenotype which is subsequently lost upon maturation to a conventional haematopoietic identity.

### 3.2.8 Divergence of the VE-cadherin\textsuperscript{+}CD45\textsuperscript{+} and VE-cadherin\textsuperscript{+}CD45\textsuperscript{−} populations within the E11.5 AGM region

The functional data described above in conjunction with arguments presented in section 1.3.9 supports the hypothesis that as LTR-dHSC emergence is initiated within intra-embryonic and extra-embryonic organs LTR-dHSCs diverge from the endothelial compartment. This hypothesis is most strongly supported by the ability of DP but not VE-cadherin\textsuperscript{SP} cells to reconstitute the haematopoietic system of adult mice. Further evidence of a possible endothelial heritage of DP cells is the expression of endothelial-like levels of Tie-2, Flk-1, PECAM-1 and Ac-LDLr. It is therefore possible that the events that result in the commitment of DP cells, or their
immediate precursors, to the haematopoietic lineage occurs at the site of origin as evidenced by the *ex vivo* recapitulation of VE-cadherin downregulation in the absence of potential influences from the hepatic environment.

### 3.2.8.1 Morphological divergence

Investigation into the morphological specification of the VE-cadherin*<sup>SP</sup>*, CD45*<sup>SP</sup>* and DP populations may provide further support for the idea that even though DP cells retain endothelial characteristics clear lineage specification has been initiated.

Evidence of morphological resolution between VE-cadherin*<sup>SP</sup>* and DP cells is observed in May-Grunwald and Giemsa stained cytospin preparations from cells purified by flow cytometry (Figure 3.9A-C). The DP population was observed to contain a high frequency of cells with blast/stem cell morphology, characterised by round cells with a high nuclear-to-cytoplasmic ratio (Figure 3.9A). Rare blast-like cells were identified within the CD45*<sup>SP</sup>* population, however, the preparation was predominated by cells with a more mature appearance as characterised by cells with large, often vacuolated, cytoplasm and small nuclei (Figure 3.9B). The VE-cadherin*<sup>SP</sup>* fraction was characterised by cells with large patchy stained nuclei and blebbing cytoplasm; the cells that characterise the CD45*<sup>SP</sup>* and DP populations (blast-like cells and myeloid cells) were not identified within the VE-cadherin*<sup>SP</sup>* population (Figure 3.9C).

### 3.2.8.2 *In vitro* function divergence
Figure 3.9: Comparative morphology of double-positive, haematopoietic and endothelial populations from the E11.5 AGM region

E11.5 AGM region was purified into VE-cadherin^CD45^ (A), VE-cadherin^CD45^ (B) and VE-cadherin^CD45^ (C) populations by flow cytometry. Cytospin preparation were made and cells were subsequently visualised with May-Grunwald-Giemsa stains. Morphological comparison of the populations reveals that cells with a haematopoietic blast-like morphology are highly enriched within the VE-cadherin^CD45^ population.

Original magnification x600.
Images are representative of two independent experiments.
To test the hypothesis that lineage commitment occurs at the site of origin, the E11.5 AGM region was purified into VE-cadherin$^{SP}$, CD45$^{SP}$, DP and DN populations and introduced into well characterised in vitro systems for the induction of haematopoietic and endothelial differentiation.

3.2.8.2.1 Myeloid CFU-C assay

In vitro clonogenic myeloid progenitor activity, as assessed by the culture of cells in methylcellulose-based medium containing a cocktail of haematopoietic growth factors (M3434), was most highly enriched within the DP population: 18±5.7 CFU-Cs were identified from 500 input cells (CFU-C frequency 1/28); 1.3±1.0 CFU-Cs were produced from 500 CD45$^{SP}$ cells (CFU-C frequency 1/384); 0 CFU-Cs from 10,000 VE-cadherin$^{SP}$ cells. DN cell derived colonies were infrequently observed: 0.3±0.6 CFU-Cs from 20,000 cells (Figure 3.10A). The vast majority of colonies produced from the DP population were characterised by either a tight (Figure 3.10B) or diffuse (Figure 3.10C) appearance.

3.2.8.2.2 Endothelial network forming assay

Assessment of endothelial differentiation/network forming capacity revealed the absence of in vitro lineage plasticity: DP and CD45$^{SP}$ cells were not capable of producing PECAM-1$^+$ tubules when co-cultured with OP-9 stromal cells in the presence of VEGF (Figure 3.10D). 3.5±0.7 and 67±9.9 tubules were produced from the co-culture of 500 and 5,000 VE-cadherin$^{SP}$ cells, respectively (Figures 3.10D-F). Extensive network formation was observed from 20,000 VE-cadherin$^{SP}$ cells (Figure 3.10G). 50,000 DN cells were able to form 1.5±0.7 PECAM-1$^+$ tubules (Figure
Figure 3.10: *In vitro* endothelial and haematopoietic differentiation potential of populations from the E11.5 AGM region

(A) The frequency of clonogenic myeloid progenitors is 14-fold more enriched within the DP fraction compared to the CD45<sup>SP</sup> population.

DP CFU-Cs formed either tight (B) or diffuse (C) haematopoietic colonies after 8 days of differentiation.

(D) *In vitro* endothelial differentiation potential was assessed by the ability of purified populations to form endothelial tubules or networks following co-culture on OP-9 stromal cells in the presence of VEGF.

(E) After 4 days endothelial tubules were produced from 500 VE-cadherin<sup>SP</sup> cells. Blue staining marks the expression of PECAM-1.

(F) Tubule formation following culture of 5,000 VE-cadherin<sup>SP</sup> cells.

(G) Extensive network formation generated from the culture of 20,000 VE-cadherin<sup>SP</sup> cells

Original magnification of all images x40. All data were collected from a minimum of 3 independent experiments. Where visible, bars indicate standard error of the mean.
3.10D). Thus, *in vitro* functional endothelial properties are largely restricted to the VE-cadherin\(^{SP}\) population.

These data demonstrate that although DP cells from the E11.5 AGM region are promiscuous by immunophenotype they have undergone clear morphological and functional divergence.

### 3.3 Discussion

As expected, the resolution of endothelial and haematopoietic lineages in the AGM region, the YS, the liver and the peripheral circulation is achieved by the mutually exclusive plasma membrane expression of VE-cadherin and CD45. These populations are further characterised by the qualitative and quantitative expression of other lineage associated proteins: the high-level expression of Tie-2, PECAM-1 and Ac-LDL receptors and the low-level of Flk-1 expression is characteristic of the endothelial compartment (VE-cadherin\(^{+}\)CD45\(^{+}\)); the ubiquitous expression of \(\alpha 4\)-integrin and Mac-1 accompanied by a low-level of PECAM-1 expression is characteristic of the haematopoietic compartment (VE-cadherin\(^{-}\)CD45\(^{+}\)).

The expression of CD41 has been presented as a marker preceding CD45 in cells committed to the haematopoietic lineage and as a marker of the onset of definitive haematopoiesis (see section 1.4.2). The data presented in this chapter demonstrates that the expression of CD41 is more highly enriched within the DP fraction; however, it is consistently detected in both the VE-cadherin\(^{SP}\) and CD45\(^{SP}\) populations. Of note is that unlike in the E13.5 liver CD41 is only detected at low
intensity and frequency in the E11.5 AGM region and the E12.5 peripheral circulation.

Within all major haematopoietic organs during the development of the LTR-dHSC pool rare populations of cells that co-express VE-cadherin and CD45 can be detected: VE-cadherin\(^+\)CD45\(^+\) (double positive/DP) cells comprise approximately 0.05±0.01% of the E11.5 AGM region, 0.03±0.008% of the E12.5 YS, 0.005±0.0013% of the peripheral circulation and 0.1±0.038% of the E13.5 liver. The co-expression of these largely mutually exclusive proteins suggests that double positive (DP) cells represent a putative intermediate during the divergence of haematopoietic cells from the endothelial lineage. This supposition is supported by the qualitative and quantitative expression of endothelial and haematopoietic lineage markers. For example, DP cells from the E11.5 AGM region express endothelial-like levels of Tie-2, Flk-1 and Ac-LDL receptors while simultaneously bearing α4-integrin and Mac-1. This phenotypic duality is also observed in DP cells from the E12.5 peripheral circulation and the E13.5 liver. Of note is that in addition to CD45 the expression of Mac-1 best delineates the endothelial, double-positive and haematopoietic populations of all the organs analysed in this study.

It was reasoned that if LTR-dHSCs arise from the endothelial compartment, from haematogenic endothelium or from the hypothesised haemangioblast, then the first LTR-dHSC to emerge is likely to bear the phenotype of both the endothelial and haematopoietic lineages. Supportive evidence is provided by the expression of the HSC-associated markers CD34, c-Kit, Sca-1 and AA4.1 on the E11.5 AGM region DP population. To functionally test this hypothesis VE-cadherin and CD45 expressing cells from major haematopoietic organs involved in LTR-dHSC
development were purified and subjected to the gold standard for dHSC detection, the *in vivo* competitive long-term repopulation assay.

Results from these experiments revealed that as LTR-dHSC expansion is initiated from intra-embryonic and extra-embryonic organs, the E11.5 AGM region and the E12.5 YS, all high-level adult repopulating capacity is restricted to cells co-expressing VE-cadherin and CD45. The restriction of E11.5 AGM region LTR-dHSCs to VE-cadherin$^+$CD45$^+$ cells is consistent with a previous report (North et al., 2002). The DP immunophenotype of LTR-dHSCs is preserved as mobilisation is facilitated via the E12.5 peripheral circulation. Marked downregulation of VE-cadherin expression does not occur in the LTR-dHSC pool until hepatic colonisation.

The addition of a period of *ex vivo* explant prior to transplantation, thus eliminating the influence of secondary organs and preventing LTR-dHSC emigration, revealed that VE-cadherin downregulation by LTR-dHSCs is not dependent on colonisation of a hepatic niche but occurs as a consequence of developmental time. These findings suggest that as LTR-dHSCs emerge they bear the hallmarks of both the endothelial and the haematopoietic lineages, implicating a recent endothelial ancestry. It must be stated that it is not possible to rule out the possibility that the downregulation of VE-cadherin in the LTR-dHSC pool occurs as a result of LTR-dHSC emergence along a non-endothelial pathway and not as a result of the loss of expression at the level of an individual cell.

Flow cytometric analysis of the E11.5-12.5 placental LTR-dHSC population suggests that a significant loss of VE-cadherin expression occurs as the stem cell pool is expanding. The role of the placenta during LTR-dHSC development remains unclear; the absence of evidence to support the capacity of the placenta to
autonomously expand LTR-dHSCs (H. Mikkola, personal communication; Ottersbach and Dzierzak, 2005) suggests that the placenta may act as niche for LTR-
dHSC expansion as the hepatic environment is developing, the loss of VE-cadherin
eexpression described above may support this hypothesis.

With regard to the three models of stem cell emergence summarised by
Godin and Cumano (2002), sub-aortic patch, haematogenic endothelium and the
haemangioblast, our data supports the idea that all nascent LTR-dHSCs emerge
through a common endothelial pathway. However, it is not possible to distinguish
between haematogenic endothelial cells and haemangioblasts as the ancestor of LTR-
dHSCs. In the absence of data describing the spatial localisation of early LTR-
dHSCs it remains unclear where these cells reside in situ. Moreover, these data do
not preclude the possibility that SAPs contribute to the early formation of LTR-
dHSCs, as proposed by Bertrand et al (2005).

Two other groups have reported findings contradicting our observation of a
mixed immunophenotype of hepatic LTR-dHSCs: North et al (2002) observed that
nearly all LTR-dHSC had downregulated VE-cadherin expression by the time the
liver was colonised at E12.5; Kim et al (2005) observed that all E13.5 hepatic LTR-
HSCs express VE-cadherin at E13.5 with downregulation not occurring until E16.5.
It is not clear from the data provided in the former study how the gating strategy was
employed during cell sorting; if VE-cadherin expression is low in the hepatic LTR-
dHSC pool the application of a conservative sort gates would prevent these cells
from being collected. The embryos used in the latter study were of a different mouse
strain, the temporal and spatial development of LTR-dHSC in the model used has not
been stringently characterised. It is therefore possible that a minor delay in the
developmental progress of the LTR-dHSC pool between the mouse strain used in this chapter (C57BL6/CBA) and the mouse strain used by Kim et al (2005) (C57BL6/6:Ka) could account for the differences observed.
4.1 Introduction

The *ex vivo* capacity for the E11.5 AGM region to initiate LTR-dHSC emergence and expansion as an intact organ is well documented (section 1.3.2-1.3.3) as is the capacity of AGM derived cell lines to support the maintenance of adult bone marrow LTR-dHSCs (section 1.3.4.). These observations suggest that the cells of E11.5 AGM region possess a valuable *in vitro* capacity which if efficiently harnessed could allow the elucidation of methods of LTR-dHSC generation *in vitro* from non-stem cell populations and possibly facilitate the *ex vivo* expansion of non-AGM region LTR-dHSCs.

Nothing is known of the roles of defined primary AGM region populations during *ex vivo* haematopoietic proliferation and differentiation; such information would prove valuable to exploit the potential of this organ. Therefore the behaviour of cells from the E11.5 AGM region was investigated within a number of different *in vitro* systems.

4.2 Results

4.2.1 Lineage restricted *in vitro* differentiation of purified E11.5 AGM region cells

4.2.1.1 Distribution of CFU-Cs
In section 3.2.8.2.11 demonstrated that the VE-cadherin^CD45^ (DP) fraction of the E11.5 AGM region has the greatest enrichment of myeloid CFU-Cs. However, significant contribution to haematopoiesis in vivo may not derive from the population with the greatest frequency of progenitors but the population which contains the greatest total number of CFU-Cs. Therefore, the absolute number of CFU-Cs present in 1.0 embryo equivalent (e.e.) of E11.5 AGM was investigated.

Quantitative analysis of colonies produced from 1.0 e.e. of purified DP, VE-cadherin^CD45^ (CD45^SP^), VE-cadherin^CD45^- (VE-cadherin^SP^) and VE-cadherin^- CD45^- (DN) populations from the E11.5 AGM region revealed that only 3.5±0.5 CFU-Cs are present in 1.0 e.e. DP cells (approximately 70 cells); 35±1.0 CFU-Cs are present in 1.0 e.e. CD45^SP^ cells (approximately 3,640 cells), 0.5±0.5 CFU-Cs are found in 1.0 e.e. VE-cadherin^SP^ cells (approximately 3,500 cells) and 7.5±0.5 CFU-Cs in 1.0 e.e. DN cells (approximately 133,000 cells) (Figure 4.1A).

Therefore, although the greatest frequency of CFU-Cs in the E11.5 AGM region is found within the DP fraction these data demonstrate that the greatest absolute number of in vitro progenitors is located within the CD45^SP^ population, which contain no LTR-dHSC activity. It is tempting to interpret these data as demonstrating the loss of VE-cadherin expression following the differentiation of LTR-dHSCs; however, it is possible that CD45^SP^ progenitors belong to a separate haematopoietic hierarchy.

Consistent with previous experiments no appreciable haematopoietic activity was detected within the endothelial lineage. Haematopoietic activity was identified within the DN fraction, however, given the large number of cells a possibility of contamination with DP and/or CD45^SP^ cells can not be ruled out.
Figure 4.1: Quantitative CFU-C disparity following purification

(A) 1.0e.e. of purified E11.5 AGM region VE-cadherin⁺CD45⁺ (DP), VE-cadherin⁻ CD45⁺ (CD45⁺), VE-cadherin⁺CD45⁻ (VEcad⁺), VE-cadherin⁻CD45⁻ (DN) cells was cultured in methylcellulose based medium. Colonies were counted after 10 days of culture.

(B) Numbers of CFU-Cs from 1.0e.e. E11.5 AGM region from either unprocessed sample (whole AGM), sample that had passed through a flow cytometer but not sorted (FC control), sample that had undergone lineage purification and subsequent proportional recombination (sort control), and purified populations cultured independently thus not interacting (purified fractions).

No significant difference was observed between whole AGM and the FC control or between the FC control or the sort control. A significant difference was observed between the sort control and the purified fractions (P<0.02).

Bars indicate standard error of the mean of three independent experiments.
4.2.1.2 Efficient CFU-C detection requires the presence of heterogeneous populations

To investigate the possibility that the differentiation capacity of haematopoietic progenitors within the E11.5 AGM is sensitive to the purification process a series of experiments were undertaken to compare the number of CFU-Cs identified from 1.0e.e. of sample from either: 1. Whole AGM without further processing; 2. AGM passed through a flow-cytometer without purification; 3. AGM populations sorted on the basis of VE-cadherin and CD45 expression and subsequently proportionally recombined (as described in section 4.2.1.1); 4. AGM populations sorted and maintained as four distinct populations (as described in section 4.2.1.1)

As illustrated in Figure 4.1B unprocessed AGM was found to contain 109±7.0 CFU-Cs. After processing through a flow cytometer 100±5.0 CFU-Cs were detected. Following recombination of purified populations 95±16 CFU-Cs were detected and 47±2.5 CFU-Cs were cumulatively detected from purified populations cultured independently of each other, representing a significant reduction in CFU-C number (P<0.01). These data suggest that the disparity in CFU-C number does not result from an experimental artefact but is likely to result from the inability of purified stem/progenitor cells to efficiently differentiate.

4.2.1.3 Mast cell restriction of purified AGM region derived stem/progenitor cells
In addition to reduced numbers, qualitative differences between the colonies produced from heterogeneous E11.5 AGM cell suspensions and purified cell populations were also noted.

Unsorted cell suspension prepared from the E11.5 AGM region was able to differentiate along erythroid and leucocytic pathways, as evidenced by the presence of CD45^Ter119^+ and CD45^Ter119^- cells, and the robust production of Mac-1^low^Gr-1^high^ neutrophils, Mac-1^high^Gr-1^low^ macrophages, c-Kit^high^Sca-1^high^/low^CD41^low^ mast cells and c-Kit^Sca-1^CD41^high^ megakaryocytes (Figure 4.2A). Detection of multilineage differentiation was also achieved by morphological investigation. The correlation of immunophenotype and lineage identity is described in Appendix 2.

In contrast, sorted DP and CD45^SP^ populations from the E11.5 AGM region were restricted to oligopotent differentiation as evidenced by the absence of erythroid cells, neutrophils, macrophages and megakaryocytes with the vast majority of cells bearing a c-Kit^high^Sca-1^high^/low^CD41^low^ immunophenotype and typical mast cell morphology (Figure 4.2B-C). Rare Mac-1^+^Gr-1^low^/neg^ cells were occasionally detected.

These observations demonstrate that cellular interaction within the E11.5 AGM region is required for multilineage differentiation. Thus, I attempted to develop an \textit{in vitro} differentiation system which would allow:

1. The analysis of the requirements for multilineage differentiation.
2. The expansion of LTR-dHSCs and CFU-Cs from phenotypically defined populations.
Figure 4.2: Lineage restriction of purified CFU-Cs

The ability of 1.0e.e. whole E11.5 AGM region (A), VE-cadherin^+CD45^+ (B) or VE-cadherin^+CD45^+ (C) cells to undergo myeloid multilineage differentiation was assessed by flow cytometry and morphological analysis after 12 days of differentiation in methylcellulose culture.

Details of lineage identification by flow cytometry are presented in Appendix 2. Neither of the purified fractions was capable of reproducing the multilineage differentiation observed from the whole AGM region; only evidence of mast cells differentiation was found, as evidenced by the co-expression of Sca-1 and c-Kit on all cells and the presentation of a heavily granulated cytoplasm.

Data presented are representative of three independent experiments. Original magnification of photomicrographs x600.
4.2.2 Liquid suspension culture

In initial experiments a highly enriched medium containing stem cell factor (SCF), Interleukin (IL)-3, IL-6, IL-7, IL-11, granulocyte-macrophage colony stimulating factor (GM-CSF) and Flt3-ligand (Flt3L) was used to culture purified E11.5 AGM population in 96-well U-bottom plates. In these conditions cells gather at the centre of the well in close contact with each other. It was hypothesised that close cellular interaction, that might be absent when low numbers of cells are cultured in methylcellulose, is necessary for multilineage differentiation.

4.2.2.1 Determining the frequency of liquid suspension colony forming units

To investigate the differentiation potential of individual progenitor cells/units I first determined the frequency of cells required for the initiation of a colony under liquid suspension culture (LSC) conditions. This was achieved by determining the number of CD45SP, VE-cadherinSP, DP and DN cells from the E11.5 AGM region required to initiate a colony using a limiting dilution approach.

Purified CD45SP, VE-cadherinSP, DP and DN cells were transferred to non-tissue culture treated U-bottom 96-wells containing growth-factor supplemented medium at a range of 1 to 5,000 cells per well. Wells were scored for the presence or absence of haematopoietic colony formation between 10-15 days of culture (Figure 4.3A). The number of cells per well resulting in only 63% of wells containing a colony was considered as containing 1 liquid suspension colony forming unit (LS-CFU), according to Poisson’s statistics.
Figure 4.3: Liquid suspension colony-initiating units are highly enriched within E11.5 AGM region VE-cadherin^CD45^ and VE-cadherin^CD45^ populations

(A) Schematic representation of the liquid suspension culture technique: cells fractions were purified from the E11.5 AGM region on the basis of VE-cadherin and CD45 expression and transferred to 96 well U-bottom plates in the presence of growth factor supplemented medium.

To calculate the number of cells required to initiate a single colony purified populations were plated at a range of 1-5,000 cells per well. After 10-15 days of culture the number of wells positive and the number negative for colony formation was scored. The minimum number of cells required to produce colonies in 67% of wells is equal to 1 liquid suspension colony forming unit (LS-CFU) (indicated by the dashed lines), according to Poisson’s statistics. LS-CFUs were present in the VE-cadherin^CD45^ population at a frequency of 1 in 25 cells (B) and 1 in 232 cells from the VE-cadherin^CD45^ population (C). Very rare LS-CFU potential was observed from cells of the VE-cadherin^CD45^- (D) and the VE-cadherin^CD45^- (E) populations; the possibility that this activity results from contamination with other cell types can not be ruled out.

Where visible, bars indicate standard error of the mean of three independent experiments.
A E11.5 AGM region

B VE-cadherin^CD45^ Cell number per well

C VE-cadherin^CD45^ Cell number per well

D VE-cadherin^CD45^- Cell number per well

E VE-cadherin^CD45^- Cell number per well

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DP cells were assessed at 1, 10, 20 and 30 sorted cells per well. As a result 1 LS-CFU was estimated per 25 input cells (Figure 4.3B), which is comparable to the frequency of CFU-Cs as determined by methylcellulose culture (1 in 28 cells) (section 3.2.8.2.1). CD45SP cells were assessed at 1, 100 and 300 cells per well. From this analysis 1 LS-CFU was estimated per 215 input cells (Figure 4.3C), which is slightly more enriched than the frequency of CFU-Cs detected using methylcellulose culture (1 in 385 cells). Rare positive wells were identified following the culture of 100-1,000 VE-cadherinSP cells (Figure 4.3D) and following the culture of 1,000-5,000 DN cells (Figure 4.3E). These data suggest that the LSC system matches, and possibly exceeds, the sensitivity of the methylcellulose culture.

4.2.2.2 Classifying LS-CFU derived colonies

To establish criteria for a comparison of the differentiation potential of DP and CD45SP LS-CFUs the gross morphology, immunophenotype and cellular morphology of colonies produced under conditions of limiting dilution was assessed. Between the DP and CD45SP populations, three types of colonies were identified after 7 days of differentiation:

1. Colony type (CT)-1 is characterised by a discrete circular gross morphology (Figure 4.4A) and is composed almost entirely of mast cells (Figure 4.4D, H).

2. CT2 lacks a discrete boundary and contains multiple cell clusters (Figure 4.4B). CT2 derived from the CD45SP population is almost entirely composed of mast cells (Figure 4.4E, H) whereas CT2 derived from DP also cells contains large numbers of granulocytes,
Figure 4.4: Characterisation of LS-CFUs from E11.5 AGM region VE-cadherin\textsuperscript{CD45\textsuperscript{+}} and VE-cadherin\textsuperscript{CD45\textsuperscript{+}} populations

At limiting dilution three colony types (CT) are identified following the differentiation of VE-cadherin\textsuperscript{CD45\textsuperscript{+}} (DP) and VE-cadherin\textsuperscript{CD45\textsuperscript{+}} (CD45\textsuperscript{SP}) cells: colony type (CT) 1 (A), CT2 (B) and CT3 (C) can be distinguished according to gross morphology following 8 days of culture. Original magnification x10. Images are representative of a minimum of four independent experiments.

Shown are the morphology of cells from CD45\textsuperscript{SP} derived CT1 (D), CD45\textsuperscript{SP} CT2 (E), DP CT2 (F) and DP CT3 (G) following May-Grunwald-Giemsa staining of cytospin preparations. Original magnification x600.

(H) Analysis of lineage composition of CTs derived from CD45\textsuperscript{SP} and DP LS-CFUs by flow cytometry demonstrates the marked difference in the differentiation potential of DP and CD45\textsuperscript{SP} cells. Data are representative of a minimum of three independent experiments. Criteria for lineage identification are presented in Appendix 2.
monocytes/macrophages, megakaryocytes and some erythroid cells (Figure 4.4F, H).

3. CT3 contains relatively few cells which are sparsely distributed (Figure 4.4C) and are almost entirely of macrophage lineage (Figure 4.4G, H).

4.2.2.3 Differentiation potential of purified LS-CFUs

Both the CD45SP and DP populations are capable of producing CT1 and CT2 but the production of CT3 is a unique capacity of the DP population. CD45SP cells produced 72%±8.0 CT1 and 28%±8.0 CT2 (Figure 4.4I). In contrast, the DP population produced 9.7%±5.7 CT1; 45%±2 CT2 and 45%±2 CT3 (Figure 4.5A). Thus, as expected, in this differentiation system the LTR-dHSC-enriched DP population showed a more potent differentiation potential than the CD45SP population.

4.2.2.4 Redirected differentiation of VE-cadherinCD45 LS-CFUs following endothelial interaction

Given the in vivo association of LTR-dHSCs and CFU-Cs with the endothelial lineage (section 3.2.8.2) and the previously documented capacity of primary endothelial cells to support haematopoiesis (section 1.3.4) the effect of co-culture of purified VE-cadherinSP cells on the differentiation of DP and CD45SP LS-CFUs was investigated.

Accordingly, DP and CD45SP cells were cultured at limiting dilution with 300 sorted VE-cadherinSP cells in the LSC system. CD45SP cells cultured with VE-
Figure 4.5: Enhanced LS-CFU differentiation following endothelial interaction

Co-culture of limiting dilution doses DP and CD45$^{SP}$ cells with 300 endothelial (VE-cadherin$^{+}$CD45$^{+}$) cells results in significant changes to CT formation:

(A) Distribution of CTs from CD45$^{SP}$ and DP cells. In the CD45$^{SP}$ fraction most LS-CFUs produced CT1 ($P<0.02$). Very few DP LS-CFUs formed CT1 ($P<0.02$); the remaining pool of DP LS-CFUs were evenly distributed between those capable of CT2 and CT3 formation.

(B) Distribution of CT formation from CD45$^{SP}$ and DP LS-CFUs in the presence of endothelium: CD45$^{SP}$ cells are predominantly directed towards CT2 formation ($P<0.02$) as are the vast majority of DP cells ($P<0.03$). Bars indicate standard error of the mean of three independent experiments.

(C) Flow cytometric analysis of CT2 lineage composition from CD45$^{SP}$ and DP LS-CFUs confirm that greater lineage heterogeneity is achieved by DP cells in the presence of endothelium. Criteria for lineage identification are described in Appendix 2.

Colony types were scored and images were taken following 10 days of differentiation. Data are representative of two independent experiments.
cadherin<sup>SP</sup> cells produced a greater proportion of CT2 but were still not capable of generating CT3 (31±1.0% CT1 and 69±1.0% CT2). Co-culture of DP and VE-cadherin<sup>SP</sup> cells resulted in an almost exclusive production of CT2 (3.0±3.0% CT1, 94±6.0% CT2 and 3.0±3.0% CT3) (Figure 4.5B). Moreover, unlike the CT2 produced from a pure population of DP cells, CT2 were now characterised by the presence of neutrophils, megakaryocytes, fewer mast cells and greater proportions of monocytes/macrophages and erythroid cells (Figure 4.5C). Of note, is that although the proportion of CT2 derived from CD45<sup>SP</sup> cells was significantly increased in the presence of endothelial cells (P<0.01) multilineage differentiation was not enhanced (Figure 4.5C).

In conclusion, interaction of DP cells with the E11.5 AGM endothelial compartment ensures successful multilineage differentiation which otherwise is restricted to mast cell or macrophage fate.

**4.2.2.4 LS-CFUs are restricted to the maintenance and expansion of CFU-Mast**

Colonies produced in LSC were further tested for their ability to maintain and expand CFU-Cs. Primary LSC colonies were harvested after 8 days and transferred to methylcellulose culture to assess CFU-C content.

This experiment was initially performed using CT2 derived from pure populations of either DP or CD45<sup>SP</sup> cells. Following 10 days of differentiation in the secondary system colonies were harvested and the lineages present were identified by flow cytometry. Although CFU-Cs were abundant in both CD45<sup>SP</sup> and DP CT2 they gave rise exclusively to mast cells (Figure 4.6A and B).
Figure 4.6: Liquid suspension culture facilitates the maintenance of oligopotent CFU-Cs

After 10 days of differentiation in LSC colonies were harvested and transferred to methylcellulose based medium. Representative images are shown of methylcellulose colonies derived from: (A) DP derived CT2, (B) CD45$\text{SP}^\text{a}$ derived CT2, (C) DP/VE-cadherin$\text{SP}^\text{b}$ co-culture derived CT2 and (D) CD45$\text{SP}^\text{c}$/VE-cadherin$\text{SP}^\text{d}$ co-culture derived CT2. Original magnification x20.

(E) Flow cytometric analysis confirms that CFU-Cs have only produced mast cells, as evidenced by the absence of erythroid, macrophage, neutrophil and megakaryocyte lineages.

Images were taken and flow cytometric analysis was performed from colonies after 12 days of differentiation in methylcellulose culture.

All data are representative of two independent experiments. Quadrants are based on appropriate isotype control staining (Figure A4.5). Values indicate the percentage of positive cells.
Figure 4.7: Adult repopulating capacity of LS-CFU derived colonies

Five pooled CT2 colonies derived from CD45SP, DP, CD45SP/VE-cadherinSP or DP/VE-cadherinSP cells after 4 days of LSC was injected into irradiated mice. No evidence of peripheral blood leucocyte chimerism was observed 12 weeks post-injection.

Data are cumulative of two independent experiments. Each point represents an individual mouse. CT denotes colony type.
Given that the presence of VE-cadherin<sup>SP</sup> cells could facilitate multilineage differentiation of LS-CFUs in a primary LSC system the ability of endothelium to maintain or expand multipotent CFU-Cs was investigated. Transfer of CT2 from DP/VE-cadherin<sup>SP</sup> and CD45<sup>SP</sup>/VE-cadherin<sup>SP</sup> combinations to methylcellulose based secondary culture again revealed the exclusive presence of CFU-Mast (Figure 4.6C-E).

Transplantation of CT2 derived from either pure or mixed populations of cells into irradiated adult mice was not able to provide any level of haematopoietic reconstitution (Figure 4.7).

These findings indicate that the LSC technique is limited by its ability to maintain only mast cell restricted CFU-Cs even under conditions which facilitate efficient multilineage myeloid differentiation. Furthermore, the system could not sustain or induce LTR-dHSC activity. Although the developed LSC system enabled the elucidation of a functional co-operation between embryonic endothelial and stem/progenitor cells this model was unlikely to be an appropriate system for the study of LTR-dHSC induction and expansion in the AGM region.

4.2.2.5 Multilineage CFU-C expansion using IMDM<sup>+</sup> medium

Based on the above experiments it became clear that the preservation of the heterogeneous cellular composition of the AGM region is likely to be essential for the in vitro analysis of LTR-dHSC development. To develop an optimised culture system, cell suspension from E11.5 AGM regions was tested in an IMDM-based
LSC using 12 different combinations of growth factors (Figure 4.8A). The criteria employed to identify the most appropriate combination of growth factors included:

1. The ability to support leucocyte, erythroid and endothelial cell survival
2. The capacity to expand CFU-C number
3. The capacity to preserve functionally heterogeneous CFU-Cs during expansion.

Comparison of the ability to support haematopoietic and endothelial cell survival during a 72 hour culture period did not reveal any marked differences between the conditions (Figure 4.8A). None of the combination of factors was able to preserve endothelial cell survival.

A quantitative comparison of CFU-Cs revealed that all combinations of factors could support CFU-Cs (Figure 4.8B). Of particular interest was that the combinations of SCF/IL-7, SCF/Flt3L, SCF/Flt3L/IL-3 and SCF/IL-3/Leukaemia Inhibitory Factor could promote an approximate 4.2-, 6.0-, 8.4- and 5.9-fold expansion, respectively.

Qualitative assessment of methylcellulose colonies confirmed that all conditions could support the expansion of multiple myeloid lineage progenitors (Figure 4.8C). Based on the ability of medium enriched with SCF, IL-3 and Flt3L (designated IMDM⁺) to effectively support the viability of input cells and promote the greatest expansion of CFU-Cs without inducing an obvious lineage bias, this medium combination was selected for further experiments.

To investigate the ability of IMDM⁺ medium to maintain or expand LTR-dHSCs following a 72 hour culture period AGM region-derived cells were injected
Figure 4.8: Differentiation of whole E11.5 AGM region in IMDM-based medium

(A) Lineage composition of E11.5 AGM region derived cells following 3 days of culture in various combinations of growth factor supplemented medium. Lineage identification of viable (7-AAD') cells was determined by flow cytometry following incubation with anti-VE-cadherin, CD45 and Ter119 antibodies. Note that endothelial cells are not present in these conditions.

(B) Number of CFU-Cs present in 0.1 equivalents of AGM derived cells following 3 days of culture as determined by secondary colony formation in methylcellulose based medium. Line indicates the number of CFU-Cs present in uncultured E11.5 AGM.

(C) Lineage composition of secondary methylcellulose colonies derived from 0.1 equivalents of AGM cells. Criteria for lineage identification are described in Appendix 2.

(D) Injection of 0.25 equivalents of AGM region per mouse after 3 days in LSC using IMDM' medium (condition 9) was not able to repopulate any irradiated adult mice after 12 weeks. Each point represents and individual mouse.

Data are representative of two independent experiments.
Comparison of the lineage composition of the E11.5 AGM following 72 hours of either organ explant or LSC reveals some interesting differences. The first is that when cultured as an intact organ at the gas-liquid interface a heterogeneous composition, comparable to that of freshly dissected organ, is maintained as evidenced by the presence of endothelial (VE-cadherin$^{SP}$), haematopoietic (CD45$^{SP}$) and DN cells (Figure 4.9A), whereas following LSC the remaining cells are predominantly haematopoietic (Figure 4.9B). Secondly, only a modest expansion of in vitro progenitors is observed following explant culture which results in the number of CFU-Cs increasing from 109±7.0 to 162±12; following LSC the number of CFU-Cs increases to 1410±150 (Figure 4.9E).

Organ reaggregation has been utilised to study the role of thymic epithelium during T-lymphocyte differentiation: reaggregation involves the formation of a solid organ containing a defined population of interest in combination with a population supportive cells that form a three-dimensional culture system (Anderson et al., 1993).

Reaggregation would hypothetically provide an ideal candidate system to study the AGM region. However, the efficiency of reaggregation is very low and in the case of the thymus presents great practical limitation (J. Sheridan personal communication). Recently the reaggregation technique for the thymus has been refined by J. Sheridan resulting in 100% efficiency when cell suspensions are cultured as a pellet instead of a cell slurry (J. Sheridan personal communication). I therefore investigated the potential of reaggregating cell suspensions of E11.5 AGM at the gas-liquid interface.

In the presence of organ explant medium (M5300) VE-cadherin$^{SP}$, CD45$^{SP}$, DP and DN populations were preserved (Figure 4.9C), however, only 42±12 CFU-Cs
Figure 4.9: Reaggregation of E11.5 AGM region facilitates the preservation of a heterogeneous lineage composition and CFU-C expansion

The lineage composition of E11.5 AGM region following 72 hours of either organ explant (A), IMDM + LSC (B), M5300 reaggregation (C) or IMDM + reaggregation (D) was determined by flow cytometry following incubation with anti-CD45 and anti-VE-cadherin antibodies. Note that preservation of both endothelial and haematopoietic compartments is only achieved following culture at the gas-liquid interface.

Contour plots are composed from $5 \times 10^4$-$1 \times 10^5$ viable cells. Quadrants are based on appropriate isotype control staining (Figure A4.7); values in quadrant indicate the percentage of positive cells. Contour plots are representative of two independent experiments.

(E) The CFU-C content of E11.5 AGM region following 72 hours of organ explant, IMDM + LSC, M5300 reaggregation or IMDM + reaggregation was assessed by secondary transfer to methylcellulose based medium. Note the significant difference in the number of CFU-Cs present between E11.5 AGM region derived cells following organ explant or M5300 based reaggregation ($P<0.01$). Colonies were scored after 10 days of culture.

Where visible, bars indicate standard error of the mean of three independent experiments. LSC denotes liquid suspension culture and reag, reaggregation.
were present in the resulting organ (Figure 4.9E) indicating that a significant loss in haematopoietic potential had occurred, compared to explant culture ($P<0.01$). Reaggregation of E11.5 AGM suspension in the presence of IMDM$^+$ medium also resulted in the preservation of VE-cadherin$^\text{SP}$, CD45$^\text{SP}$ DP and DN populations (Figure 4.9D). Unlike in the presence of M5300 medium large numbers of CFU-Cs (2180±60) were generated (Figure 4.9E). These data encouraged further investigation of the haematopoietic activity of AGM reaggregates.

4.2.3.1 Development of IMDM$^+$ reaggregates during a 96 hour culture period

A study of the temporal lineage composition of IMDM$^+$ reaggregates during a 96 hour period reveals that following an initial decrease in cell number during the first twenty-four hours 	extit{ex vivo} from 140,000±12,000 cells to 73,000±1,581 cells, cell number increases to 120,000±13,692 after 48 hours, 130,000±21,615 after 72 hours and 180,000±33,375 by 96 hours (Figure 4.10A). Examples of the changes observed in the size and gross structure of reaggregates during 	extit{ex vivo} culture is shown in Figure 4.10B.

During the culture period there is no overall increase in the number of endothelial cells present in the reaggregate: the VE-cadherin$^\text{SP}$ fraction appears to develop proportionally to the whole organ as evidenced by the initial decrease in cell number from 3,500±300 to 770±17 within 24 hours of culture which is restored to 3,564±660 cells by 96 hours (Figure 4.10A). In contrast, the haematopoietic compartment undergoes marked expansion: preceded by an initial decrease from 3,640±312 to 1,314±28 viable cells after 24 hours the number of haematopoietic
Figure 4.10: AGM reaggregate development in IMDM+ medium

(A) Changes in the cell number of IMDM+ reaggregates over a 96 hour culture period. Lineage identity and viability of cells was detected by flow cytometry according to the expression of CD45 and VE-cadherin, and the exclusion of 7-AAD.

(B) Representative images of reaggregates after 24 and 96 hours in culture. Original magnification x4.

Where visible, bars indicate standard error of the mean of three independent experiments.
cells increases to 8,460±965, 19,500±3,242 and 32,130±5,957 following 48, 72 and 96 hours, respectively (Figure 4.10A).

4.2.3.2 CFU-C expansion during reaggregation

IMDM⁺ AGM reaggregates are able to facilitate a rapid expansion of CFU-Cs. At 0 hours (fresh E11.5 AGM region) 118±13 CFU-C are detected per organ, after 24 hours 360±50 CFU-Cs are detected, increasing to 1,200±190 after 48 hours, 2,180±60 after 72 hours and 5,441±118 after 96 hours (Figure 4.11A).

The expansion of CFU-Cs within IMDM⁺ reaggregates does not occur in a lineage restricted manner as indicated by an approximately proportional increase in unipotent and multipotent progenitors: CFU-Macrophage (Mac), CFU-Mast and CFU-Granulocyte Macrophage (GM) demonstrated a 65-fold (increasing from 23±1.1 CFU-Cs to 1,489±337), 52-fold (26±3.6 CFU-Cs to 1,361±321) and 40-fold (31±19 CFU-Cs to 1,249±299) expansion during a 96 hour culture period (Figure 4.11B). A marked expansion of BFU-Erythroid (E) and CFU-Granulocyte Erythroid Macrophage Megakaryocyte (GEMM) was observed at a lower fold increase: the number of BFU-E increased from 27±1.5 to 339±88 and CFU-GEMM, from 19±1.4 to 250±50, which in both cases represents a 13-fold increase (Figure 4.11B).

Reaggregation of E11.5 AGM region in the presence of IMDM⁺ medium allows the preservation of heterogeneous lineage composition and facilitates the expansion of in vitro haematopoietic progenitors.

4.2.3.3 Organisation of reaggregate structure
Figure 4.11: Expansion of functionally heterogeneous CFU-Cs following reaggregation

E11.5 AGM region was reaggregated using IMDM+ medium. After 0, 24, 48, 72 or 96 hours of culture cells were transferred to methylcellulose based medium.

A) Quantitative changes in CFU-Cs over 96 hours of culture.
B) Qualitative expansion CFU-Cs over 96 hours of culture.

Colonies were counted or scored after 8 days of culture.

Where visible, bars indicate standard error of the mean of three independent experiments.
Following 96 hours of reaggregation AGM region-derived cells organise into an organ comprising a central body with outward protrusions (Figure 4.10B). The study of thin sections revealed that haematopoietic cells (PECAM-1^CD45^+) are distributed throughout the reaggregate with areas of greatest concentration occurring close to the periphery (Figure 4.12A). Extensive endothelial structures are also observed which organise into a vascular bed (VB), characterised by multiple layers of endothelial cells (PECAM-1^CD45^-) that are arranged beneath the periphery of the reaggregate, and vascular networks (VN) that penetrate the body of the reaggregate (Figure 4.12B).

Merging the images produced following anti-CD45 and anti-PECAM-1 staining reveals that the protrusions generally contain few haematopoietic cells and are devoid of any endothelium (Figure 4.12C). The vascular bed does not extend to the surface of the reaggregate and is always separated from it by a few layers of cells. Haematopoietic cells can be seen within the areas of VN formation; however, areas of apparent colonial growth containing CD45^{high} cells occur frequently in close contact with the peripheral VB. Utilised a method for the three-dimensional (3-D) study of whole organs following antibody staining, Z-stack renderings from 100-180 1.0μm optical sections were generated. The distribution of haematopoietic cells across the periphery of the reaggregate and the absence of endothelial cells within the protrusions was confirmed by 3-D analysis of reaggregate structure (Figure 4.12D). Rotation of Z-stacks and detailed investigation of optical sections through the reaggregate surface to an approximate depth of 150μm allowed a more complete visualisation of the VB which was found to encapsulate the majority of the reaggregate (Figure 4.12E). Study of the space between the VB and the reaggregate
Figure 4.12: Organisation of endothelial and haematopoietic components in IMDM reaggregates following 96 hours in culture

(A) Confocal analysis reveals that CD45 expression (green) is concentrated in the periphery of the reaggregate. Original magnification x200. Bar represents 70μm.

(B) PECAM-1 (red) is expressed in two major structures: in the vascular bed (VB) and in the vascular network (VN). Original magnification x200. Bar represents 70μm.

(C) Overlaying images of CD45 (green) and PECAM-1 (red) expression reveal areas of haematopoietic clustering around the VB. Nuclei are stained with DAPI (blue). Original magnification x200. Bar represents 70μm.

Three-dimensional view of a reaggregate obtained following Z-stack reconstruction from whole mount confocal images. Shown are surface (D) and internal (E) views of a reaggregate (original magnification x100; bars represent 150μm).

Images A-C are representative of observations made from a population of 4 reaggregates. Images D and E are representative of observations made from a population of 5 reaggregates. VB denotes vascular bed; VN, vascular network.

Representative examples of appropriate isotype control staining are provided in Figure A4.8.
periphery revealed this region to be tightly packed with haematopoietic cells (Figure 4.12E). The non-haematopoietic and non-endothelial composition of E11.5 AGM region reaggregates has not yet been investigated.

4.2.3.4 Haematopoietic development within the reaggregation system

The impact of reaggregation on the progression of haematopoietic development was investigated according to expression of plasma membrane proteins. Although the reaggregate system is clearly conducive to leucopoiesis, as indicated by the increase in CD45+ cells (Figures 4.9A and 4.10A), the low percentage of CD45-Ter119+ cells suggests that erythropoiesis is not favoured (Figure 4.13A).

Haematopoietic (CD45+) cells in the E11.5 AGM region demonstrate a PECAM-1low phenotype (see Figure 3.2B) this characteristic is preserved within the reaggregate (Figure 4.13B). Unlike within the E11.5 AGM region no PECAM-1highCD45+ cells were observed which in the fresh organ correspond to VE-cadherin+CD45+ (DP) cells (Figures 3.2B and 4.13B). The endothelial population (VE-cadherinSP) retained a PECAM-1high phenotype (Figure 4.13B).

Within the E11.5 AGM region few haematopoietic cells express Tie-2 (see Figure 3.2B) but during the hepatic phase of haematopoiesis Tie-2 becomes upregulated (see Figure 3.8B). In the reaggregate system Tie-2 expression is absent from CD45 expressing cells (Figure 4.13C). Although Tie-2 continues to be expressed within the endothelial population it has been markedly downregulated (Figures 3.8B and 4.13C). Consistent with the E11.5 AGM region, AA4.1 is absent from CD45 expressing cells but is maintained at low level with the endothelial lineage (Figure 4.13D).
periphery revealed this region to be tightly packed with haematopoietic cells (Figure 4.12E). The non-haematopoietic and non-endothelial composition of E11.5 AGM region reaggregates has not yet been investigated.

4.2.3.4 Haematopoietic development within the reaggregation system

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Haematopoietic (CD45$^+$) cells in the E11.5 AGM region demonstrate a PECAM-1$^{\text{low}}$ phenotype (see Figure 3.2B) this characteristic is preserved within the reaggregate (Figure 4.13B). Unlike within the E11.5 AGM region no PECAM-1$^{\text{high}}$CD45$^+$ cells were observed which in the fresh organ correspond to VE-cadherin$^+$CD45$^+$ (DP) cells (Figures 3.2B and 4.13B). The endothelial population (VE-cadherin$^{\text{SP}}$) retained a PECAM-1$^{\text{high}}$ phenotype (Figure 4.13B).

Within the E11.5 AGM region few haematopoietic cells express Tie-2 (see Figure 3.2B) but during the hepatic phase of haematopoiesis Tie-2 becomes upregulated (see Figure 3.8B). In the reaggregate system Tie-2 expression is absent from CD45 expressing cells (Figure 4.13C). Although Tie-2 continues to be expressed within the endothelial population it has been markedly downregulated (Figures 3.8B and 4.13C). Consistent with the E11.5 AGM region, AA4.1 is absent from CD45 expressing cells but is maintained at low level with the endothelial lineage (Figure 4.13D).
Figure 4.13: Analysis of haematopoietic development following reaggregation
Expression of haematopoietic and endothelial lineage markers in IMDM reaggregates following 96 hours of culture considered in the context of CD45 expression. Each plot is composed from 1x10^4 viable (7-AAD^-) cells. Quadrants are based on appropriate isotype control staining (Figure A4.9). Values in quadrants indicate the percentage of positive cells. Contour plots are representative examples of two independent experiments.
The expression of Mac-1 and Gr-1 is largely restricted to the haematopoietic compartment. Consistent with E13.5 hepatic haematopoiesis the expression of Mac-1 has been downregulated by the majority of cells (compare Figures 3.8B and 4.13E). Following the culture period c-Kit becomes expressed on most CD45+ cells; all cells co-expressing Sca-1 and c-Kit are haematopoietic, possibly reflecting differentiation along the mast cell pathway (Figure 4.13F and Appendix 2). α4-integrin continues to be expressed by the majority of haematopoietic cells and CD41 becomes upregulated on a fraction of the population (Figure 4.13G); unlike in the E11.5 AGM region and in the E13.5 liver, CD41 becomes restricted to the haematopoietic lineage (Figures 3.2B and 3.8B). No CD3ε+ T-lymphocytes are detected after the culture period, and although B220 is expressed by many of the haematopoietic cells the lymphoid nature of these cells is yet to be confirmed (Figure 4.13H).

On balance the nature of the haematopoietic cells present in the reaggregated organ after the culture period is substantially different from that of the original AGM region and is not entirely consistent with a progression to hepatic haematopoiesis.

4.2.3.5 Generation of LTR-dHSCs following reaggregation

The potential of reaggregation to facilitate the maintenance and expansion of LTR-dHSCs: reaggregates were generated in the presence of organ explant medium (M5300; of which hydrocortisone (HC) is an indispensable component), IMDM+ or IMDM+ supplemented with HC. Following 96 hours of culture a 0.5 reaggregate dose (r.d.) from each condition was injected into an irradiated adult recipient. After a minimum of 12 weeks post-injection the peripheral blood of recipient mice was assessed for leucocyte chimerism by flow cytometry.
Reaggregates cultured in the presence of M5300(HC) were able to reconstitute the haematopoietic system of 6 out of 8 experimental mice, resulting in a 50% mean peripheral blood leucocyte chimerism (PBLC) (range 16-77%). All 9 mice that received IMDM+HC reaggregates were successfully repopulated with an 85% mean PBLC (range 81-90%) and all 10 mice that received IMDM+ reaggregates were also repopulated with an 83% mean PBLC (range 70-87%) (Figure 4.14).

Based on both the proportion of repopulated mice and the mean PBLC achieved by each of the conditions tested these data indicate that a greater number of LTR-dHSCs are produced in the presence of IMDM+ medium then with M5300(HC) medium. Furthermore, IMDM+ appears to circumvent the requirement for HC, as evidenced by the absence of any marked difference in the mean PBLC between mice that had received IMDM+HC and IMDM+ transplants.

4.11 Marked expansion of LTR-dHSCs occurs between 72 and 96 hours of culture

Preliminary investigations into the dynamics of LTR-dHSC expansion within E11.5 AGM reaggregates revealed that a rapid expansion of LTR-dHSCs occurs between 72 and 96 hours of culture.

0.5 r.d. of reaggregates generated in the presence of IMDM+ medium were injected into irradiated adult mice following 24, 48, 72 and 96 hours of culture. 12 weeks post-injection mice were assessed for contribution of donor cells to peripheral blood leucocytes.
Figure 4.14: Enhanced maintenance of LTR-dHSCs following reaggregation in the presence of IMDM\(^+\) medium

0.5 doses of reaggregate produced using M5300(HC) IMDM\(^+(HC)\) or IMDM\(^+\) medium were injected into irradiated adult mice with 2x10\(^4\) competitor bone marrow cells. The peripheral blood of recipient mice was analysed for chimerism 12 weeks post injection. Each plot represents an individual mouse.

The mean peripheral blood leucocyte chimerism (PBLC) was significantly different between the experimental groups that received M5300 reaggregate and those that received IMDM\(^+\) based reaggregates (\(P<0.01\)). No significant difference in the mean PBLC was observed between recipients of IMDM\(^+(HC)\) and IMDM\(^+\) reaggregates.

Data presented are cumulative of two independent experiments.

r.d. denotes reaggregate dose; HC, hydrocortisone.
Previous reports document that a minimum of 1.0 equivalent of E11.5 AGM region is required to achieve successful reconstitution of an adult mouse (Gekas et al., 2005; Kumaravelu et al., 2002; Orelio et al., 2004). After 24 hours a 0.5 r.d. was able to repopulate 2 out of 4 mice resulting in a 50% mean PBLC (Figure 4.15A). Given that the entire experimental group had received a total of 2.0 r.d, the number of LTR-dHSCs present in 1.0 r.d. is not likely to be greater than 1. After 48 hours only 1 out of 4 mice were successfully transplanted (52% PBLC), again suggesting that no marked expansion in LTR-dHSCs had occurred (Figure 4.15A).

By 72 and 96 hours post reaggregation all 4 mice injected were successfully repopulated and demonstrated a mean PBLC of 60% (range 21-75%) and 85% (range 83-87%), respectively (Figure 4.15A). Differences in PBLC achieved following 72 and 96 hours of culture suggests that there is a substantial increase in LTR-dHSCs numbers from 72 to 96 hours, however, any approximation of the absolute number of LTR-dHSCs in these experiments is impossible. Therefore, it is likely that an expansion of LTR-dHSCs is initiated between 48-72 hours post-reaggregation and continues until at least 96 hours post-reaggregation.

4.2.3.7 1 to 140 LTR-dHSCs in 96 hours

To accurately assess the magnitude of LTR-dHSC expansion following 96 hours of culture limiting dilution experiments were performed.

6 out of 6 mice that had received 0.3 r.d. were successfully repopulated (85% mean PBLC), 8 out of 8 mice were repopulated with 0.1 r.d. (71% mean PBLC), 11 out of 12 mice were repopulated with 0.05 r.d. (58% mean PBLC) and 9 out of 12 mice that had received 0.01 r.d. (46% mean PBLC) (Figure 4.15B). Calculations
Figure 4.15: Rapid in vitro expansion of LTR-dHSCs

(A) 0.5 doses of reaggregate produced using IMDM⁺ medium following 24, 48, 72 and 96 hours of culture were injected into irradiated adult mice with 2x10⁴ competitor bone marrow cells. The peripheral blood of recipient mice was analysed for chimerism 12 weeks post injection. Each plot represents an individual mouse. A significant difference in mean peripheral blood leucocyte chimerism (PBLC) was observed between 72 and 96 hours (P<0.05).

(B) To determine the number of LTR-dHSCs present in IMDM⁺ reaggregates following 96 hours of culture 0.3-0.01r.d. was injected into irradiated adult mice with 2x10⁴ competitor bone marrow cells. The peripheral blood of recipient mice was analysed for chimerism 12 weeks post injection. Each plot represents an individual mouse.

Based on calculations using L-Calc software approximately 119 LTR-dHSCs (range of 98-140) are present in 1.0 reaggregate dose (r.d.) after 96 hours. Of note is the significant reduction in mean peripheral blood leucocyte chimerism achieved between 0.3r.d. and 0.1r.d. (P<0.02) and between 0.1r.d. and 0.01r.d. (P<0.04).

Data presented are cumulative of two independent experiments.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Dose injected</th>
<th>Small colonies ± SD</th>
<th>Large colonies ± SD</th>
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</thead>
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<tr>
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<td>-</td>
<td>2.8±1.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0</td>
<td>3.7±2.1</td>
</tr>
<tr>
<td>M5300</td>
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<tr>
<td></td>
<td>0.05</td>
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<td>0.5±0.7</td>
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<tr>
<td></td>
<td>0.5</td>
<td>*</td>
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</tr>
<tr>
<td></td>
<td>0.1</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>IMDM⁺</td>
<td>0.05</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
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<td>0</td>
<td>7.7±3.9</td>
</tr>
</tbody>
</table>

¹1% FCS/PBS injected

*Individual colonies indistinguishable due to confluent growth

Small colony = ≤5mm diameter; Large colony = ≥6mm diameter

Table 4.1: CFU-S content of E11.5 AGM region reaggregates

Cells from M5300(HC) or IMDM⁺ reaggregates were into irradiated adult mice. Spleenic colonies were counted at 11 days post injection.

Data are representative of two independent experiments. r.d. denotes reaggregate dose.
Figure 4.16: Rapid \textit{in vitro} expansion of CFU-S in E11.5 AGM region reaggregates

(A) Image of spleens dissected from recipient mice 11 days after receiving an injection of (from left to right) 0.01, 0.05 and 0.1 reaggregate doses of IMDM\textsuperscript{+} reaggregate or 1% FCS/PBS (control solution).

(B) To confirm the donor identity of CFU-S, 0.01r.d. of IMDM\textsuperscript{+} reaggregate generated from the AGM regions of embryos ubiquitously expressing green fluorescent protein (GFP) was injected into irradiated adult mice. Shown are representative examples of spleens from mice injected with reaggregate cells after 96 hours of culture (left) and control solution (right). From an experimental group of 8 recipients 18 out of 19 splenic colonies were GFP\textsuperscript{+}.

(C) Large numbers of colonies were generated following the injection of IMDM\textsuperscript{+} reaggregate resulting in confluent growth so colony number could not be accurately counted. Therefore, the mass of spleens from recipient mice was measured 11 days post injection. Significant differences were observed between the spleen mass of mice that received 0.5-0.05 reaggregate doses of M5300(HC) and IMDM\textsuperscript{+} reaggregates ($P<0.01$) after 96 hours of culture.

Data presented are cumulative of two independent experiments. Bars indicate standard deviation. HC denotes hydrocortisone.
not be accurately scored, however, injection of 0.01r.d. resulted in the production of
7.7±3.9 large colonies and 0 small colonies (Table 4.1 and Figure 4.16A).

To determine the origin of CFU-S following 96 hours of culture 0.01r.d. of
AGM region reaggregates generated from embryos ubiquitously expressing GFP
were injected into recipient mice. 11 days after injection spleens were examined;
95% of colonies identified expressed GFP and were thus derived from the
reaggregate (Figure 4.16B).

Recording the mass of spleens from experimental mice confirmed that a
greater number of CFU-S were present in IMDM+ derived reaggregates than from
M5300(HC) derived reaggregates: control mice had a spleen mass of 4.0±0.8mg;
mice that received 0.5, 0.1, and 0.05 doses of M5300 reaggregate had spleen masses
of 5.8±1.7mg, 5.3±0.5mg and 3.7±0.5mg, respectively; mice that received 0.5, 0.1,
0.05 and 0.01 doses of IMDM+ reaggregate had spleen masses of 14±2.1mg,
13±2.7mg, 11±2.6mg and 6.1±1.6, respectively (Figure 4.16C). These data
demonstrate that the reaggregation of E11.5 AGM in the presence of IMDM+
medium is able to facilitate significant and rapid expansion of late developing CFU-S.

Previously published data reports that the E11.5 AGM region is capable of
expanding late developing CFU-S from 6.0±0.9 to 31±5.7 during whole organ
explant in the presence of M5300(HC) medium (Medvinsky and Dzierzak, 1996;
Medvinsky et al., 1993). Here, the failure to achieve CFU-S expansion following the
disruption of AGM structure is reported (only a basal number of CFU-S11 was
preserved, approximately 7.4 CFU-S per reaggregate).

In the presence of IMDM+ approximately 770 CFU-S were produced per
reaggregate suggesting that IMDM+ was able to compensate for the effects of
structural disruption. In conclusion using the IMDM\textsuperscript{+} reaggregation system allowed the expansion of both LTR-dHSCs and CFU-S. Given the inability to distinguish CFU-S derived from LTR-dHSCs and those of alternative origin it is not possible to conclude whether IMDM\textsuperscript{+} medium expands multiple members of the definitive haematopoietic hierarchy or if both primitive and definitive haematopoietic cells can respond to IMDM\textsuperscript{+}.

4.3 Discussion

Although the E11.5 AGM region is capable of generating multipotent LTR-dHSCs \textit{in vitro} differentiation of the stem cell-enriched VE-cadherin\textsuperscript{+}CD45\textsuperscript{+} population in methylcellulose medium is restricted to the mast cell lineage.

To test the hypothesis that interactions from heterogeneous populations might be required to facilitate multilineage differentiation a novel liquid suspension culture (LSC) system was developed and characterised. This system allows functionally heterogeneous colony forming units to be detected with greater sensitivity in the absence of supportive stoma. Using the LSC method liquid suspension-colony forming units (LS-CFUs) from the DP and CD45\textsuperscript{SP} populations are detected at the same frequency as CFU-Cs in methylcellulose culture.

In methylcellulose both the DP (LTR-dHSC enriched) and CD45\textsuperscript{SP} (haematopoietic) populations appeared to be restricted to mast cell differentiation. Under LSC conditions multilineage differentiation capacity of DP cells was revealed: in contrast to the CD45\textsuperscript{SP} population that was largely restricted to mast cell differentiation the DP population is composed of LS-CFUs of heterogeneous differentiation potential. In LSC, this heterogeneity is characterised by the formation
of mast cell colonies (colony type 1), colonies enriched with neutrophils, monocyte/macrophages, mast cells, megakaryocytes and erythroid cells (colony type 2) and colonies containing only macrophages (colony type 3).

Upon co-culture with endothelial (VE-cadherin$^{SP}$) cells enhanced multilineage differentiation was achieved from DP but not CD45$^{SP}$ cells. This was evidenced by the increased frequency of colony type 2 from the DP fraction. Under similar conditions CD45$^{SP}$ cells continued to produce mast cells with the rare appearance of macrophages.

Thus the described LSC protocol enabled the recapitulation of the myeloid multilineage differentiation potential of the DP population characteristic for these cells in vivo. Moreover, these data suggest that endothelial cells are required to either facilitate further maturation of DP cells to multipotent haematopoietic progenitors or to produce factors allowing successful differentiation. Further detailed studies are required to investigate the mechanism of endothelial support.

Although multilineage differentiation could be achieved during LSC subsequent transfer of cells to methylcellulose culture showed a restricted mast cell differentiation potential. Importantly, LTR-dHSCs were not maintained under LSC conditions. Using cellular suspensions of whole E11.5 AGM region CFU-Cs of multilineage differentiation potential could be maintained following LSC, however, LTR-dHSCs remained undetectable.

Because the original aim of this study was to learn how to harness the most potent haematopoietic capacity of the AGM region as a tool to study LTR-dHSC emergence and expansion a novel in vitro system based on the organ explant technique was developed. Reaggregating whole E11.5 AGM region at the gas-liquid
interface resulted in the preservation of the heterogeneous lineage composition of the AGM region and the \textit{ex vivo} expansion of functionally heterogeneous CFU-Cs. More importantly, significant expansion of LTR-dHSCs was achieved during a 96 hour period of culture.

Investigation of the three-dimensional structure of E11.5 AGM region reaggregates formed in the presence of IMDM$^+$ revealed extensive vascular network (VN) and vascular bed (VB) formation. Furthermore, haematopoietic activity was preferentially associated with the peripheral VB possibly implicating an interaction between the endothelium and haematopoietic cells. The importance of vascular-haematopoietic interactions was hypothesised previously by Li et al (2003). Further investigations will be required to discover if this association is physiologically relevant.

\textit{Ex vivo} expansion of LTR-dHSCs from the E11.5 AGM region has only been achieved using organ explant culture which results in 12 LTR-dHSCs being produced from 1 stem cell following 72-120 hours of culture (Kumaravelu et al., 2002). In this chapter time course and limiting dilution experiments have demonstrated that between 48 and 96 hours of culture a massive expansion of LTR-dHSCs occurs in the IMDM$^+$ reaggregate resulting in the formation of 98-140 LTR-dHSCs. Expansion of CFU-Cs and LTR-dHSCs is accompanied by a massive expansion of CFU-S indicating that LTR-dHSC expansion does not appear due to the inhibition of differentiation. Differential identification of LTR-dHSCs, CFU-S, CFU-C and mature cells within the reaggregate system will facilitate the analysis of stem and progenitor cell niches.
The ability to preserve and enhance LTR-dHSC expansion following disruption of the AGM structure means that sophisticated experiments can now be performed including:

1. Identification of embryonic lineages with the potential to give rise to LTR-dHSCs.

2. Studying mechanisms of LTR-dHSC expansion (e.g. de novo induction, pre-LTR-dHSC maturation or amplification of pre-existing LTR-dHSCs).


The reaggregation system will be particularly useful once exact serum-free culture conditions are defined.
Chapter 5: Results 3

Dorsoventral polarity of LTR-dHSC regulation

5.1 Introduction

The emergence and expansion of the LTR-dHSC pool during embryogenesis follows a precise temporal and spatial pattern: LTR-dHSCs sporadically appear in the E10.5 AGM region but robust detection does not occur until the AGM region initiates LTR-dHSC expansion at E11.5 (section 1.3). The first LTR-dHSCs are anatomically restricted to the dorsal aorta/para-aortic mesenchyme (Ao) of the AGM region but by E12.5 LTR-dHSCs are found in both the Ao/Pa-M and urogenital ridges (UGR) (de Bruijn et al., 2000b).

It is widely hypothesised that haematopoietic stem cell activity in the human and murine embryo is localised to the ventral wall of the Ao. As discussed in section 1.3.8 this hypothesis is based on:

1. Reports of intra-aortic haematopoietic clusters (IAHCs) on the ventral lumen of the Ao, commonly interpreted as haematopoietic cell budding from the endothelial lining
2. The presence of sub-aortic patches in the ventral para-aortic mesenchyme (Pa-M).

To date no direct experimentation has been presented to challenge or support this hypothesis. To address the possibility of an anatomical polarity of haematopoietic activity the E11.5 AGM region was initially dissected into the proposed regions of interest: the Ao and UGR were dissected as illustrated in Figure 5.1A. Subsequently,
the Ao was bisected into ventral (AoV) and dorsal (AoD) portions (Figures 5.1B, A.5 and A.6).

5.2 Results

5.2.1 Spatial distribution of clonogenic progenitors in the E11.5 AGM region

5.2.1.1 Quantitative distribution of CFU-Cs

The distribution of CFU-Cs within the E11.5 AGM region was investigated to identify any anatomical foci of haematopoietic progenitor activity. To this end 0.5 embryo equivalents (e.e.) of cell suspension from whole AGM region, Ao, UGR, AoV or AoD were cultured in methylcellulose based medium (M3434); colonies were counted after eight days of culture. CFU-Cs are present in both the Ao and UGR, however, a significantly greater portion of the CFU-C pool is localised to the Ao ($P<0.03$): 0.5e.e. of whole E11.5 AGM region contains $61\pm17$ CFU-Cs; the Ao contains $41\pm7.7$ CFU-Cs; $18\pm6.5$ CFU-Cs are present in the UGR (Figure 5.1C). Although some bias in favour of the AoV was observed it had no statistical significance: 0.5e.e. of AoV was found to contain $25\pm5.7$ CFU-Cs while $18\pm4.4$ CFU-Cs were found in the AoD (Figure 5.1C).

5.2.1.1 Qualitative distribution of CFU-Cs

Methylcellulose colonies from sub-dissected AGM regions were qualitatively analysed after 8-10 of differentiation. Criteria for the identification of burst forming unit-erythroid (BFU-E), CFU-Mast, CFU-Macrophage (CFU-Mac), CFU-
Figure 5.1: Distribution of CFU-Cs in the E11.5 AGM region

(A) Schematic representation of the dissection of dorsal aorta/para-aortic mesenchyme (Ao) and urogenital ridges (UGR) from the E11.5 AGM region.

(B) Schematic representation of the bisection Ao into ventral (AoV) and dorsal (AoD) aspects. Note the presence of the notochord which allows the unambiguous distinction between ventral and dorsal aspects after bisection.

(C) Quantitative distribution of CFU-Cs from sub-regions of the E11.5 AGM region. 0.5e.e. of whole AGM region, Ao, UGR, AoV and AoD were cultured in the presence of growth factor supplemented methylcellulose based medium. After 8 days of culture the number of colonies was scored. A significant difference was observed between the number of CFU-Cs identified in the Ao and UGR ($P<0.03$). No significant difference was observed between the AoV and AoD.

(D) The qualitative distribution of CFU-Cs was scored between 8-10 days of differentiation. The criteria for colony identification are described in Appendix 3. No significant differences were observed between the AoV and AoD with respect to BFU-E, CFU-Mac, CFU-Mast and CFU-GM. A significant difference was observed in the number of CFU-GEMM ($P<0.02$).

BFU-E denotes burst forming unit-erythroid; CFU, colony forming unit; Mac, macrophage; GM, granulocyte/macrophage; GEMM, granulocyte/erythroid/macrophage/megakaryocyte.

Where visible, bars indicate standard error of the mean of three independent experiments.
Granulocyte/Macrophage (CFU-GM), and Granulocyte/Erythrocyte/Macrophage/Megakaryocyte (CFU-GEMM) are outlined in Appendix 3.

No obvious anatomical difference in the qualitative distribution of CFU-Cs was observed (Figure 5.1D): from 0.5e.e. of whole AGM region 13±0.7 BFU-E, 16±4.5 CFU-Mac, 9.0±1.3 CFU-Mast, 16±9.3 CFU-GM and 8.0±1.0 CFU-GEMM were identified; in the Ao 6.8±0.9 BFU-E, 11±1.8 CFU-Mac, 7.7±0.4 CFU-Mast, 8.9±3.3 CFU-GM and 6.0±1.3 CFU-GEMM; in the UGR 2.4±0.9 BFU-E, 7.2±3.0 CFU-Mac, 4.5±0.3 CFU-Mast, 2.2±1.9 CFU-GM and 1.4±0.4 CFU-GEMM; from the AoV 5.4±1.4 BFU-E, 7.1±1.6 CFU-Mac, 4.2±1.4 CFU-Mast, 4.8±1.4 CFU-GM and 3.8±0.4 CFU-GEMM and from the AoD 2.7±0.3 BFU-E, 6.3±2.2 CFU-Mac, 4.2±0.7 CFU-Mast, 2.7±1.1 CFU-GM and 1.9±0.1 CFU-GEMM. Interestingly, CFU-GEMM were more abundant in the AoV than in the AoD (P<0.02).

Therefore only minor qualitative difference distinguishes the ventral and dorsal aspects of the E11.5 AGM region. Although this difference concerns multipotent CFU-GEMM this does not provide strong support for a polarity in haematopoietic activity as would be expected if ventrally located intra-aortic haematopoietic clusters were highly enriched for clonogenic haematopoietic activity.

5.2.2 Ex vivo CFU-C expansion is restricted to the ventral aspect of the dorsal aorta/para-aortic mesenchyme

The presence of haematopoietic progenitor cells in the AoV and AoD does not necessarily mean that they originated locally in these regions. It is possible that haematopoietic progenitor cells are generated exclusively from either the AoV or AoD and subsequently migrate. To investigate this possibility Ao, AoV and AoD
were dissected from the E11.5 AGM region and explanted at the gas-liquid interface in the presence of M5300 organ explant medium (containing hydrocortisone).

5.2.2.1 Quantitative changes in CFU-Cs following explant culture

In total, 50±14 CFU-Cs were produced from 0.5 e.e. of Ao following 72 hours of explant culture (representing a 23% increase in CFU-C number). From independently cultured AoV 44±2.8 CFU-Cs were generated (a 74% increase) and from independently cultured AoD 5.4±0.7 CFU-Cs was detected, corresponding to a 70% decrease (Figure 5.2A). Thus, although at E11.5 both AoV and AoD contained approximately the same number of CFU-Cs following explant only the AoV has the capacity to expand CFU-Cs.

5.2.2.2 Qualitative CFU-C changes following explant culture

Following explant culture changes in the type of colony produced was observed. From 0.5 e.e. cultured Ao 5.0±2.5 BFU-Es were detected, representing a decrease of 21%; CFU-Mac increased by 40% to 16±7.8; CFU-Mast increased by 69% to 13±3.3; CFU-GM increased by 12% to 10±2.5; 5.8±2.8 CFU-GEMMs were detected, a decrease of 3.3% (Figure 5.2B). None of the described changes in the type of CFU-C are statistically significant.

Cultured AoV produced a similar decrease in BFU-E and CFU-GEMM while significantly expanding in all other CFU-Cs. AoV contained 2.5±0.5 BFU-E (54% decrease; not statistically significant); 15±1.3 CFU-Mac (111% increase; P=0.01); 11±3.1 CFU-Mast (164% increase; P=0.05); 12±2.5 CFU-GM (150% increase; P=0.03); 3.3±0.1 CFU-GEMM (13% decrease; not statistically significant) (Figure
Figure 5.2: CFU-Cs expansion during ex vivo culture of Ao, AoV and AoD

(A) Following 72 hours of explant culture 0.5e.e. E11.5 Ao, AoV or AoD was transferred to methylcellulose based medium. The appearance of myeloid colonies was scored after 8 days of culture. No significant quantitative difference was observed between the Ao and AoV.

(B) Qualitative distribution of CFU-C following explant culture was assessed between 8-10 days of culture. No significant qualitative differences were observed between the Ao and AoV.

BFU-E denotes burst forming unit-erythroid; CFU denotes colony forming unit; Mac denotes macrophage; GM denotes granulocyte/macrophage; GEMM denotes granulocyte/erythroid/macrophage/megakaryocyte.

Where visible, bars indicate standard error of the mean of four independent experiments.
A

Input organ (0.5e.e.)

B

Input organ (0.5e.e.)
5.2B). Following explant culture of AoD contained: 0 BFU-E; 2.7±1.4 CFU-Mac (57% decrease; not significant); 1.6±0.9 CFU-Mast (62% decrease; \( P<0.02 \)); 0.9±0.6 CFU-GM (67% decrease; not significant); 0.3±0.3 CFU-GEMM (84% decrease; \( P<0.01 \)) (Figure 5.2B).

Based on these data the expansion of all CFU-Cs during explant culture are attributable to the activity of the AoV. This suggests that the ventral aspect of the AGM region does indeed possess unique haematopoietic potential.

### 5.2.3 Dorsoventral polarity of LTR-dHSC emergence and expansion

#### 5.2.3.1 In vivo restriction of LTR-dHSCs to the AoV

The E11.5 AGM region was sub-divided into the Ao and UGR and directly injected into irradiated adult mice. Injection of a 2.0e.e. Ao resulted in the high-level multi-lineage haematopoietic reconstitution of 5 out of 7 mice and the establishment of a 46% mean peripheral blood leucocyte chimerism (PBLC) (range 20-79%) (Figure 5.3A). Injection of 2.0e.e. UGR resulted in the repopulation of only 1 out of 7 mice (Figure 5.3A). Thus, consistent with previously published observations (de Bruijn et al., 2000a) we find that the vast majority of LTR-dHSCs are restricted to the region of the dorsal aortal/para-aortic mesenchyme (\( P<0.05 \)).

Injection of AoV and AoD revealed that LTR activity is largely restricted to the ventral aspect of the Ao: injection of 2.0e.e. of AoV into irradiated adult recipients resulted in the successful repopulation of 11 out of 14 mice (mean PBLC of 64%; range 5-81%) while injection of a 2.0e.e. dose of AoD repopulated only 2 out of 12 mice (mean PBLC of 22%) (Figure 5.3B).
Figure 5.3: Dorsoventral polarity in LTR-dHSC localisation in the E11.5 AGM region  
(A) Distribution of LTR-dHSC between the E11.5 dorsal aorta (Ao) and urogenital ridges (UGR).  
(B) Distribution of LTR-dHSCs between the ventral (AoV) and dorsal (AoD) aspects of the Ao.  

Each point represents an individual recipient mouse. Only mice demonstrating a peripheral blood leucocyte chimerism ≥5% at 12 weeks post-transplantation were considered to be reconstituted. Data are cumulative of three independent experiments. e.e. denotes embryo equivalent.
Figure 5.4: Ventral induction of LTR-dHSC emergence

The E10.5 dorsal aorta was bisected into ventral (AoV) and dorsal (AoD) aspects, cultured at the gas-liquid interface for 72 hours and transplanted into irradiated adult mice at a dose of 2.0e.e. per recipient.

Each point represents an individual recipient mouse. Only mice demonstrating a peripheral blood leucocyte chimerism $\geq 5\%$ at 12 weeks post-transplantation were considered to be reconstituted. Data are cumulative of three independent experiments. e.e. denotes embryo equivalent.
increase the sensitivity of the assay read out each recipient received a 0.3e.e. dose of explanted organ.

31 out of 35 mice were successfully repopulated following injection with AoV resulting a mean PBLC of 64% (PBLC 64%; range 5-81%). 1 out of 36 mice that received 0.3e.e. of AoD was successfully repopulated (PBLC 7%) (Figure 5.5). These data demonstrate that expansion of AGM region-derived LTR-dHSCs occurs exclusively in the ventral portion of the Ao.

5.2.4 Dorsoventral distribution of cells co-expressing haematopoietic and endothelial lineage markers

Within the E11.5 AGM region the CD45-expressing population is composed of dominating PECAM-1^low and minor PECAM-1^high subsets (Figure 5.6A). The co-expression of VE-cadherin and CD45, which distinguishes E11.5 AGM region LTR-dHSCs from the non-stem cell haematopoietic compartment, is restricted to the PECAM-1^high population: 83%±5.1 of PECAM-1^highCD45^+ expressed VE-cadherin. Of note is that the entire PECAM-1^highCD45^+ population bear a VE-cadherin^low phenotype (Figure 5.6B). No significant expression of VE-cadherin was observed in the PECAM-1^lowCD45^+ population (Figure 5.6C); 79%±4.6 PECAM-1^highCD45^-cells co-expressed VE-cadherin; based on previous analyses (section 3.2.2.1) a minor population of VE-cadherin^-CD45^- cells is expected to express PECAM-1 thus accounting for the minor population of PECAM-1^highCD45^-VE-cadherin^- cells (Figure 5.6D).

An apparent difference in the sensitivity of whole mount confocal microscopy relative to flow cytometry results in the inefficient visualisation of
Figure 5.5: Dorsoventral polarity of LTR-dHSC expansion

To assess the capability of E11.5 AoV and AoD to facilitate LTR-dHSC expansion organs were cultured at the gas-liquid interface for 72 hours then injected into irradiated adult mice at 0.3e.e., a dose that would only repopulate the majority of mice if LTR-dHSC expansion had occurred.

Each point represents an individual recipient mouse. Only mice demonstrating a peripheral blood leucocyte chimerism $\geq 5\%$ at 12 weeks post-transplantation were considered to be reconstituted. Data are cumulative of three independent experiments. e.e. denotes embryo equivalent.
Figure 5.6: Co-expression of PECAM-1, VE-cadherin and CD45 in the E11.5 AGM region

(A) Pattern of CD45 and PECAM-1 expression in the E11.5 AGM region

Co-expression of VE-cadherin is observed in the vast majority of the PECAM-1-high CD45⁻ (A) and PECAM-1-highCD45⁺ (B) populations but not in the PECAM-1-lowCD45⁺ (C) population.

The presented contour plot is representative of three independent experiments. Gates are based on appropriate isotype control staining (Figure A4.10). Values indicated the percentage of positive cells.
PECAM-1\textsuperscript{low} expression, therefore restricting detection to PECAM-1\textsuperscript{high} cells (an experimental observation that is illustrated in Figure 5.7).

5.2.4.1 Confocal imaging of the AoV

A three-dimensional (3-D) study of the ventral aspect of the dorsal aorta/para-aortic mesenchyme (AoV) was undertaken to investigate the localisation of PECAM-1\textsuperscript{high}CD45\textsuperscript{+}(VE-cadherin\textsuperscript{+}) cells, the population enriched for LTR-dHSCs. Particular attention was paid to the endothelial structure of the Ao.

Figure 5.7A depicts a representative example of a Z-stack constructed from whole mount preparations of AoV after staining with anti-CD45 (green) and anti-PECAM-1 (red) antibodies. Ao can be seen in the central portion of the figure and is flanked by endothelial networks that precede the UGR (Figure 5.7A and B). The para-aortic mesenchyme (Pa-M), which separates the Ao from the flanking endothelial networks, is largely composed of cells that lack any detectable CD45 or PECAM-1 expression. Vasculature can be seen branching from the Ao (*) to the endothelial networks of the UGR (†) (Figure 5.7C).

Haematopoietic cells are predominantly distributed within the Pa-M with rare cells being localised to the lumen of the ventral wall of the dorsal aorta (Figure 5.7A and C). Importantly, the PECAM-1\textsuperscript{high}CD45\textsuperscript{+} immunophenotype is exclusive to cells in contact with the luminal side of the Ao (arrow head). CD45 expressing cells within the Pa-M do not express PECAM-1 (Figure 5.7B and C).

Confirmation of the restriction of PECAM-1\textsuperscript{high}CD45\textsuperscript{+} cells to the aortic lumen was made following the examination of transverse sections from whole E11.5 AGM regions (Figure 5.8).
Figure 5.7: Whole mount confocal microscope imaging of the ventral aspect of the E11.5 dorsal aorta

Three-dimensional (3-D) model of the organisation of haematopoietic (CD45 expressing; shown in green) and endothelial (PECAM-1 expressing; shown in red) compartments of the ventral aspect of the dorsal aorta (AoV) was generated using 120-180 1.0μm optical sections of whole organ.

(A) Study of the luminal view of the AoV reveals low numbers of PECAM-1^{high}CD45^{+} cells. Original magnification x200; bar represents 75μm.

(B) Rotation of the 3-D model reveals that no cells in the sub-aortic region co-express CD45 and high-levels of PECAM-1. Original magnification x200; bar represents 75μm.

(C) Enlargement of the AoV luminal view (dashed box) clearly illustrating the presence of cells co-expressing CD45 and high levels of PECAM-1 on the luminal side of the dorsal aorta (arrow head) with PECAM-1^{low/NEG} cells being largely restricted to the sub-aortic regions (arrow). Of note is the branching vasculature (*) that connect the vascular networks of the UGR to the Ao (†). Original magnification x200; bar represents 75μm.

Data presented are representative examples of 12 individual organs from five independent experiments. Appropriate isotype control staining is provided in Figure A4.11.

Ao denotes dorsal aorta and Pa-M, para-aortic mesenchyme.
Figure 5.8: Distribution of endothelial and haematopoietic cells within the E11.5 AGM region

Serial 10μm transverse sections were made from E11.5 AGM (n=3). Provided here is a representative example of the distribution of haematopoietic cells within the AGM region. Note that within the region of the Ao and para-aortic mesenchyme PECAM-1^{high}CD45^{+} cells are restricted to the luminal side of the Ao.

(A) Expression of CD45.

(B) Expression of PECAM-1

(C) Overlay of CD45 (green) and PECAM-1 (red) expression

(D) Enlargement of boxed area in (C).

Appropriate isotype control staining is provided in Figure A4.12. Original magnification x200; bar represents 70μm.

Ao denotes dorsal aorta; Cv denotes cardinal vein; Ms denotes mesentery; S-Cv denotes sub-cardinal vein.
Based on these observations it is possible to conclude that during the endothelial stage of LTR-dHSC development stem cells are likely to be in contact with the aortic lumen of the AoV. These data are unable to address questions regarding the exact spatial origin of emerging LTR-dHSCs which is likely to be a dynamic process requiring more sophisticated investigation then can be achieved by analysing still images.

5.2.5 Regulation of haematopoietic activity in the dorsal aorta

5.2.5.1 AoV and AoD transcriptional activity

As a first step in identifying elements that might regulate LTR-dHSC generation within the E11.5 Ao the expression of transcripts for factors known to play significant roles in the induction of haematopoiesis, development of the definitive haematopoietic hierarchy and LTR-dHSC expansion was investigated by RT-PCR from preparations of E11.5 AoV and AoD.

Bone morphogenetic protein 4 (BMP4) is differentially expressed between the AoV and AoD with nearly all detectable signal being localised to the AoV (Figure 5.10A). A low Sonic hedgehog signal was observed only from the AoD. Investigation into the transcriptional expression of Noggin, Chordin and Gremlin, whose protein products are potent inhibitors of BMP4 signalling (Hsu et al., 1998; Larrain et al., 2000; Re'em-Kalma et al., 1995; Smith and Harland, 1992), revealed that Noggin is found in both the AoV and AoD with a possible bias towards the AoV; in contrast a consistent bias of Chordin expression was observed in the AoD. Low levels of Gremlin transcript were occasionally detected with no apparent anatomical bias.
GATA-2 transcripts are exclusively expressed in the AoV. GATA-3 transcripts were found at high-level in the AoV and with lower frequency in the AoD (Figure 5.9A). Identifying the precise spatial pattern of GATA-3 expression in the AoV and AoD might be of particular interest given its supportive role in the development of definitive haematopoiesis (see section 1.4.4.2.3.). Both Hoxb4 and Runx1 transcripts were detected with greatest frequency in the AoV.

In situ hybridisation and antibody staining on E11.5 AGM sections will be required to complement these observations for the deduction of more useful information regarding the distribution and co-localisation of the above transcripts and their respective protein products. Such studies might facilitate the identification of any anatomical hotspots of haematopoietic regulation.

5.2.5.2 Expansion of AoV-derived CFU-Cs in response to Noggin

As a preliminary step in elucidating the mechanistic processes of dorsoventral haematopoietic regulation the effect of exogenous soluble factors on ex vivo CFU-C maintenance and expansion during AoV and AoD organ explant culture was pursued. To this end explants were cultured in the presence of exogenous BMP4 or Noggin at concentrations of 10, 50 or 100ng/ml. Following 72 hours of culture 0.5e.e. of organ was transferred to secondary M3434 culture to induce the terminal differentiation of CFU-Cs.

5.2.5.2.1 Effect of exogenous BMP4
Figure 5.9: Role of BMP4 signalling during \textit{ex vivo} CFU-C maintenance

(A) Representative example of three independent RT-PCR analyses for the expression of factors essential for haematopoietic development, or stem cell expansion, from total AoV or AoD RNA. Ribosomal L32 served as a control. The role of BMP4 signalling during \textit{ex vivo} CFU-C maintenance was investigated by adding exogenous recombinant BMP4 or Noggin to explants of AoV (B) and AoD explants (C): BMP4 or Noggin was added to cultures at 10, 50 or 100 ng/ml. After 72 hours of culture cells were transferred to methylcellulose culture. Colonies were counted after 8 days of culture.

At 10 ng/ml of Noggin and 50 ng/ml of BMP4 the number of CFU-Cs in the AoV was significantly decreased ($P<0.04$); at 100 ng/ml of Noggin a significant increase in AoV CFU-Cs was observed ($P<0.02$). No significant changes in the numbers of CFU-C in the AoD were observed.

Bars indicate standard error of the mean of three independent experiments.
The addition of 10ng/ml of BMP4 during AoV explant culture resulted in the formation of $28 \pm 17$ CFU-Cs, $50$ng/ml in $16 \pm 3.0$ CFU-Cs and 100ng/ml in $23 \pm 9.3$ CFU-Cs (Figure 5.9B). Paired control experiments resulted in the production of $34 \pm 2.0$, $43 \pm 7.0$ and $38 \pm 0.5$ CFU-Cs, respectively (Figure 5.9B). Therefore BMP4 does not increase CFU-Cs activity in the AoV, moreover, 50ng/ml BMP4 significantly decreases the number of CFU-Cs ($P<0.04$).

No significant change in the number of CFU-Cs generated during AoD explant culture was observed in the presence of BMP4: in the presence of 10ng/ml $4.8 \pm 0.3$ CFU-Cs were detected ($5.3 \pm 3.3$ in control experiments); $2.0 \pm 0$ CFU-Cs in the presence of 50ng/ml ($5.0 \pm 3.0$ in control experiments); $7.5 \pm 0.5$ CFU-Cs in the presence of 100ng/ml ($6.3 \pm 4.3$ in control experiments) (Figure 5.9C).

### 5.2.5.2.2 Effect of exogenous Noggin

In the presence of 10ng/ml of Noggin the AoV CFU-C pool decreased to $15 \pm 3.8$ ($P<0.02$). No significant difference was observed in the presence of 50ng/ml which yielded $25 \pm 10$ CFU-Cs; a significant expansion was observed in the presence of 100ng/ml which resulted in the formation of $98 \pm 12$ CFU-Cs ($P=0.02$) (Figure 5.9B).

No significant changes in the CFU-C content of AoD following explant was observed in the presence of 10, 50 and 100ng/ml as evidenced by the detection of $5.3 \pm 3.3$, $6.5 \pm 0$ and $5.5 \pm 2.0$ CFU-Cs, respectively (Figure 5.9C).

These data suggests that the addition of BMP4 inhibitors facilitates the expansion of CFU-Cs by symmetric division, *de novo* progenitor formation or the
induction of stem/progenitor cell differentiation. The high concentration of Noggin required to facilitate expansion may reflect the high level of BMP4 expressed in the AoV, as revealed by RT-PCR. The effect of exogenous BMP4 and Noggin on ex vivo LTR-dHSC expansion has yet to be determined.

5.3 Discussion

The dorsoventral distribution of CFU-Cs does not correlate with the distribution of LTR-dHSCs. In the E11.5 AGM region CFU-Cs are detected in the UGR and Ao yet the vast majority of LTR-dHSCs are restricted to the Ao. Bisection of Ao into the AoV and AoD does not reveal any significant quantitative or qualitative differences in the distribution of CFU-C however the vast majority of LTR-dHSCs are restricted to the AoV. Explant and transplantation of E10.5 and E11.5 AoV and AoD revealed that LTR-dHSCs emerge and expand exclusively within the AoV. Furthermore, E11.5 Ao, AoV and AoD explant experiments demonstrate that all CFU-C expansion in the Ao is attributable to the AoV.

Interestingly, although CFU-Cs are present in the AoD following explant culture they either differentiate or die. This indicates that the AoD is unable to sustain the independent production of CFU-Cs or LTR-dHSCs. Given that in the Ao the AoV is the focus of LTR-dHSC and CFU-C generation it is reasonable to speculate that downstream progeny migrate away from parental stem cells that compartmentalised in the AoV. If this is accurate a question is raised: why are LTR-dHSCs not as freely distributed as CFU-Cs? Reasons for this may include the low number of LTR-dHSCs present in the AGM region at any one time (Kumaravelu et al., 2002), the possibility that CFU-Cs are a more robust haematopoietic unit then
LTR-dHSCs or that within the AGM region CFU-Cs and LTR-dHSCs derive independently of each other.

We (section 3.2.4.1) and other investigators (North et al., 2002) have identified the first LTR-dHSC in the VE-cadherin\(^+\)CD45\(^+\) population of the E11.5 AGM region, a population also contained a high frequency of CFU-Cs. For technical reasons a surrogate PECAM-1\(^{\text{high}}\)CD45\(^+\)(VE-cadherin\(^+)\) immunophenotype was used to identify this LTR-dHSC/CFU-C enriched population. Three-dimensional imaging and analysis of transverse sections revealed that the PECAM-1\(^{\text{high}}\)CD45\(^+\)(VE-cadherin\(^+)\) immunophenotype is restricted to rare cells on the luminal wall of the dorsal aorta. Given that the \textit{in vivo} repopulation studies described above place LTR-dHSCs exclusively within the AoV the first LTR-dHSCs are likely to localise to the luminal wall of the AoV.

Expression of \textit{GATA-2}, \textit{GATA-2}, \textit{Hoxb4} and \textit{Runx1} are known to substantially impact the development of definitive haematopoiesis and regulate the \textit{ex vivo} expansion of LTR-dHSCs (see sections 1.4.3 and 1.5). Interestingly transcripts of these genes are either exclusively or preferentially expressed in the AoV.

Both the AoV and AoD are highly enriched for transcripts for the BMP4 antagonists \textit{Noggin} and \textit{Chordin}. Based on the enrichment of \textit{BMP4} mRNA in the AoV it was hypothesised that the addition of exogenous BMP4 to explants of AoD might facilitate CFU-C maintenance or expansion. This appeared not to be the case. A similar response to BMP4 was observed during the explant culture of the AoV. Based on data from RT-PCR analysis the inability to induce a positive response may
have resulted from the presence of Noggin and Chordin. These experiments should be repeated with the addition of a Noggin and Chordin antagonist.

In the presence of a low concentration of Noggin (10ng/ml) a significant decrease in the number of CFU-Cs was observed following AoV culture. In the presence of a high concentration of Noggin (100ng/ml) a significant increase in CFU-C number was observed. These data provide foundation for the hypothesis that the proliferation of CFU-Cs is regulated in a dose-dependent manner by BMP4 signalling. Further experiments are required to investigate the impact of exogenous factors on the LTR-dHSC compartment of the AoV.
Chapter 6: General summary

During the course of this thesis some of the most pressing questions surrounding the *in vivo* and *in vitro* emergence and expansion of long-term definitive haematopoietic stem cells (LTR-dHSCs) and progenitor cells have been addressed: are LTR-dHSCs be induced from non-haematopoietic cells? Can the expansion of LTR-dHSCs observed *in vivo* be recapitulated *in vitro*? What factors regulate embryonic LTR-dHSC/progenitor cell expansion? It is likely that elucidating the ontogeny of LTR-dHSCs and the regulation of *in vivo* induction and expansion will facilitate practical therapeutic applications.

6.1 Ancestry of early LTR-dHSCs

During the investigation of a possible endothelial phase of LTR-dHSC development we have demonstrated that LTR-dHSCs emerge from the AGM region and YS bearing a dual endothelial and haematopoietic immunophenotype. This duality, predominantly based on the co-expression of VE-cadherin and CD45, is preserved during circulation but lost following hepatic colonisation. *Ex vivo* culture of primary haematopoietic organs, the AGM region and the YS, for a period of 72 hours demonstrates that the loss of an endothelial-like immunophenotype occurs in the absence of the hepatic microenvironment and thus is likely to be a consequence of developmental time. These findings provide support for a common pathway of extra-embryonic and intra-embryonic LTR-dHSC emergence as evidenced by the co-expression of VE-cadherin and CD45 on AGM region-derived and YS-derived LTR-dHSCs during their respective phases of stem cell expansion.
Flow cytometric analysis revealed a broad expression of endothelial markers in the VE-cadherin$^+$CD45$^+$ LTR-dHSC population of the E11.5 AGM region; however, in vitro experiments revealed that a clear functional division exists between the endothelial and haematopoietic compartments.

Taken together, it is likely that as LTR-dHSCs transgress through the endothelial stage of development within primary embryonic haematopoietic organs they temporarily preserve an endothelial phenotype. This phenotypic similarity suggests, but does not prove, an endothelial origin of LTR-dHSCs.

6.2 Reaggregation: a means to harness the environment of the E11.5 AGM region

Experimentation with purified E11.5 AGM region populations using a novel liquid suspension culture (LSC) technique has revealed that a functional interaction between the VE-cadherin$^+$CD45$^+$ (DP) and the VE-cadherin$^+$CD45$^-$ (endothelial) populations occurs in vitro to facilitate multilineage myeloid differentiation. This finding alludes to a possible functional benefit of haematopoietic and endothelial cell interaction in vivo.

Using the LSC technique it was not possible to maintain multilineage CFU-Cs or LTR-dHSCs from purified population of E11.5 AGM region, even in the presence of multiple haematopoietic growth factors. Using a refined growth factor supplemented medium (IMDM$^+$) expansion of functionally heterogeneous CFU-Cs from whole E11.5 AGM was possible yet the preservation of LTR-dHSC activity remained elusive.
A major undertaking in the field of experimental haematology is the race to produce an *in vitro* system that can be used to induce or facilitate the expansion of LTR-dHSC. Combing the previously described organ explant system, which allows a 10-fold increase in LTR-dHSC number, with a modified reaggregation technique I have developed a highly efficient system that enables the most rapid *in vitro* expansion of LTR-dHSCs to date.

Following 96 hours of reaggregate culture a 98-140-fold expansion of LTR-dHSCs was observed, which is more efficient than other currently published *in vitro* systems in which it has been reported that adult bone marrow LTR-dHSCs can be expanded 5-50 fold during similar periods of *ex vivo* culture (Antonchuk et al., 2002; Krosl et al., 2003; Reya et al., 2003; Zhang and Lodish, 2005). It will be important to determine whether this novel embryo-based system has a capacity to expand a defined number of LTR-dHSCs from non-embryonic sources of stem cells such as murine adult bone marrow, mobilised adult LTR-dHSCs and even sources of human HSCs such as cord blood.

### 6.3 Ventral polarity of LTR-dHSC induction, residence and expansion

It has long been hypothesised that definitive haematopoiesis, particularly the emergence of LTR-dHSCs, is initiated in the ventral (AoV) portion of the dorsal aorta. In this thesis I have provided the first direct evidence to demonstrate the *in situ* ventral polarity of LTR-dHSC distribution. Furthermore, I have shown that between E10.5-11.5 all the components required for the induction and expansion of LTR-dHSCs are uniquely present in the AoV.
Preliminary experiments have been performed that implicate the role of BMP4 signalling in the regulation of haematopoiesis in the AoV. Further experiments combining organ explant culture supplemented with exogenous factors followed by *in vivo* transplantation will enable molecular pathways involved in the initiation and expansion of embryonic LTR-dHSCs to be unveiled.

In conclusion, the AGM region is a unique organ that is capable of facilitating the emergence of LTR-dHSCs from non-stem cell populations and expanding a LTR-dHSC pool. Importantly, this activity can be recapitulated *ex vivo*. Within this thesis I have: 1. explored the induction of LTR-dHSCs from the endothelial compartment; 2. pioneered a method that allows the environment of the E11.5 AGM region to induce rapid LTR-dHSC expansion; and 3. identified the ventral portion of the dorsal aorta as a focus of stem cell emergence, maintenance and expansion.
Appendix 1: Publication

Development;132:4179-4191
dorsal aorta (Jordan, 1917; Medvinsky et al., 1996; North et al., 1999; Tavian et al., 1996) and the umbilical cord (North et al., 1999) are often interpreted as haematopoietic cells budded off from the endothelial lining. Occasional disruption of the endothelial basal membrane underlying such clusters suggest active involvement of local endothelium (Tavian et al., 1999). Labelling of chick embryonic endothelium in ovo resulted in the subsequent appearance of labelled haematopoietic cells, consistent with an endothelial origin of haematopoiesis (Jaffredo et al., 2000; Jaffredo et al., 1998). Recent data have suggested the origin of HSCs from sub-endothelial patches/mesenchyme (Bertrand et al., 2005; North et al., 2002). However, the relation of these cells to the endothelial lineage has yet to be unveiled. There is also some experimental evidence suggesting the existence of the haemangioblast in the adult bone marrow (Bailey and Fleming, 2003; Pelosi et al., 2002). Furthermore, key regulators of vascular and angiogenesis, VEGF and angiopoietin 1, play crucial roles in the maintenance of HSCs in adult bone marrow (Gerber et al., 2002; Takakura et al., 1998). Angiopoietin signalling may be involved in embryonic development of HSCs (Hsu et al., 2000; Yuasa et al., 2002). Although some controversy remains, a lineage relationship between haematopoietic and endothelial differentiation is currently widely accepted.

The definitive haematopoietic hierarchy develops from definitive HSCs (Kondo et al., 2003), the appearance of which in the embryo follows a complex developmental pattern (de Bruijn et al., 2000; Gekas et al., 2005; Kumaravelu et al., 2002; Medvinsky et al., 1996; Muller et al., 1994; Ottersbach and Dzierzak, 2005). By late E10.5-E11.5, the first definitive HSCs appear in the AGM region, the umbilical vessels and then slightly later in the YS. Recently, the E10.5-E13 placenta has also been identified as an early abundant reservoir of HSCs (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). Of note, high level repopulating activity (>5%) appears only from E11 (Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996). The growing number of definitive HSCs in embryonic blood from E11.5 correlates with rapid liver colonization (Christensen et al., 2004; Ema and Nakaochi, 2000; Kumaravelu et al., 2002; Morrison et al., 1995). As shown by the organ culture approach, the AGM region by E11.5 acquires the capacity to initiate/expand HSCs but by E12.5 HSC generation is overtaken by the YS (Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996), which supports the suggestion that the YS also contributes to definitive haematopoiesis (Toles et al., 1989; Weissman et al., 1978; Yoder et al., 1997). The capacity of the placenta to expand HSCs is an important issue that has yet to be investigated. Collectively, these data show a dramatic increase in HSC numbers between E11.5 and E12.5 in the entire conceptus, including the foetal liver and the placenta, implying a massive initiation of new HSCs in the embryo (Gekas et al., 2005; Kumaravelu et al., 2002).

Here, using in vivo and in vitro techniques (Fig. 1), we have explored the evolution of the endothelial character of definitive HSCs during their initiation, migration and hepatic colonization. At initiation, HSCs reside exclusively within a population defined by the co-expression of both endothelial and haematopoietic markers. The HSCs largely retain endothelial markers during circulation until they colonise the liver where VE-cadherin is downregulated. However, the

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**Materials and methods**

**Animals**

C57BL/6xCBA F1 mice were bred in animal facilities at the University of Edinburgh. Donor tissues from Ly-5.2/5.2 embryos were isolated and transplanted into irradiated Ly-5.1/5.1 or Ly-5.1/5.2 adult recipients as described previously (Kumaravelu et al., 2002). In some cases, Ly-5.1/5.2 embryos were transplanted into Ly-5.2/5.2 recipients. The day of discovery of the vaginal plug was designated as day 0.5. Embryos were scored according to Thelier criteria (http://genex.hgu.mrc.ac.uk/intro.html). Animals were kept in compliance with Home Office regulations.

**Tissue preparation and organ culture**

E11.5 AGM, E12.5 YS and E13.5 FL were dissected from embryos. Special care was taken to remove large vessels from the YS. Peripheral blood (PB) was collected from E12.5 embryos as previously described (Kumaravelu et al., 2002). Dissected organs were incubated in 0.1% collagenase-dispase (Roche)/PBS (Sigma) at 37°C for 40 minutes and then dissociated by gentle manual pipetting. Prior to antibody labelling PB and FL suspensions were depleted of erythrocytes using anti-Ter119 antibody conjugated magnetic microbeads (Miltenyi Biotec). Organ cultures were set up as described previously (Kumaravelu et al., 2002). E11.5 and E12.5 placenta were prepared as previously described (Gekas et al., 2005).

**Flow cytometry**

Monoclonal antibodies against the following antigens were used: AA4.1-APC, α4-integrin-FITC, B220-biotin, CD3ε-PE, CD4-PE, CD8α-Biotin, CD34-FITC/PE, CD41-FITC, CD45-PE/FITC, KIT
Progressive divergence of definitive haematopoietic stem cells from the endothelial compartment does not depend on contact with the foetal liver

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Summary

The yolk sac and the para-aortic splanchnopleura/aorta-genital ridges-mesonephros (P-Sp/AGM) region are the main sites of haematopoietic activity in the mouse embryo at the pre-liver stage of development. By day 11.5 of gestation, the AGM region is capable of autonomous initiation and expansion of definitive haematopoietic stem cells (HSCs). By day 12.5, HSC activity in the AGM region is reduced whilst a second wave of HSCs begins to emerge in the yolk sac. We show here that HSCs emerging in both locations are marked by co-expression of the endothelial-specific marker VE-cadherin and the pan-leukocyte antigen CD45. Phenotypic characterisation using CD31, TIE2, FLK1, Ac-LDL receptors, and CD34 markers demonstrated significant similarities between this VE-cadherinCD45 'double-positive' population and endothelial cells suggesting a common origin for these cells. The double-positive fraction also expressed the stem cell markers Kit, Seal and AA4.1. Long-term transplantation experiments demonstrated that the double-positive population, which constituted less than 0.05% of the day 11.5 AGM region and the day 12.5 yolk sac, is highly enriched for HSCs. In vitro assays showed that this population is also enriched for myeloid progenitors. During foetal liver colonization, circulating HSCs remained within the VE-cadherin+ cell fraction, although their phenotypic similarity with endothelial cells became less prominent. Upon liver colonisation the majority of HSCs downregulated VE-cadherin, expression of which was completely lost in the adult bone marrow. Partial loss of VE-cadherin expression in HSCs can be observed extra-hepatically in the advanced AGM region by E12.5. Similarly, the CD34+K1T population in the placenta, recently identified as a reservoir of HSCs, partly lose VE-cadherin expression by E12.5. By culturing isolated E11.5 AGM region and E12.5 yolk sac we show that the developmental switch from a 'primary' VE-cadherinCD45 to a more 'advanced' VE-cadherinCD45+ phenotype does not require contact of HSCs with the liver and is probably a function of developmental time.

Key words: AGM region, Yolk sac, Stem cells, VE-cadherin, Mouse

Introduction

Two distinct haematopoietic systems have been identified during embryogenesis: embryonic and adult/definitive (Dzierzak and Medvinsky, 1995; Baron, 2003; Godin and Cumano, 2002). The transitory embryonic hierarchy emerges by embryonic day 7 (E7) in the yolk sac (YS). Its main component is a population of large nucleated erythrocytes that originate mainly from mesodermal FLK1+VE-cadherin cells (Fujimoto et al., 2001). Later during organogenesis, FLK1 expression is restricted to the embryonic endothelium (Yamaguchi et al., 1993). VE-cadherin is an essential endothelial-specific protein that mediates inter-cellular adhesion of endothelial cells in both embryonic and adult vasculature (Breier et al., 1996; Carmeliet et al., 1999).

A large body of data suggests that endothelial and haematopoietic cells have a common origin in development. Endothelial and haematopoietic cells share a number of common markers (Godin and Cumano, 2002). Experiments using ES cell differentiation models have shown that adult-type clonogenic haematopoietic progenitors can originate from the endothelial VE-cadherinCD45 cells (Fujimoto et al., 2001). The relationship between endothelial and haematopoietic cells has also been clonally explored using an ES cell blast colony assay based on FLK1 and PECAM/CD31 expression (Chung et al., 2002; Ema and Rossant, 2003; Fehling et al., 2003; Kennedy et al., 1997; Lacaud et al., 2002).

FLK1 progenitor cells committed to endothelial-haematopoietic differentiation have recently been localised to the primitive streak of the E7.0-7.5 mouse embryo (Huber et al., 2004). In the more advanced embryo, VE-cadherin+ cells are also a source of haematopoietic cells (Nishikawa et al., 1998a). Immunohistochemical analysis suggests that some cells of the embryonic dorsal aorta co-express endothelial-specific VE-cadherin and haematopoietic markers (Breier et al., 1996; Carmeliet et al., 1999; Fraser et al., 2003). Clusters of haematopoietic cells adhered to the endothelium of the
Table 1. Statistical analysis of VE-cadherin and CD45 expression in major E11.5-E13.5 haematopoietic organs during key stages of HSC development

<table>
<thead>
<tr>
<th>Embryo age</th>
<th>Organ</th>
<th>n</th>
<th>VE-cadherin*CD45</th>
<th>VE-cadherin-CD45*</th>
<th>VE-cadherin<em>CD45</em></th>
<th>VE-cadherin CD45*</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.5</td>
<td>AGM</td>
<td>7</td>
<td>2.5±0.4 (1.3-4.0)</td>
<td>2.6±0.4 (1.6-4.4)</td>
<td>0.05±0.01 (0.01-0.1)</td>
<td>95±0.8 (91-96)</td>
</tr>
<tr>
<td></td>
<td>Yolk sac</td>
<td>4</td>
<td>6.9±1.3 (3.5-9.6)</td>
<td>2.4±0.5 (1.3-3.4)</td>
<td>0.05±0.02 (0.01-0.08)</td>
<td>91±1.6 (87-95)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>6</td>
<td>10±0.07 (8.9-12.0)</td>
<td>4.1±0.7 (2.9-6.3)</td>
<td>1.2±0.2 (0.5-2.1)</td>
<td>84±1.3 (80-87)</td>
</tr>
<tr>
<td>12.5</td>
<td>AGM</td>
<td>4</td>
<td>2.1±0.2 (1.4-2.4)</td>
<td>1.5±0.3 (0.9-2.1)</td>
<td>0.02±0.01 (0.01-0.04)</td>
<td>96±0.4 (96-98)</td>
</tr>
<tr>
<td></td>
<td>Yolk sac</td>
<td>3</td>
<td>5.3±2.4 (2.4-10.0)</td>
<td>1.3±0.3 (0.7-1.9)</td>
<td>0.03±0.01 (0.02-0.04)</td>
<td>93±2.2 (89-96)</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>6</td>
<td>1.2±0.6 (0.2-4.0)</td>
<td>0.035±0.005 (0.017-0.045)</td>
<td>0.005±0.002 (0.001-0.008)</td>
<td>99±0.6 (96-100)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>5</td>
<td>5.5±0.3 (4.5-6.5)</td>
<td>1.3±0.2 (0.8-1.7)</td>
<td>0.31±0.04 (0.22-0.42)</td>
<td>93±0.4 (92-94)</td>
</tr>
<tr>
<td>13.5</td>
<td>Liver</td>
<td>5</td>
<td>3.5±0.4 (2.2-4.4)</td>
<td>1.2±0.4 (0.5-2.8)</td>
<td>0.1±0.04 (0.02-0.22)</td>
<td>95±0.13 (95-96)</td>
</tr>
</tbody>
</table>

of the endothelial (VE-cadherin*CD45*), double-positive (DP) (VE-cadherin*CD45*) and haematopoietic (VE-cadherin*CD45*) populations (Fig. 2B). To this end, the endothelial markers TIE2, FLK1, PECAM1, receptors for Ac-LDL and CD34 were included into the staining panel. FACS analysis revealed similarity between the endothelial and the DP populations with respect to expression of these markers (Fig. 2B). Of note, PECAM1 and Ac-LDL receptors were also expressed at low level in the haematopoietic population.

We further characterised the DP population for the expression of the HSC markers KIT (also known as C-KIT) (Ikuta and Weissman, 1992; Sanchez et al., 1996; Uchida and Weissman, 1992) and SCA1 (Chen et al., 2003; Uchida and Weissman, 1992) (Fig. 2B). Similar to the endothelial

VE-cadherin*CD45*

VE-cadherin CD45*

Fig. 3. Morphology of E11.5 AGM endothelial, double-positive and haematopoietic populations. Cells with blast morphology are highly enriched within the VE-cadherin*CD45* population (May-Grunwald-Giemsa staining of cytospin preparations).
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(Also known as C-KIT)-PE/ APC, FLK1-PE, GR1-Biotin, Ly5.1-PE, Ly5.2-FTTC, MAC1-PE, SCA1-PE, TIE2-PE, VE-cadherin-biotin and CD16/32-purified. Biotin was detected using APC- or PE-conjugated streptavidin. Appropriate isotype controls were used. Dead cells were excluded using 7-AAD. Reagents were purchased from eBioscience and Pharmingen.

MoFlo (DakoCytomation) and FACStar (Beckton-Dickinson) flow cytometers were used for sorting. A FACScalibur (Beckton-Dickinson) was used for flow cytometric analysis. Data analysis was performed using FlowJo software (TreeStar).

Long-term repopulation assay

Competitive transplantation experiments were set up as previously described (Kumaravelu et al., 2002). The transplanted embryonic cells are expressed in HSC equivalents (HSC.e.): one HSC.e. is that phenotype contained in one organ. The transplanted bone marrow cells are expressed throughout the paper in embryo equivalents (e.e.), and stained with May-Grunwald and Giemsa stains (BDH). Images were taken with an Axiovert S 10 microscope (Zeiss) using Openlab software. Images were prepared using Adobe Photoshop.

Cytological examination

Following cell sorting, E11.5 AGM fractions were centrifuged at 1000 rpm for 4 minutes (Cytospin 3, Shandon) onto poly-L-lysine-coated slides (BDH). Preparations were fixed in methanol for 2.5 minutes and stained with May-Grunwald and Giemsa stains (BDH). Images were taken with an Axiovert S100 microscope (Zeiss) using Openlab software (Improvis). Images were prepared using Adobe Photoshop.

Clonogenic myeloid progenitor assay

Sorted cells from E11.5 AGM were cultured in methylcellulose medium (M3434, Stem Cell Technologies) according to the manufacturer’s instructions. Haematopoietic colonies were scored between 10 and 12 days.

Endothelial tube/network forming assay

Endothelial network formation was assessed using OP9 stromal cells in the presence of 50 ng/ml VEGF (Fraser et al., 2003; Nishikawa et al., 1998b). The number of PECAM 1 tubules was counted after 4 days. A two-step anti-PECAM 1 staining protocol was followed to identify endothelial development; following a primary anti-PECAM 1 antibody incubation (BD Bioscience) endothelium was visualised using a secondary antibody conjugated with alkaline phosphatase (Southern Biotechnology Associates: 3030-04) and a Vector Blue Alkaline Phosphatase Substrate Kit III (Vector: SK-5300). Pictures were taken with an Olympus IX50 microscope using ViewFinder software. Images were prepared using Adobe Photoshop.

Single-cell progenitor assay

OP9 haematopoietic differentiation assay was performed as described previously (Nishikawa et al., 1998b) with slight modifications. Briefly, confluent OP9 layers in 96-well plates were seeded with flow cytometrically sorted cells and incubated in α-MEM medium supplemented with FCS (10%), EPO (1 U/ml), IL3 (200 U/ml), and G-CSF-conditioned medium (1%). Following 7 days of culture, the wells containing round cells on top of the OP9 layer were counted. The haematopoietic identity of these cells was confirmed by flow cytometric analysis following CD45 antibody staining.

Results

E11.5 AGM cells co-expressing VE-cadherin and CD45 are highly enriched for endothelial and stem cell markers

To investigate the relationship between endothelial and haematopoietic populations two principal markers were initially used. Flow cytometric analysis demonstrated that although VE-cadherin and CD45 expression are largely mutually exclusive, a rare (0.05±0.01%) population of co-expressing cells could be detected in the E11.5 AGM region (Fig. 2A and Table 1). To explore the possible developmental link between the endothelial and haematopoietic lineages in more depth, we undertook further phenotypic characterisation.

Fig. 2. (A) Flow cytometric analysis of E11.5 AGM region demonstrating endothelial (VE-cadherin"CD45"; R1), haematopoietic (VE-cadherin"CD45"; R3) and double-positive (DP) (VE-cadherin"CD45"; R2) cell populations. The contour plot shown is a representative example compiled from approximately 10e.e. of E11.5 AGM (1.5×10^6 cells). (B) The endothelial affiliation of the double-positive population extends beyond the expression of VE-cadherin, demonstrated by endothelial-like levels of TIE2, Flk1, PECAM1, Ac-LDL, CD34 and SCA1 expression. The DP and the endothelial populations largely share endothelial and stem cell markers. Isotype control (white) and specific antibody staining (grey) are presented. The analysis of DP, endothelial and haematopoietic populations was made using data from 200-700, 15,000-20,000 and 9000-30,000 live cells, respectively. All data are representative of two to four experiments for each marker analysed.
Table 2. VE-cadherin is expressed by HSCs within primary haematopoietic organs and during migration, but is progressively downregulated in the liver and the adult bone marrow

<table>
<thead>
<tr>
<th>Donor organ</th>
<th>Population transplanted</th>
<th>Embryo equivalent (e.e.)/HSC equivalent (HSC.e.) transplanted</th>
<th>Reconstituted/transplanted</th>
<th>Mean chimerism range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E11.5 AGM</td>
<td>VE-cadherin CD45⁺</td>
<td>2.6e.e.</td>
<td>4/12</td>
<td>36 (5-60)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>4.0-5.0e.e.</td>
<td>0/15</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>4.0-5.0e.e.</td>
<td>0/10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>0.5-2.5.e.e.</td>
<td>0/10</td>
<td>-</td>
</tr>
<tr>
<td>E12.5 AGM</td>
<td>CD45⁺</td>
<td>1.0-2.0e.e.</td>
<td>6/11</td>
<td>69 (8-90)</td>
</tr>
<tr>
<td></td>
<td>CD45⁺</td>
<td>1.0-2.0e.e.</td>
<td>0/10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>1.0-3.0e.e.</td>
<td>8/17</td>
<td>61 (5-85)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>1.0-2.0.e.e.</td>
<td>6/15</td>
<td>61 (16-89)</td>
</tr>
<tr>
<td>E12.5 Yolk sac</td>
<td>CD45⁺</td>
<td>1.0.e.e.</td>
<td>3/3</td>
<td>74 (61-87)</td>
</tr>
<tr>
<td></td>
<td>CD45⁺</td>
<td>1.25.e.e.</td>
<td>1/4</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>0.4-0.8.e.e.</td>
<td>7/10</td>
<td>33 (5-87)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>0.7-1.0.e.e.</td>
<td>0/10</td>
<td>-</td>
</tr>
<tr>
<td>E12.5 PB</td>
<td>VE-cadherin CD45⁺</td>
<td>1.5-3.0.e.e.</td>
<td>8/12</td>
<td>54 (10-90)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>1.5-3.0.e.e.</td>
<td>1/10</td>
<td>84</td>
</tr>
<tr>
<td>E13.5 Liver</td>
<td>VE-cadherin CD45⁺</td>
<td>0.02.e.e.</td>
<td>4/5</td>
<td>30 (6-42)</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>0.02.e.e.</td>
<td>5/5</td>
<td>70 (61-76)</td>
</tr>
<tr>
<td>Adult bone marrow</td>
<td>VE-cadherin CD45⁺</td>
<td>2-10 HSC.e.</td>
<td>0/13</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>2-10 HSC.e.</td>
<td>7/10</td>
<td>10</td>
</tr>
</tbody>
</table>

*Chimerism observed in peripheral blood leucocytes by flow cytometry. Only recipients demonstrating at least 5% chimerism after 12 weeks were regarded as reconstituted.

†Either male donor cells were transplanted into female recipient mice or Ly-5.1/5.2 cells were transplanted into Ly-5.2/5.2 recipient mice.

‡Chimerism determined in peripheral blood leucocytes by either Y-chromosome PCR or flow cytometry.

(Nishikawa et al., 1998b). We found that endothelial tubule and network formation was largely restricted to the endothelial (VE-cadherin CD45⁺) population: 500 endothelial cells formed about four PECAM1⁺ tubules, while 5000 endothelial cells formed ~60 tubules and 20,000 endothelial cells resulted in extensive network formation (Fig. 4G-I). By contrast, only two tubules were formed with 50,000 VE-cadherin CD45⁺ plated cells and no endothelial capacity was observed within the haematopoietic or DP populations (Fig. 4G). Therefore, despite clear phenotypic similarity with the endothelium, the DP population has functionally diverged from the endothelial compartment.

**Definitive HSCs in the E11.5 AGM region reside within the DP cell fraction**

E11.5 AGM region was flow sorted on the basis of VE-cadherin and CD45 expression. In line with previous reports (North et al., 2002) all HSC activity was detected within the VE-cadherin CD45⁺ population. From a cohort of 12 adult recipients, four were repopulated with the DP population, with a range of 5-60% peripheral blood leucocyte chimerism (average of 36%) (Table 2). As one E11.5 AGM region harbours 1 HSC (Kumaravelu et al., 2002), the frequency of HSCs in the DP population is estimated at 1 in 70 cells (Table 3).

No high-level reconstituting capacity was observed within the VE-cadherin CD45⁺ fraction. However, six out of 15 injected recipients demonstrated long-term (greater than 3 months) low-level chimerism, typically between 0.1-2.0% (data not shown). No repopulating activity was seen from either of the endothelial or double-negative fractions, even when twice the embryo equivalents were delivered.

It has been shown that VE-cadherin⁺ cells from the E9.5 YS are restricted in their capacity for effective myeloid differentiation

**Table 3. HSCs are highly enriched within the VE-cadherin-expressing populations**

<table>
<thead>
<tr>
<th>Embryo age</th>
<th>Organ</th>
<th>Cells per organ (1×10⁵)*</th>
<th>HSCs per organ †</th>
<th>HSC candidate phenotype</th>
<th>Estimated HSC frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.5</td>
<td>AGM</td>
<td>1.4±0.12</td>
<td>1</td>
<td>VE-cadherin CD45⁺</td>
<td>-1/70</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>-</td>
</tr>
<tr>
<td>12.5</td>
<td>YS</td>
<td>2.1±0.2</td>
<td>2</td>
<td>VE-cadherin CD45⁺</td>
<td>-1/64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>28.7±2.9</td>
<td>3</td>
<td>VE-cadherin CD45⁺</td>
<td>-1/48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>-</td>
</tr>
<tr>
<td>13.5</td>
<td>FL</td>
<td>63.6±2.5</td>
<td>260</td>
<td>VE-cadherin CD45⁺</td>
<td>-1/127</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VE-cadherin CD45⁺</td>
<td>-1/2,226</td>
</tr>
</tbody>
</table>

*Dead cells excluded according to 7-AAD uptake.
†Previously determined by limiting dilution analysis in unsorted organs (Kumaravelu et al., 2002).
In vitro endothelial and haematopoietic differentiation potential of E11.5 AGM fractions sorted according to VE-cadherin (VEcad) and CD45 expression. (A) The frequency of clonogenic myeloid progenitors is 14-fold higher within the DP fraction compared with the haematopoietic (VE-cadherin+CD45+) population. Tight (B) and diffuse colonies (C) are predominantly derived from VE-cadherin+CD45+ cells. (D) Clonal analysis of sorted VE-cadherin+CD45+ cells using OP9 assay reveals that haematopoietic progenitors are present at a frequency of 7.6%. (E) Cobble-stone areas (arrow) were frequently observed beneath the stoma and floating haematopoietic cells (asterisk), shown at higher magnification in F. (G) In vitro endothelial network formation was largely restricted to the VE-cadherin+CD45+ population. After 4 days of co-culture with OP9 stromal cells, 60 PECA1+ tubules (blue staining) were produced from 5000 endothelial cells (H) and extensive network formation was seen from 20,000 cells (I). All data were collected from a minimum of three replicate experiments.

We then investigated whether co-culture with the OP9 stromal cell line would unveil haematopoietic potential in non-DP cells. In limiting dilution experiments, we found that clonogenic progenitors in the E11.5 AGM region resided exclusively within the DP population, at an average frequency 7.6% (Fig. 4D-F). Other cell fractions failed to produce any detectable numbers of haematopoietic cells.

**Endothelial network formation is restricted to the VE-cadherin+CD45+ population**

To investigate the possibility that the DP population might encompass the in vivo haemangioblast, we tested the in vitro endothelial differentiation/network forming capacity of the four E11.5 AGM fractions using a well established assay.

nuclear-to-cytoplasmic ratio, characteristic for blast/stem cells. The haematopoietic population consisted mainly of more mature cells with large, often vacuolated, cytoplasm and small nuclei of variable shape.

In agreement with the high frequency of progenitor-like cells in the DP cytospin, the greatest frequency of clonogenic cells (CFU-C) detected by the methylcellulose assay were concentrated in the DP population. Approximately one out of 28 DP cells were capable of producing cell colonies (Fig. 4A-C). The frequency of CFU-Cs within the haematopoietic population was approximately 14 times lower (1/385 cells) (Fig. 4A). Neither endothelial nor double negative (VE-cadherin+CD45+) cells were capable of generating haematopoietic colonies.
of foetal liver cells, equating to approximately five stem cells (Kumaravelu et al., 2002) (Table 2). While all five recipients of the haematopoietic fraction were repopulated, only four out of five mice transplanted with the DP fraction were successfully reconstituted. No obvious difference was observed in multi-lineage differentiation capacity between VE-cadherin<sup>CD45</sup> and VE-cadherin<sup>CD45<sup>+</sup></sup> HSCs isolated from the E13.5 liver (data not shown). We also found that both VE-cadherin<sup>+</sup> and VE-cadherin<sup>−</sup> HSCs were capable of repopulating secondary recipients (Fig. 5B).

It is difficult to assess accurately the actual numbers of HSCs in each of these fractions given the limited number of experimental animals, but it is possible to compare approximate numbers between the two cell populations. The haematopoietic (VE-cadherin<sup>CD45</sup>) fraction was able to reconstitute all five of the animals tested so we can predict that there were more than five HSCs present in the transplanted fraction. However, only four out of the five recipients of the DP cell population were reconstituted, indicating a frequency of fewer than five HSCs in that fraction. The observation that the level of reconstitution in each recipient transplanted with the haematopoietic fraction was, on average, twofold higher than with the DP cells (Table 2) supports these comparative predictions. Presuming that the competitive pressure from host and carrier cells in both experimental groups was similar, we conclude that the total number of HSC/RUs in the transplanted haematopoietic population was higher than in the DP population.

Finally we tested by transplantation of purified populations whether adult bone marrow HSCs expressed VE-cadherin. We

---

**Table 2**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>E12.5 yolk sac</th>
<th>E12.5 peripheral blood&lt;sup&gt;*&lt;/sup&gt;</th>
<th>E13.5 liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>VE-cadherin&lt;sup&gt;CD45&lt;/sup&gt;</td>
<td>2.2</td>
<td>0.04</td>
<td>2.5</td>
</tr>
<tr>
<td>Haematopoietic</td>
<td>5.0</td>
<td>38</td>
<td>2.3</td>
</tr>
</tbody>
</table>

<sup>*</sup>Ter119 depleted

---

**Fig. 6.** (A) VE-cadherin<sup>CD45</sup> cells can be detected in all organs involved in the emergence (E12.5 yolk sac), migration (E12.5 blood) and expansion (E13.5 liver) of HSCs. The percentage of cells in each quadrant is indicated. Data are representative examples from six experiments. Each contour plot is composed from 1×10<sup>5</sup> YS, 1×10<sup>5</sup> (Ter119<sup>+</sup>) PB and 3×10<sup>5</sup> FL. Quadrants are based on appropriate isotype control staining (see Fig. S1 in the supplementary material). (B) Circulating double-positive cells of E12.5 peripheral blood show attenuated expression of endothelial markers. Each analysis was made using data from 80-110 DP, 1000-4000 endothelial or 8000-20,000 haematopoietic cells. (C) Within the E13.5 liver, some HSCs remain associated with the DP fraction. However, the majority of HSCs reside within the haematopoietic (VE-cadherin<sup>CD45</sup>) fraction. This is reflected in the upregulation of essential stem cells markers (TIE2, KIT, SCA1 and MAC1) in the haematopoietic population. Each analysis was made using data from 1000-4000 DP, 8000-20,000 endothelial or 30,000-70,000 haematopoietic cells. All data are representative of 2-4 experiments for each marker analysed. Isotype control (white) and specific antibody staining (grey) are presented.
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Fig. 5. (A) High-level chimerism could be detected in all major adult haematopoietic organs following the transplantation of VE-cadherin-expressing HSC candidates from the E11.5 AGM region, E12.5 peripheral blood and E13.5 foetal liver. There is significant contribution to short-lived granulocytes/monocytes (MAC-1GR1) and pro/pre-B-cells (B220CD43) within the bone marrow, and immature CD4CD8 thymic T-cells 12 weeks after transplantation. (B) Successful haematopoietic reconstitution of secondary recipients demonstrates the long-term self-renewal potential of VE-cadherin+ stem cells. The percentage of cells in each gate/quadrant is indicated.

Definitive HSCs in the E12.5 YS express VE-cadherin

Other organs actively involved in HSC development were also shown to contain rare double-positive cells: E12.5 YS (0.03%±0.01); E12.5 PB (0.005%±0.002) and E13.5 foetal liver (0.1%±0.04) (Fig. 6A and Table 1). Between E11.5 and E13.5 the liver hosts the largest population of VE-cadherin+CD45+ cells. Although the absolute number of DP cells increases between E11.5 and E13.5 (see Table S1 in the supplementary material), the frequency decreases from 1.2% of total E11.5 cells to 0.3% at E12.5, and 0.1% by E13.5 (Table 1), reflecting a dilution by other proliferative cells.

At the peak of HSC activity in the YS at E12.5 (Kumaravelu et al., 2002), sorted VE-cadherin+ and VE-cadherin− cell populations were transplanted into irradiated adults. Out of 10 recipients that received transplants of VE-cadherin+ cells, seven mice were repopulated at the range of 5-87% with an average of 33% (Table 2). No reconstituting capacity was detected within the VE-cadherin− fraction. CD45+ and CD45− fractions from E12.5 YS were also separately transplanted. Three out of three mice were repopulated with CD45+ cells (61-87% range of chimerism with an average of 74%), whereas one out of four recipients was repopulated with the CD45− fraction, possibly reflecting contamination with CD45− cells.

We conclude that as in the E11.5 AGM all definitive HSCs in the E12.5 YS reside within the DP population. As the E12.5 YS contains about two HSCs (Kumaravelu et al., 2002), the DP population in this tissue is highly enriched for HSC activity (1/64 cells) (Table 3). Thus, HSCs emerging both in the E11.5 AGM and E12.5 YS co-express VE-cadherin and CD45.

Downregulation of VE-cadherin in E12.5 AGM HSCs

By E12.5 the HSC activity in the AGM region decreases (Kumaravelu et al., 2002). At this more advanced stage (in contrast to E11.5), the AGM region contains some VE-cadherin− cells that are capable of repopulating (Table 2): all HSCs in E12.5 AGM region however remain CD45+. These data suggest that VE-cadherin is downregulated as HSC development progresses.

VE-cadherin phenotype of placental CD34+Klr fraction

Recent reports have shown that HSCs in the placenta reside within CD34+KIT+ fraction (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). We therefore analysed VE-cadherin and CD45 expression in this placental population (Fig. 7A-C). At E11.5 the entire CD34+KIT+ population was VE-cadherin+CD45+ (Fig. 7B). At E12.5, the CD34+KIT+ population retains CD45+ but only 30±3.0% remain VE-cadherin+ (Fig. 7C), indicating that, as in the AGM region, downregulation of VE-cadherin at the phenotypic level may occur in the placental HSC fraction.

Most definitive HSCs in the E13.5 foetal liver and all HSCs in the adult bone marrow are VE-cadherin negative

HSCs born in extra hepatic sites colonise the foetal liver via the circulation. Therefore we investigated the phenotype of HSCs present in the E13.5 foetal liver. Following the purification of VE-cadherin+CD45+ and VE-cadherin CD45− fractions each recipient received 0.02 embryo equivalent (e.e.)
A further increase in CD41 representation was observed in the endothelial, haematopoietic and DP populations of the E13.5 liver (Fig. 6C). Growth in numbers of CD41 haematopoietic cells during development may correlate with progressive production of megakaryocytes, or with the broad expression in embryonic haematopoietic cells.

### Developmental downregulation of VE-cadherin within the HSC pool is not dependent on the liver microenvironment

To determine whether direct contact with the liver was required for the loss of VE-cadherin expression, we explored whether VE-cadherin- HSCs would develop in organ explants in vitro.

We therefore used an organ culture model which we have previously shown to be permissive for expansion of HSC in the E11.5 AGM region and E12.5 YS (Kumaravelu et al., 2002), thus allowing us to test whether VE-cadherin-negative HSCs would develop independently of the liver micro environment.

We found that by the end of the culture period, significant fractions of VE-cadherin-negative HSCs emerged in both the isolated AGM and the yolk sac (Table 4, see also Fig. S2 in the supplementary material). The proportions of mice reconstituted with VE-cadherin* and VE-cadherin* AGM fractions were similar (seven out of 11 mice and seven out of nine mice reconstituted, respectively). Efficient repopulation was also observed with yolk sac VE-cadherin* and VE-cadherin* fractions (seven out of 10 mice and 10 out of 10 mice, respectively) (Table 4). Assuming that the ex vivo generation of HSCs reflects the in vivo pathway with reasonable fidelity, it is therefore likely that the downregulation of VE-cadherin within the HSC pool is a function of an intrinsic developmental time. This process, as hypothesised by the authors, is presented in Movie 1 (see supplementary material).

### Discussion

In the mouse embryo, HSCs first emerge by late E10.5-E11.5 in the AGM region and umbilical vessels and, perhaps with a slight delay, in the YS (Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Detailed temporal analysis revealed that the peak of HSC activity in the E11.5 AGM region is overtaken subsequently by E12.5 YS (Kumaravelu et al., 2002), reinforcing the idea that some HSCs have an extra-embryonic origin (Moore and Metcalf, 1970; Toles et al., 1989; Weissman et al., 1978; Yoder et al., 1997). The recent identification of the placenta as an abundant niche for HSCs suggests a potentially important role in embryonic development of HSCs (Gekas et al., 2005; Ottersbach and Dzierzak, 2005).

The idea that the haematopoietic and endothelial systems have a common cellular origin was originally based on: (1) morphological similarities observed between developing haematopoietic and endothelial cells in YS blood islands (Sabin, 1920), (2) clusters of haematopoietic cells that are believed to be budding from the floor of the dorsal aorta; and (3) a large number of shared genetic markers (Godin and Cumano, 2002). Haematopoietic development via an endothelial differentiation pathway has been analyzed experimentally using in vitro differentiation assays based on Flk1 and VE-cadherin expression (Chung et al., 2002; Kennedy et al., 1997; Lacaud et al., 2002; Nishikawa et al., 1998a; Nishikawa et al., 1998b). Cells with haemangioblastic characteristics have been reported in the early primitive streak (Huber et al., 2004) and the adult haematopoietic system (Bailey et al., 2004; Pelosi et al., 2002).

We have focused on cells co-expressing VE-cadherin and CD45, reasoning that if the definitive haematopoietic system originates from the embryonic endothelium or the haemangioblast, then the first founder HSC may inherit endothelial features. Previous data corroborate this idea (de Bruijn et al., 2002; North et al., 2002). We have followed here the evolution of the dual endothelial and haematopoietic character of highly repopulating definitive HSCs through key developmental stages: (1) initiation in the E11.5 AGM and E12.5 YS; (2) circulation in peripheral blood (E12.5); and (3) settling in the foetal liver (E13.5).

VE-cadherin and CD45 are predominantly mutually exclusive with DP cells representing a minority of about 0.05% in embryonic haematopoietic tissues. We used flow cytometric cell sorting to purify populations based on VE-cadherin and CD45 expression and, using in vitro methylcellulose assay found that the highest frequency of haematopoietic progenitors was present in the DP population. We also confirmed that long-term highly repopulating HSCs in the E11.5 AGM reside within this fraction. Furthermore, at the peak of stem cell activity in the E12.5 YS, HSCs also reside within the VE-cadherin expressing fraction. The enrichment of HSCs in the DP populations is very high: the E11.5 AGM and E12.5 YS contain one HSC in 70 and one HSC in 64 DP cells, respectively. Thus, HSC emergence in both extra-hepatic haematopoietic organs is associated with VE-cadherin expression.

Further phenotypic characterization revealed striking similarity between the DP and endothelial cells in the E11.5 AGM region. Both the endothelial and DP populations are largely TIE2*FLK1*PECAM1*Ac-LDL-receptor*+. The haematopoietic fractions are either negative for these markers or express them at low level. The analysis of other 'HSC markers'
found that all repopulating activity was associated with the VE-cadherin-negative fraction (Table 2).

Thus, although HSCs express VE-cadherin at their initiation in the E11.5 AGM region and E12.5 YS, adult bone marrow HSCs do not express this marker. As both VE-cadherin+ and VE-cadherin− HSCs exist in E12.5 AGM, and placenta and E13.5 liver these data collectively suggest that VE-cadherin expression is progressively lost as a function of HSC maturation.

Definitive HSCs preserve VE-cadherin expression while travelling via the embryonic circulation

We then determined if loss of VE-cadherin in the HSC pool occurs before or after liver colonization. Following erythrocyte depletion, VE-cadherin+ and VE-cadherin− fractions were flow sorted from E12.5 circulation and transplanted into irradiated mice. Although eight out of 12 mice that received VE-cadherin+ cells showed 10-90% leukocyte chimerism (average of 54%), only one out of the 10 recipients of the VE-cadherin−cells were successfully repopulated (Table 2). Therefore, as in the E11.5 AGM and E12.5 YS the majority if not all of circulatory HSCs continue expression of VE-cadherin before colonising the liver.

Progressive phenotypic divergence of HSCs from the endothelial compartment

The emergence of extra-hepatic HSCs occurs in close ontogenic relation with the endothelial compartment. We therefore analysed how the HSCs and endothelial compartments phenotypically diverge during crucial developmental stages, in the E12.5 blood and the E13.5 liver.

In the E11.5 AGM region, TIE2 expression was observed in both endothelial and DP populations, but not in haematopoietic cells (Fig. 2B). A similar but less pronounced tendency was observed in the E12.5 circulation (Fig. 6B). However, in the E13.5 liver a significant proportion of haematopoietic cells (15%) expressed TIE2 (Fig. 6C), which is consistent with the appearance of HSCs in this fraction and expression of TIE2 in foetal liver HSCs (Hsu et al., 2000).

The vast majority of cells in the endothelial populations of the E11.5 AGM region and the E13.5 liver expressed Flk1 (Shalaby et al., 1997) at low level (Fig. 2A; Fig. 6C). The majority of DP cells also demonstrated a FLK1low phenotype in these locations. Expression of FLK1 in circulatory cells was negligible. No marked FLK1 expression was observed in the haematopoietic population in any of the organs analyzed.

PECAM1 was expressed to a certain extent in all of the cell fractions during development (Fig. 2B; Fig. 6B,C). However, in haematopoietic cells PECAM1 was expressed at significantly lower level than in the endothelial and the DP fractions. The DP population in all tissues showed bimodal (PECAM1low and PECAM1high) staining profile. Significant expression of PECAM1 in E12.5 circulation was observed only in the DP fraction (Fig. 6B). Interestingly, HSCs at the pre-definitive stage in the yolk sac and in the adult bone marrow are PECAM1 positive (Baumann et al., 2004).

Ac-LDL uptake was observed at high level by the endothelial and the DP fractions in all tested locations (Fig. 2B; Fig. 6B,C). Lower level of uptake was found in the haematopoietic fractions with the exception of the E13.5 liver which did not take up any Ac-LDL (Fig. 6C).

The endothelial populations in all of the organs investigated showed a strong expression of KIT. Both in the E11.5 AGM region and in the E13.5 liver the DP populations were also significantly enriched for KIT+ cells in comparison with haematopoietic cells (Fig. 2B; Fig. 6C). A significant proportion of cells within the DP fraction of the E12.5 circulation also expressed KIT+ cells (Fig. 6B). In general, the level of KIT expression increased over time among all cell fractions.

SCA1 expression is absent in the early yolk sac and only appears on the emergence of definitive haematopoiesis (de Bruijn et al., 2002; Lu et al., 1996). The endothelial and the DP fractions expressed SCA1 at higher levels than the haematopoietic fraction. By E12.5 both the endothelial and DP populations within circulation comprise substantial subsets of SCA1high cells (Fig. 6B). The level of SCA1 expression in circulating DP cells is remarkably high. In the E13.5 foetal liver SCA1 is expressed at elevated levels in all three populations (Fig. 6C).

Within the E12.5 circulation and the E13.5 foetal liver MAC1 continues to mark both the DP and the haematopoietic populations (Fig. 6B,C). MAC1 is fully excluded from the endothelial compartment of the AGM region, peripheral blood or liver (Fig. 2B; Fig. 6B,C).

In the E11.5 AGM region α4-integrin expression marks ∼3% of endothelial cells but in the DP and haematopoietic populations virtually all cells express α4-integrin. In the E12.5 circulation, the expression in the endothelial fractions is absent and is restricted to ∼52% of the DP population (Fig. 6B). In the E13.5 liver the proportion of α4-integrin-expressing cells is markedly increased in all three populations (Fig. 6C).

Very few cells in the E11.5 AGM were CD41 positive (Fig. 2B). In the E12.5 circulation CD41 expression in the DP and the haematopoietic fractions was markedly increased (Fig. 6B).
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Divergence of haematopoietic stem cells from endothelium

region in vivo has also shown that VE-cadherin is progressively downregulated in HSCs with time.

We also tested multi-lineage contribution of VE-cadherin\(^*\) and VE-cadherin\(^*\) foetal liver HSCs into recipient haematopoietic system. Although no obvious differences have been observed between these two cell types, it needs to be elucidated further if the emergence of ‘advanced’ VE-cadherin CD45\(^*\) HSCs is associated with the appearance of some novel functional characteristics.

In summary, we show that definitive HSCs emerging in pre-liver embryonic sites are uniquely promiscuous. Not only does this fraction co-express the predominantly mutually exclusive VE-cadherin and CD45 determinants, but it also bears cardinal markers of endothelial and haematopoietic/stem cell differentiation. We show that this promiscuity is largely retained in circulatory HSCs. However, after seeding the foetal liver, a significant proportion of HSCs lose their endothelial identity. We show that this process does not depend on immediate contact with the liver, but rather appears to be a function of developmental time.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/113/18/4179/DC1

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Appendix 2: Flow cytometric analysis of methylcellulose colonies

To enable the rapid detection of myeloid multilineage differentiation in methylcellulose based medium (M3434) haematopoietic colonies derived from 0.5 embryo equivalents of whole E11.5 AGM region were purified by flow cytometry according to the plasma membrane expression of CD45, Ter119, c-Kit, Sca-1, CD41, Mac-1 and Gr-1; cytospin preparations were produced from purified fractions and subsequently visualised with May-Grunwald-Giemsa stains for morphological investigation.

Flow cytometric analysis and sorting experiments were performed following 8 days of differentiation.

Data presented are representative of two independent experiments.
Figure A2.1. The CD45\textsuperscript{+}Ter119\textsuperscript{+} fraction is exclusively comprised of erythroid cells (A); the leucocytic lineages are all found in the CD45\textsuperscript{+}Ter119\textsuperscript{−} fraction (B).
Figure A2.2. The mast cell lineage can be purified on the basis of c-Kit^{high}CD41^{low} expression (A) and the megakaryocytic lineage on the basis of c-Kit^{high}CD41^{high} expression (B).
Figure A2.3. Mast cells are purified in both the Sca-1<sup>low</sup>c-Kit<sup>high</sup> (A) and Sca-1<sup>high</sup>c-Kit<sup>high</sup> (B) fractions; no obvious difference in the morphology of Sca-1<sup>low</sup> and Sca-1<sup>high</sup> mast cells was observed. The Sca-1<sup>low</sup>c-Kit<sup>−</sup> fraction is composed on macrophages, neutrophils and erythroid lineages (C); the Sca-1<sup>high</sup>c-Kit<sup>−</sup> fraction, of the monocyte/macrophage lineage (D).
Figure A2.4. Neutrophils are exclusively found in the Mac-1$^{low}$Gr-1$^{high}$ fraction (A); macrophages, in the Mac-1$^{high}$Gr-1$^{low}$ fraction (B); the Mac-1$^{low}$Gr-1$^{low/neg}$ fraction comprises a mix of neutrophils, macrophages and blast-like cells (C).
Appendix 3: Scoring E11.5 AGM region methylcellulose colonies

To ensure the accuracy of colony identification in M3434 by gross morphology a qualitative study of E11.5 AGM region derived colonies was undertaken. In contrast to adult bone marrow colonies, which are generally scored between 10-14 days of differentiation, E11.5 AGM derived colonies are distinguishable between 7-9 days of differentiation.

All images were taken from colonies after 8 days of differentiation. Original magnification of cytospin images x600.
Figure A3.1. BFU-E produce colonies of a typical gross morphology characterised by clusters of pigmented red cells (Ai and ii). The presence of erythroid cells and megakaryocytes (Meg) (Bi) or erythroid cells (Ery) and macrophages (Mac) (Bii) is confirmed by the analysis of cytospin preparation under high magnification. Aii is a magnification of the boxed region in Ai.
Figure A3.2. CFU-Mac produce small dispersed colonies (Ai and ii) that are composed of large heavily vacuolated cells (B). Aii is a magnification of the boxed region in Ai.
Figure A3.3. Colonies derived from CFU-Mast are heterogeneous characterised by either a large dispersed colony (Ai and ii) composed of large cells that are apparently vacuolated (C) or a mix of tightly packed and dispersed cells that are reminiscent of CFU-GM (B) that are generally composed of heavily granulated cells (Di-ii). CFU-Mast is detected at higher frequency in the E11.5 AGM region than in the adult bone marrow; the gross morphology of CFU-Mast and CFU-GM can similae. Aii is a magnification of the boxed region in Ai.
Figure A3.4. CFU-GM derived colonies have a typical morphology characterised by a dense epicentre and diffuse periphery (A). Distinction between CFU-GM and CFU-Mast achieved through the identification of macrophages (Mac) and polymorphonuclear neutrophils (Neutro) (B).
Figure A3.5. CFU-GEMM produce either small or large colonies. A characteristic large colony is shown in (A). Granulocytes, erythroid cells (Ery), macrophages (Mac), megakaryocytes (Meg) and blast cells (Blast) can be identified in cytospin preparations (Bi-iii). Aii is a magnification of the boxed region in Ai.
Appendix 4: isotype control staining
Figure A4.1: E11.5 AGM region control 1
A representative example of E11.5 AGM region incubated with rat IgG2β,κ and rat IgG2α,κ antibodies. X-axis: FITC conjugated antibody; Y-axis: Biotinylated antibody visualised using APC conjugated streptavidin.

This contour plot is composed from approximately 1x10^5 viable (7-AAD^-) cells. Values indicate the percentage of positive cells.
Figure A4.2: Yolk sac, peripheral blood and foetal liver controls
Representative examples of E12.5 yolk sac (A), E12.5 peripheral blood (B) and E13.5 liver (C) incubated with rat IgG2a,κ and rat IgG2α,κ antibodies. X-axis: FITC conjugated antibody; Y-axis: Biotinylated antibody visualised using APC conjugated streptavidin.

Contour plot A is composed from approximately 5x10^4 viable (7-AAD^-) cells, contour plot B, from approximately 2x10^4 non-erythroid viable cells and contour plot C, from 2.1x10^5 viable cells. Values indicate the percentage of positive cells.
Figure A4.3: Placenta controls

Representative examples of E11.5 (A) and E12.5 placenta (B) incubated with rat IgG2β,κ and rat IgG2α,κ antibodies. X-axis: PE conjugated antibody; Y-axis: APC conjugated streptavidin.

Contour plots A and B are composed from approximately 1x10^5 viable (7-AAD^-) cells. Values indicate the percentage of positive cells.
Figure A4.4: Adult haematopoietic organ isotype control staining

(A) A representative example of erythrocyte depleted adult peripheral blood incubated with mouse IgG2α,κ antibodies. X-axis: FITC conjugated antibody; Y-axis: PE conjugated streptavidin.

(B) Representative examples of erythrocyte depleted adult bone marrow incubated with rat IgG2β,κ antibodies. X-axis: PE conjugated antibody; Y-axis: Biotinylated antibody visualised using APC conjugated streptavidin.

(C) Representative examples of erythrocyte depleted adult bone marrow incubated with rat IgG2α,κ antibodies. X-axis: Biotinylated antibody visualised using APC conjugated streptavidin; Y-axis: PE conjugated antibody.

(D) A representative example of erythrocyte depleted adult spleen incubated with rat IgG2α,κ and hamster IgG1,κ antibodies. X-axis: Biotinylated antibody visualised using APC conjugated streptavidin; Y-axis: PE conjugated antibody.

(E) A representative example of adult thymus thymocytes incubated with rat IgG2α,κ and rat IgG2β,κ antibodies. X-axis: PE conjugated; Y-axis: APC conjugated antibody.

Each contour plot is composed from approximately $1 \times 10^4$ viable (7-AAD−) cells. Values indicate the percentage of positive cells.
Figure A4.5: CFU-C derived colonies flow cytometry control

Following the differentiation of whole E11.5 AGM region in methylcellulose based medium for 10 days colonies were pooled and incubated with either:

(A) Rat IgG2β,κ antibodies. X-axis: PE conjugated; Y-axis: FITC conjugated.
(B) Rat IgG2β,κ antibodies. X-axis: PE conjugated; Y-axis: APC conjugated.
(C) Rat IgG2β,κ antibodies. X-axis: FITC conjugated; Y-axis: APC conjugated.

Representative examples of each condition are presented. Each contour plot is composed from approximately 3x10^4 viable (7-AAD^-) cells. Values indicate the percentage of positive cells.
Figure A4.6: Liquid suspension culture colony initiating unit flow cytometry controls

Following the differentiation of VE-cadherin$^+$CD45$^+$ under conditions of liquid suspension culture well containing colony type 2 were pooled and incubated with either:

(A) Rat IgG2β,κ antibodies. X-axis: PE conjugated; Y-axis: FITC conjugated.
(B) Rat IgG2β,κ antibodies. X-axis: PE conjugated; Y-axis: APC conjugated.

Representative examples of each condition are presented. Each contour plot is composed from approximately $3\times10^4$ viable (7-AAD$^-$) cells. Values indicate the percentage of positive cells.
Figure A4.7: E11.5 AGM region ex vivo culture comparison controls

72 hours after either explant culture (A), liquid suspension culture (B) or reaggregation in the presence of IMDM+ medium derivative of the E11.5 AGM region were incubated with rat IgG2β,κ and IgG2α,κ antibodies. X-axis: FITC conjugated; Y-axis: Biotinylated antibody visualised using APC conjugated streptavidin.

Representative examples of each condition are presented. Contour plot is composed from approximately 1.0x10^5 viable (7-AAD⁻) cells, contour plot B, from 1.5x10^4 viable cells and contour plot C, from 2x10^4 viable cells. Values indicate the percentage of positive cells.
Figure A4.8: IMDM$^+$ reaggregate confocal microscopy controls

(A) A representative example of a 10$\mu$m section incubated with APC conjugated rat IgG2$\beta$,$\kappa$ (green) and PE conjugated rat IgG2$\alpha$,$\kappa$ (red). Original magnification x200; bar represents 75$\mu$m.

(B) A representative example of IMDM$^+$ reaggregate incubated with APC conjugated rat IgG2$\beta$,$\kappa$ (green) and PE conjugated rat IgG2$\alpha$,$\kappa$ (red). Original magnification x100; bar represents 75$\mu$m.

In all images nuclei are stained with DAPI (blue).
Figure A4.10. E11.5 AGM region flow cytometry control 2.

A representative example of E11.5 AGM region incubated with rat IgG2β,κ and rat IgG2α,κ antibodies. X-axis: APC conjugated antibody; Y-axis: PE conjugated.

This contour plot is composed from approximately $6 \times 10^4$ viable (7-AAD-) cells. Values indicate the percentage of positive cells.
Figure A4.11 Ventral dorsal aorta whole mount confocal microscope control
Ventral dorsal aorta from the E11.5 AGM region was incubated with rat IgG2β,κ (green) and rat IgG2α,κ (red) antibodies. Nuclei are stained with DAPI (blue). Original magnification x200; bar represents 75μm.

Presented image is representative of five independent experiments.
Figure A4.12 Thin section confocal microscopy control

A 10μm section through the E11.5 AGM region was incubated with rat IgG2β,κ (green) and rat IgG2α,κ (red) antibodies. In all images nuclei are stained with DAPI (blue). Original magnification x200; bar represents 75μm.

Presented image is representative of three independent experiments.
Appendix 5: Bisection of the dorsal aorta from the E11.5 AGM region

Images A-B; C-D; E-F are representative confocal photomicrographs of aortae bisected along the mid-line resulting in the separation of dorsal and ventral aspects. PECAM-1 expression is represented in red (PE); CD45, green (APC); nuclei (DAPI), blue. Note the presence of the notochord (Nc) on the dorsal aspect.

Bar represents 70μm.

Images are representative of the bisection of four individual organs.
Figure A6: E11.5 dorsal aorta isotype control

(A-D) Representative confocal microscopy images of bisected incubated with rIgG2β,κ-APC (green) and rIgG2β,κ-PE (red). Nuclei are stained with DAPI (blue). Bar represents 70μm.
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