

# Progressive divergence of definitive haematopoietic stem cells from the endothelial compartment does not depend on contact with the foetal liver

Samir Taoudi\*, Aline M. Morrison\*, Hirofumi Inoue, Ruby Gribi, Janice Ure and Alexander Medvinsky†

MRC Centre Development in Stem Cell Biology, Institute for Stem Cell Research, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JQ, UK

\*These authors contributed equally to this work

†Author for correspondence (e-mail: a.medvinsky@ed.ac.uk)

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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-cadherin<sup>+</sup>CD45<sup>+</sup> 'double-positive' population and endothelial cells suggesting a common origin for these cells. The double-positive fraction also expressed the stem cell markers Kit, Sca1 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<sup>+</sup> 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<sup>+</sup>KIT<sup>+</sup> 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-cadherin<sup>+</sup>CD45<sup>+</sup> to a more 'advanced' VE-cadherin<sup>-</sup>CD45<sup>+</sup> 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<sup>+</sup>VE-cadherin<sup>-</sup> 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

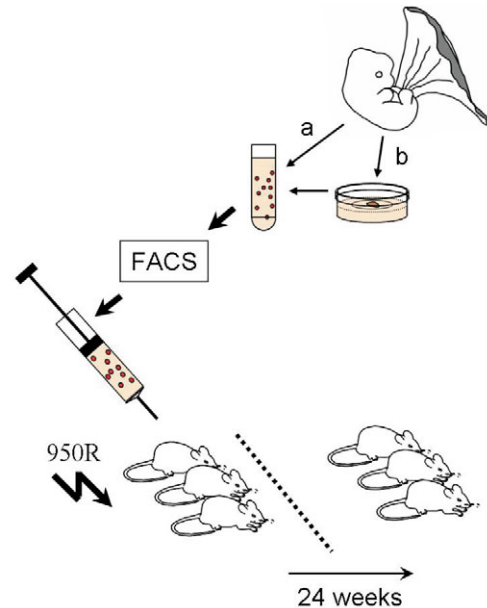
using ES cell differentiation models have shown that adult-type clonogenic haematopoietic progenitors can originate from the endothelial VE-cadherin<sup>+</sup>CD45<sup>-</sup> cells (Fujimoto et al., 2001). The relationship between endothelial and haematopoietic cells has also been clonally explored 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<sup>+</sup> progenitor cells committed to endothelio-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<sup>+</sup> 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

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 budding 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 vasculo- 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 Nakauchi, 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



**Fig. 1.** Candidate cell fractions from E11.5-E13.5 embryonic organs were purified by flow cytometry and transplanted into lethally irradiated adult recipients. High-level (>5%) long-term multi-lineage contribution to haematopoiesis was determined after 24 weeks. Path 'a' represents the transplantation of freshly dissected organs, whereas path 'b' indicates the additional 3-day explant culture step prior to transplantation.

developmental switch of HSCs from the VE-cadherin positive to negative phenotype does not require contact with the liver.

## 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 Thelie criteria [for example, E11-E11.5 (41-47 somite pairs) is equal to stages 18 and 19] (<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,  $\alpha$ 4-integrin-PE, B220-biotin, CD3 $\epsilon$ -PE, CD4-PE, CD8 $\alpha$ -Biotin, CD34-FITC/PE, CD41-FITC, CD45-PE/FITC, KIT

(also known as C-KIT)-PE/APC, FLK1-PE, GR1-Biotin, Ly5.1-PE, Ly5.2-FITC, 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 throughout the paper in embryo equivalents (e.e.), defined as a unit of (sorted) cells equivalent to the number of cells of that phenotype contained in one organ. The transplanted bone marrow cells are expressed in HSC equivalents (HSC.e.): one HSC.e. is equivalent to 10,000 nucleated bone marrow cells, which harbours 1 HSC (Kumaravelu et al., 2002).

### 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 (Improvion). 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 tubule/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 PECAM1<sup>+</sup> tubules was counted after 4 days. A two-step anti-PECAM1 staining protocol was followed to identify endothelial development; following a primary anti-PECAM1 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.

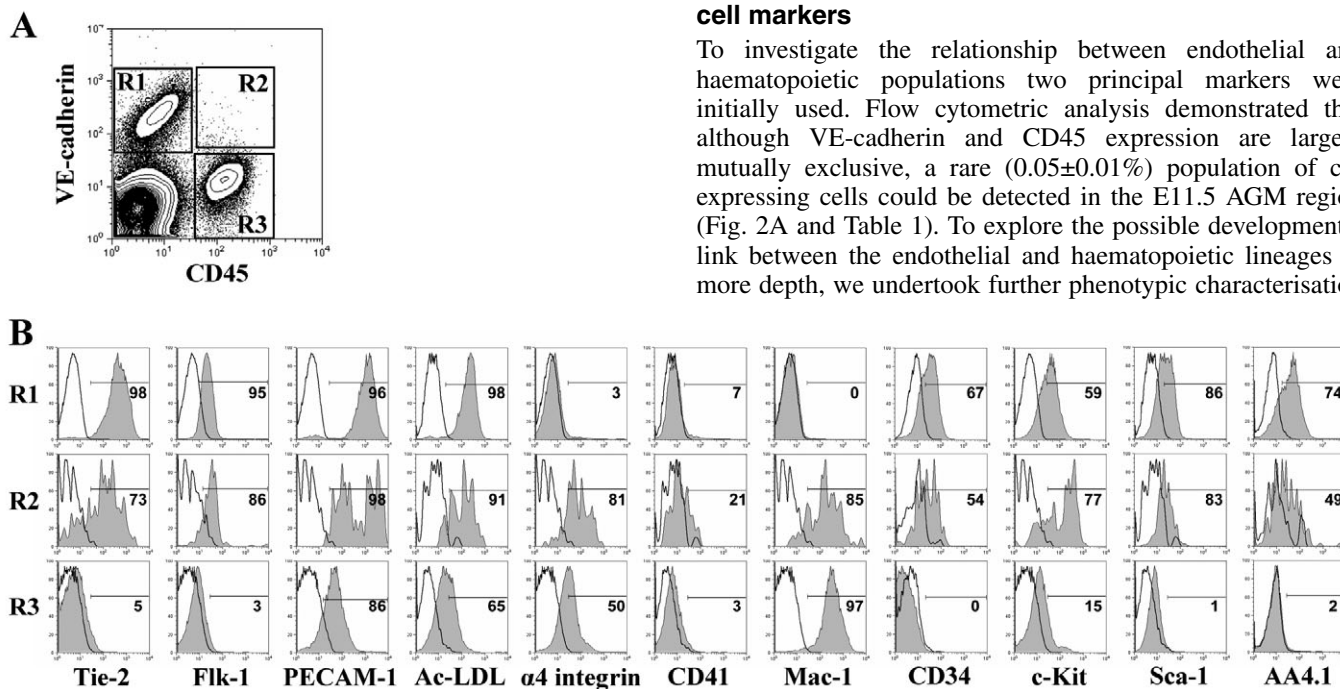
### Single-cell progenitor assay

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  $\alpha$ -MEM medium supplemented with FCS (10%), EPO (1U/ml), IL3 (200U/ml), Pokeweed mitogen spleen-conditioned medium (2%) 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 ( $0.05 \pm 0.01\%$ ) 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<sup>+</sup>CD45<sup>-</sup>; R1), haematopoietic (VE-cadherin<sup>-</sup>CD45<sup>+</sup>; R3) and double-positive (DP) (VE-cadherin<sup>+</sup>CD45<sup>+</sup>; R2) cell populations. The contour plot shown is a representative example compiled from approximately 10e.e. of E11.5 AGM ( $1.5 \times 10^6$  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 1. Statistical analysis of VE-cadherin and CD45 expression in major E11.5-E13.5 haematopoietic organs during key stages of HSC development**

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

of the endothelial (VE-cadherin<sup>+</sup>CD45<sup>-</sup>), double-positive (DP) (VE-cadherin<sup>+</sup>CD45<sup>+</sup>) and haematopoietic (VE-cadherin<sup>-</sup>CD45<sup>+</sup>) 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

compartment, the entire VE-cadherin<sup>+</sup>CD45<sup>+</sup> population expressed KIT. However, in contrast to both the endothelial and haematopoietic lineages, this fraction was greatly enriched for KIT<sup>high</sup> cells. Unlike the haematopoietic population, all endothelial and DP cells expressed low levels of SCA1. AA4.1 and CD34, also associated with the embryonic HSCs phenotype (Bertrand et al., 2005; Petrenko et al., 1999; Sanchez et al., 1996), were expressed at a similar level in both the endothelial and the DP populations but were not expressed in the haematopoietic fraction (Fig. 2B).

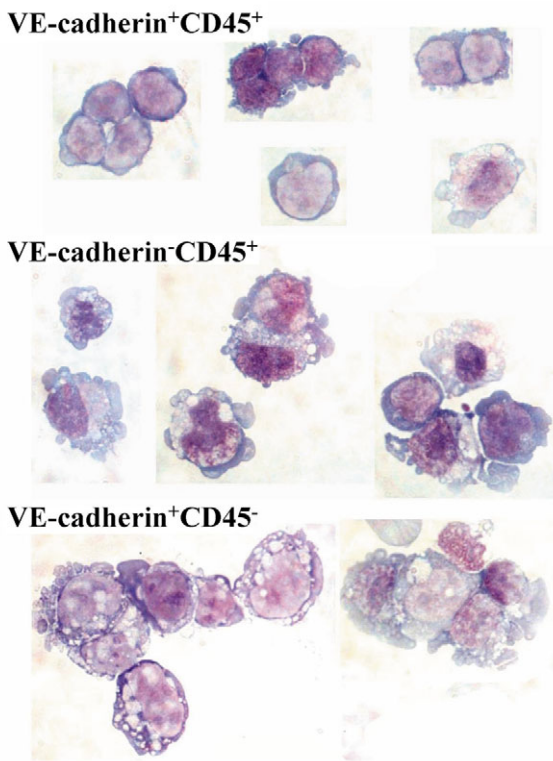
We then analyzed the expression of MAC1, which is present in some AGM HSCs and in all foetal liver HSCs (Morrison et al., 1995; Sanchez et al., 1996) and found that the majority of the DP cells express high levels of MAC1. Virtually all cells of the haematopoietic population were also MAC1<sup>+</sup>.

To understand more precisely the identity of the DP fraction markers associated with early haematopoietic differentiation were also examined.  $\alpha$ 4-Integrin labels a large number of adult haematopoietic cells (Arroyo et al., 1996) and marks haematogenic endothelial activity (Ogawa et al., 1999). In the E11.5 AGM region  $\alpha$ 4-integrin expression marks ~3% of endothelial cells but in the DP and haematopoietic populations virtually all cells express  $\alpha$ 4-integrin (Fig. 2B). Finally, the expression of CD41, a traditional megakaryocyte/platelet marker implicated in early haematopoietic development was also analysed (Bertrand et al., 2005; Emambokus and Frampton, 2003; Ferkowicz et al., 2003; Mikkola et al., 2003; Mitjavila-Garcia et al., 2002). A fraction of the DP population was found to contain CD41-positive cells.

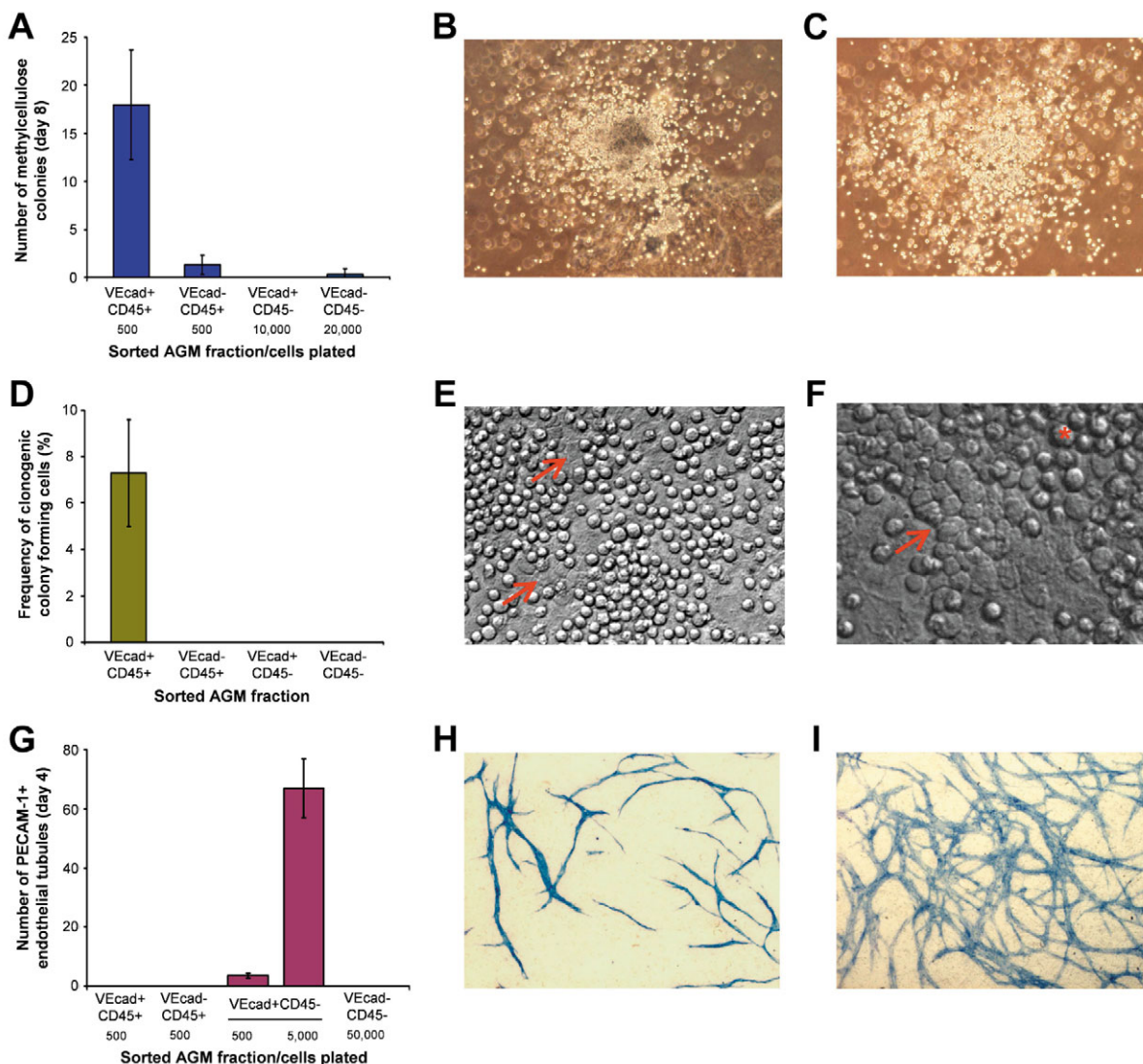
Thus, the DP population is uniquely promiscuous. Not only does this fraction co-express the predominantly mutually exclusive VE-cadherin and CD45 determinants, but it also bears cardinal markers of endothelial and haematopoietic/stem cell differentiation.

### E11.5 AGM clonogenic myeloid progenitors are enriched in the DP fraction

We next investigated the morphology of the E11.5 AGM endothelial (VE-cadherin<sup>+</sup>CD45<sup>-</sup>), haematopoietic (VE-cadherin<sup>-</sup>CD45<sup>+</sup>) and DP (VE-cadherin<sup>+</sup>CD45<sup>+</sup>) populations. Cytospin preparations from sorted fractions revealed that the endothelial fraction contained mainly cells with large patchy stained nuclei and blebbing cytoplasm (Fig. 3). Vacuolated cytoplasm of endothelial cells outlining the embryonic dorsal aorta was noticed previously on histological sections (North et al., 1999). The majority of DP cells demonstrated a high



**Fig. 3.** Morphology of E11.5 AGM endothelial, double-positive and haematopoietic populations. Cells with blast morphology are highly enriched within the VE-cadherin<sup>+</sup>CD45<sup>+</sup> population (May-Grunwald-Giemsa staining of cytospin preparations).



**Fig. 4.** 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<sup>-</sup>CD45<sup>+</sup>) population. Tight (B) and diffuse colonies (C) are predominantly derived from VE-cadherin<sup>+</sup>CD45<sup>+</sup> cells. (D) Clonal analysis of sorted VE-cadherin<sup>+</sup>CD45<sup>+</sup> 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<sup>+</sup>CD45<sup>-</sup> population. After 4 days of co-culture with OP9 stromal cells, 60 PECAM1<sup>+</sup> 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.

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<sup>-</sup>CD45<sup>-</sup>) cells were capable of generating haematopoietic colonies.

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<sup>+</sup>CD45<sup>-</sup> 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

**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**

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

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

<sup>†</sup>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.

<sup>‡</sup>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<sup>+</sup>CD45<sup>-</sup>) population: 500 endothelial cells formed about four PECAM1<sup>+</sup> 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<sup>-</sup>CD45<sup>-</sup> 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<sup>+</sup>CD45<sup>+</sup> population. From a cohort of 12 adult recipients, four were repopulated with the DP population, with a range of 5-60% peripheral blood leukocyte 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<sup>-</sup>CD45<sup>+</sup> 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.

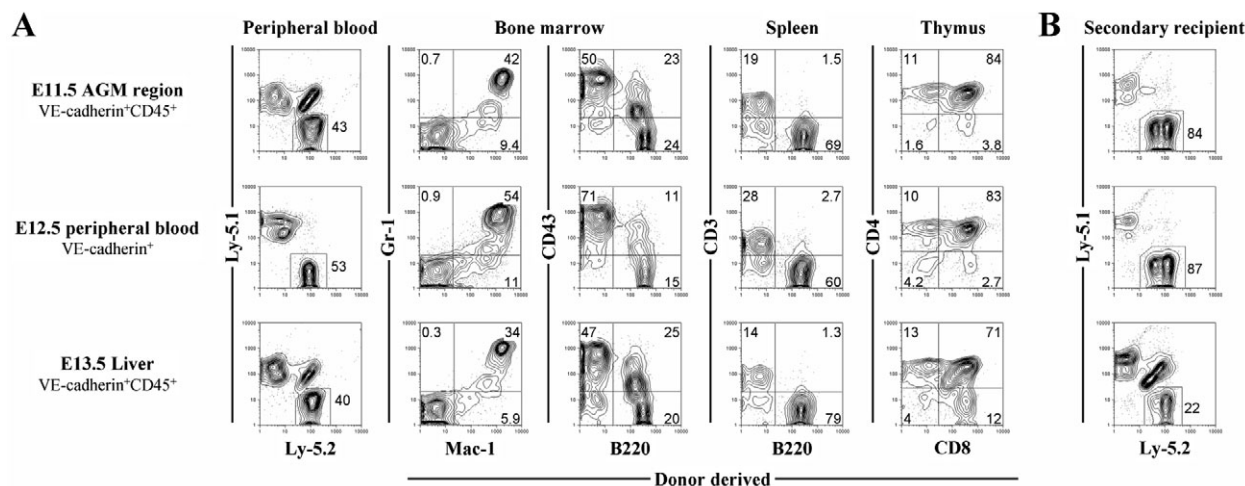
It has been shown that VE-cadherin<sup>+</sup> cells from the E9.5 YS are restricted in their capacity for effective myeloid differentiation

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

Embryo age	Organ	Cells per organ (1 × 10 <sup>5</sup> )*	HSCs per organ <sup>†</sup>	HSC candidate phenotype	Estimated HSC frequency
11.5	AGM	1.4 ± 0.12	1	VE-cadherin <sup>+</sup> CD45 <sup>+</sup>	~1/70
				VE-cadherin <sup>-</sup> CD45 <sup>+</sup>	–
12.5	YS	2.1 ± 0.2	2	VE-cadherin <sup>+</sup> CD45 <sup>+</sup>	~1/64
				VE-cadherin <sup>-</sup> CD45 <sup>+/–</sup>	–
	PB	28.7 ± 2.9	3	VE-cadherin <sup>+</sup> (CD45 <sup>+</sup> )	~1/48
				VE-cadherin <sup>-</sup> (CD45 <sup>+/–</sup> )	–
13.5	FL	63.6 ± 2.5	260	VE-cadherin <sup>+</sup> CD45 <sup>+</sup>	~1/127
				VE-cadherin <sup>+</sup> CD45 <sup>+</sup>	~1/2,226

\*Dead cells excluded according to 7-AAD uptake.

<sup>†</sup>Previously determined by limiting dilution analysis in unsorted organs (Kumaravelu et al., 2002).



**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 (MAC1<sup>+</sup>GR1<sup>+</sup>) and pro/pre-B-cells (B220<sup>+</sup>CD43<sup>+</sup>) within the bone marrow, and immature CD4<sup>+</sup>CD8<sup>+</sup> thymic T-cells 12 weeks after transplantation. (B) Successful haematopoietic reconstitution of secondary recipients demonstrates the long-term self-renewal potential of VE-cadherin<sup>+</sup> stem cells. The percentage of cells in each gate/quadrant is indicated.

following engraftment in neonatal animals (Fraser et al., 2002). On occasions, we also observed a bias towards lymphoid contribution from E11.5 AGM and E12.5 PB HSCs. Therefore, as long-lived lymphoid cells can circulate in the absence of HSCs, we performed a more detailed analysis. We found long-term production of short-lived donor-derived MAC1<sup>+</sup>GR1<sup>+</sup> granulocytes especially in bone marrow (Fig. 5A). We also demonstrated donor-derived CD43<sup>+</sup>B220<sup>+</sup> pre/pro-B-cells in bone marrow and CD4<sup>+</sup>CD8<sup>+</sup> cells in the thymus, demonstrating long-term contribution of donor HSCs. Furthermore, successful secondary transplantation demonstrates contribution into the stem cell compartment of the primary recipient (Fig. 5B).

### 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<sup>+</sup>CD45<sup>+</sup> 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<sup>+</sup> and VE-cadherin<sup>-</sup> cell populations were transplanted into irradiated adults. Out of 10 recipients that received transplants of VE-cadherin<sup>+</sup> 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<sup>-</sup> fraction. CD45<sup>+</sup> and CD45<sup>-</sup> fractions from E12.5 YS were also separately transplanted. Three out of three mice were repopulated with CD45<sup>+</sup> cells (61–87% range of chimerism with an average of 74%), whereas one out of four recipients was repopulated with the CD45<sup>-</sup> fraction, possibly reflecting contamination with CD45<sup>+</sup> 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<sup>-</sup> cells that are capable of repopulating (Table 2): all HSCs in E12.5 AGM region however remain CD45<sup>+</sup>. These data suggest that VE-cadherin is downregulated as HSC development progresses.

### VE-cadherin phenotype of placental CD34<sup>+</sup>KIT<sup>+</sup> fraction

Recent reports have shown that HSCs in the placenta reside within CD34<sup>+</sup>KIT<sup>+</sup> 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<sup>+</sup>KIT<sup>+</sup> population was VE-cadherin<sup>+</sup>CD45<sup>+</sup> (Fig. 7B). At E12.5, the CD34<sup>+</sup>KIT<sup>+</sup> population retains CD45<sup>+</sup> but only 30±3.0% remain VE-cadherin<sup>+</sup> (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

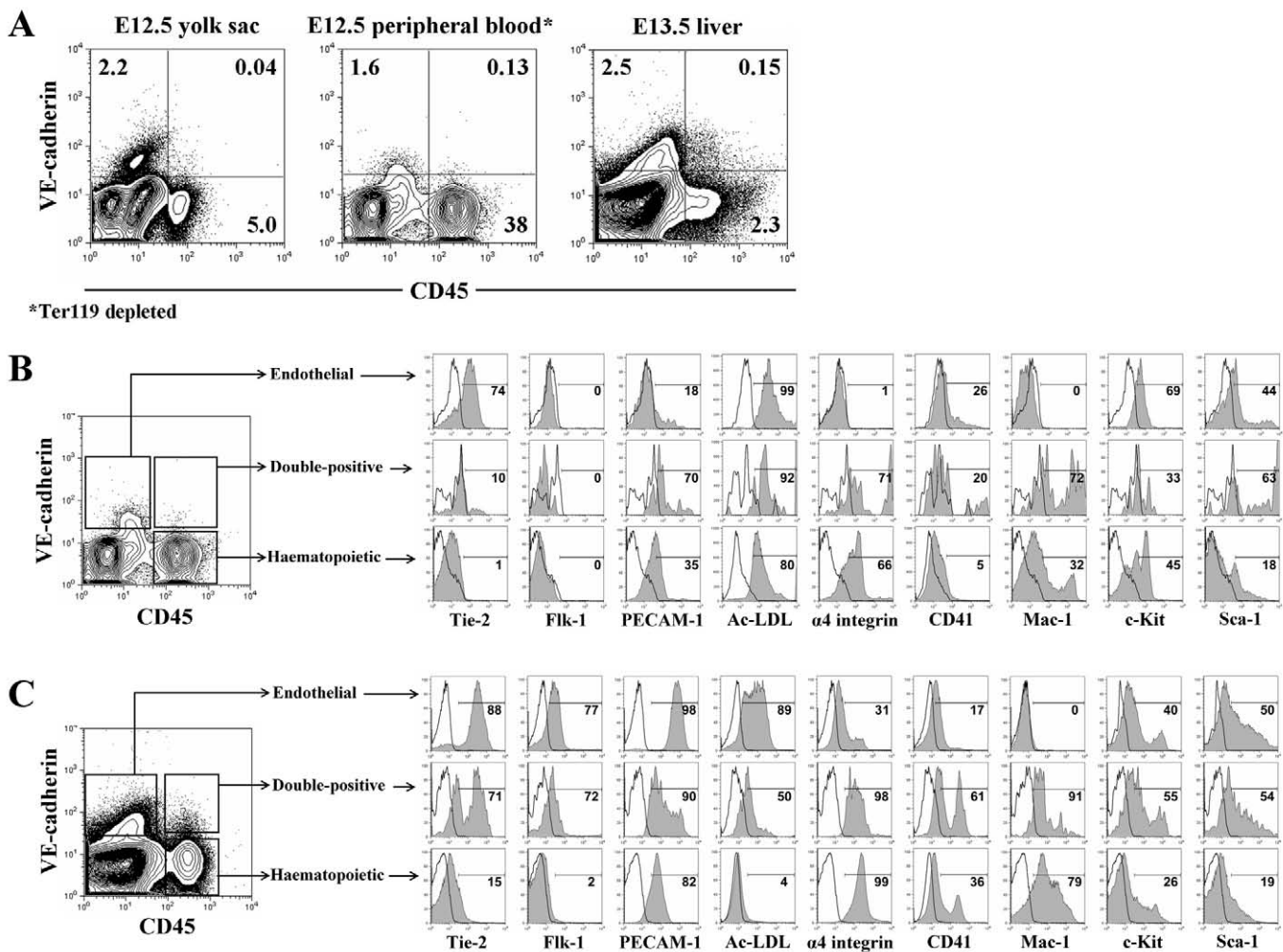
HSCs born in extra hepatic sites colonise the foetal liver via the circulation. Therefore we investigated the phenotype of HSCs present in the E13.5 foetal liver. Following the purification of VE-cadherin<sup>+</sup>CD45<sup>+</sup> and VE-cadherin<sup>-</sup>CD45<sup>+</sup> fractions each recipient received 0.02 embryo equivalent (e.e.)

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>+</sup>CD45<sup>+</sup> and VE-cadherin<sup>-</sup>CD45<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>-</sup>CD45<sup>+</sup>) fraction was able to

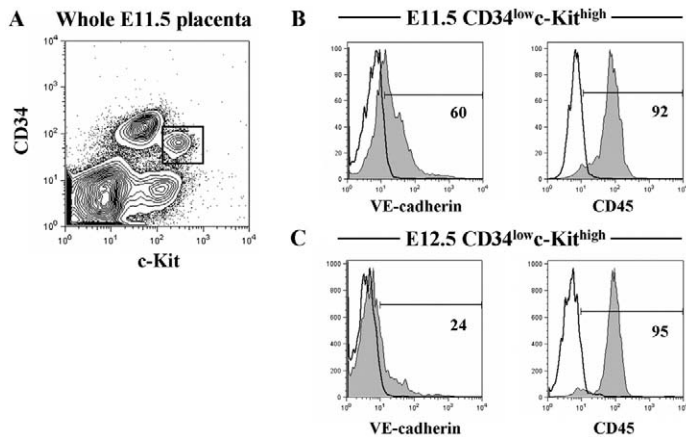
reconstitute all five of the animals tested so we can predict that there were more than five HSCs present in the transplanted fraction. However, only four out of the five recipients of the DP cell population were reconstituted, indicating a frequency of fewer than five HSCs in that fraction. The observation 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



**Fig. 6.** (A) VE-cadherin<sup>+</sup>CD45<sup>+</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  $1 \times 10^6$  YS,  $1 \times 10^5$  (Ter119<sup>-</sup>) PB and  $3 \times 10^6$  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 8000–13,000 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>-</sup>CD45<sup>+</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.





**Fig. 7.** VE-cadherin and CD45 expression within the E11.5 and E12.5 placental CD34<sup>+</sup>KIT<sup>+</sup> population. (A) CD34 and KIT expression profile of the E11.5 placenta. CD34<sup>+</sup>KIT<sup>+</sup> were gated (black box) and analysed for VE-cadherin and CD45 expression. Contour plot is composed from  $1 \times 10^5$  cells. (B) All E11.5 CD34<sup>+</sup>KIT<sup>+</sup> cells co-express VE-cadherin and CD45. (C) At E12.5, the placenta CD34<sup>+</sup>KIT<sup>+</sup> population has markedly lost VE-cadherin expression. Analyses in B and C were made using data from from 2000-4000 gated cells. All data are representative of three experiments. Isotype control (white) and specific antibody staining (grey) are presented.

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<sup>+</sup> and VE-cadherin<sup>-</sup> 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<sup>+</sup> and VE-cadherin<sup>-</sup> fractions were flow sorted from E12.5 circulation and transplanted into irradiated mice. Although eight out of 12 mice that received VE-cadherin<sup>+</sup> cells showed 10-90% leukocyte chimerism (average of 54%), only one out of the 10 recipients of the VE-cadherin<sup>-</sup> 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 FLK1<sup>low</sup> 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 (PECAM1<sup>low</sup> and PECAM1<sup>high</sup>) 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<sup>+</sup> 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<sup>+</sup> 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 SCA1<sup>high</sup> 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  $\alpha 4$ -integrin expression marks ~3% of endothelial cells but in the DP and haematopoietic populations virtually all cells express  $\alpha 4$ -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  $\alpha 4$ -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).

**Table 4. Downregulation of VE-cadherin in HSCs from E11.5 AGM and E12.5 yolk sac explants following 3 days of culture**

Donor organ	Population transplanted	Embryo equivalent (e.e.) transplanted	Reconstituted/transplanted	Mean chimerism/range (%)*
Cultured E11.5 AGM	VE-cadherin <sup>+</sup>	0.3e.e.	7/11	56 (5-85)
	VE-cadherin <sup>-</sup>	0.3-0.4e.e.	7/9	45 (5-82)
Cultured E12.5 YS	VE-cadherin <sup>+</sup>	0.3-0.5e.e.	7/10	40 (5-85)
	VE-cadherin <sup>-</sup>	0.3-0.5e.e.	10/10	55 (31-86)

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

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<sup>-</sup> 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<sup>+</sup> and VE-cadherin<sup>-</sup> 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<sup>+</sup> and VE-cadherin<sup>-</sup> 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<sup>+</sup>FLK1<sup>+</sup>PECAM1<sup>+</sup>Ac-LDL-receptor<sup>+</sup>. The haematopoietic fractions are either negative for these markers or express them at low level. The analysis of other 'HSC markers'

has shown that the DP fraction contains particularly high levels of KIT, SCA1, CD34 and AA4.1. Endothelial cells also but to a lesser extent are enriched for these markers. Close similarity between the endothelium and the DP fraction of the E11.5 AGM region, with respect to the above markers, again strongly suggests an ontogenetic link between these populations. However, in contrast to true AGM-derived endothelial cells, the DP cells did not form endothelial tubules in vitro, suggesting their functional divergence from the vasculature. Furthermore, by E11.5 endothelial cells are no longer capable of producing haematopoietic cells in vitro. By this stage, haematopoietic differentiation is associated exclusively with the DP fraction, whereas during E8-E10, such activity is clearly associated with the endothelial fraction (Fraser et al., 2003). Thus, despite phenotypic similarity between the endothelial and DP populations, a clear functional divergence develops in the embryo. The divergence of the endothelial and the DP cells can also be observed at the morphological level, with the DP fraction being enriched for cells with a blast-like morphology.

It is interesting that the DP population is entirely positive for an early embryonic HSC marker MAC1<sup>+</sup> (Morrison et al., 1995; Sanchez et al., 1996), which is entirely absent from the endothelial fraction, suggesting that MAC1 marks a diverging point between the segregating endothelial and HSC/haematopoietic compartments.

We then tested if HSCs that moved away from their site of origin lose their endothelial character. We have found that long-term repopulating HSCs which enter the circulation by E12.5 remain VE-cadherin<sup>+</sup>. The DP cells continue expressing TIE2, PECAM1 and Ac-LDL receptor, but expression of FLK1 becomes negligible. Among stem cell markers, SCA1 is expressed at particularly high level and the expression of a foetal HSC marker MAC1 is also remarkably high (Fig. 6C).

By E13.5 HSC activity within the foetal liver becomes associated predominantly, but not entirely, with the VE-cadherin negative fraction. [The discrepancy with the previous report (North et al., 2002) may result from differences in gating strategies or sensitivities of the flow cytometers used.] The phenotypic similarities between the endothelial and the DP fractions remain evident but expression of Ac-LDL receptor becomes significantly lower in the DP cells (Fig. 7B). Interestingly, a fraction of cells in the haematopoietic population, which contains most liver HSCs, becomes TIE2<sup>+</sup>, consistent with previous reports on TIE2 expression in HSCs (Arai et al., 2004; Hsu et al., 2000). In accordance with this, stem cell markers (SCA1 and KIT) are also upregulated in the haematopoietic fraction in the E13.5 liver (compared with the haematopoietic fractions in the AGM region and circulation).

As downregulation of VE-cadherin in the liver occurs only at the post-migratory stage, we tested if direct contact with the liver was required for VE-cadherin downregulation. To this end we analysed the in vitro development of HSCs in isolated E11.5 AGM region and E12.5 YS using an organ culture system (Kumaravelu et al., 2002). By the end of the culture period at least 50% of HSCs lost VE-cadherin expression. Therefore, progressive divergence of HSCs from the endothelial compartment does not require specific contact with the foetal liver and probably depends on developmental time. In line with this, the analysis of the advanced E12.5 AGM

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<sup>+</sup> and VE-cadherin<sup>-</sup> 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<sup>-</sup>CD45<sup>+</sup> 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/132/18/4179/DC1>

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**Table S1. Absolute number of VE-cadherin<sup>+</sup>CD45<sup>+</sup> cells within the foetal liver between E11.5 and E13.5**

Embryonic age	Cells per organ ( $1 \times 10^5$ )*	VE-cadherin <sup>+</sup> CD45 <sup>+</sup> cells/organ
E11.5	2.0±0.1	2460±430
E12.5	35.9±3.5	11,100±1300
E13.5	63.6±2.5	6360±2400

\*Dead cells excluded according to 7-AAD uptake.