

## DEVELOPMENT AND DISEASE

# Blood-forming potential of vascular endothelium in the human embryo

Estelle Oberlin, Manuela Tavian, István Blazsek and Bruno Péault\*

INSERM Unité 506, Villejuif, France

\*Author for correspondence (e-mail: peault@vjf.inserm.fr)

Accepted 16 May 2002

### SUMMARY

Hematopoietic cells arise first in the third week of human ontogeny inside yolk sac developing blood vessels, then, one week later and independently, from the wall of the embryonic aorta and vitelline artery. To address the suggested derivation of emerging hematopoietic stem cells from the vessel endothelium, endothelial cells have been sorted by flow cytometry from the yolk sac and aorta and cultured in the presence of stromal cells that support human multilineage hematopoiesis. Embryonic endothelial cells were most accurately selected on CD34 or CD31 surface expression and absence of CD45, which guaranteed the absence of contaminating hematopoietic cells. Yet, rigorously selected endothelial cells yielded a progeny of

myelo-lymphoid cells in culture. The frequency of hemogenic endothelial cells in the yolk sac and aorta reflected the actual blood-forming activity of these tissues, as a function of developmental age. Even less expected, a subset of endothelial cells sorted similarly from the embryonic liver and fetal bone marrow also exhibited blood-forming potential. These results suggest that a part at least of emerging hematopoietic cells in the human embryo and fetus originate in vascular walls.

Key words: Human embryo, Endothelium, Hematopoietic stem cell, Yolk sac, Aorta, Liver, Bone marrow

### INTRODUCTION

The first tissues that terminally differentiate in the course of higher vertebrate ontogeny are yolk sac blood-filled vessels in which both endothelial and hematopoietic cells arise at the expense of solid cords of extra-embryonic mesoderm. Peripheral cells in these mesodermal cords flatten into endothelium while the inner cell mass hollows into a lumen save cell clusters, also known as blood islands, that adhere to the newly formed vascular wall and sustain initial hematopoiesis (Sabin, 1920; Murray, 1932).

A second, and presumably last, wave of hematopoietic stem cell emergence takes place autonomously within the embryo in the region of the dorsal aorta, as documented in bird (reviewed by Dieterlen-Lièvre, 1994), mouse (reviewed by Cumano and Godin, 2001) (Ling and Dzierzak, 2002) and human embryos (Tavian et al., 2001). In all these species, incipient intra-embryonic blood-forming activity was assessed functionally but was also correlated with the transient presence of hematopoietic cells identified on tissue sections. In avian embryos, progenitors are localized inside then underneath the embryonic aorta (reviewed by Dieterlen-Lièvre, 1994). Discrete clusters of hematopoietic cells are also associated with the ventral wall of the dorsal aorta and vitelline artery in the mouse (Garcia-Porrero et al., 1995; Wood et al., 1997; North et al., 1999) and human embryo where they are significantly larger (Tavian et al., 1996; Tavian et al., 1999). In the latter

species, embryonic endothelium-adherent hematopoietic cells display surface antigens and express genes that typify primitive progenitors (Tavian et al., 1996; Labastie et al., 1998; Watt et al., 2000).

Hematopoietic stem cells thus always emerge, in early development, in close physical association with endothelial cells, be it in the extra-embryonic yolk sac or inside the embryo. Direct filiation of hematopoietic cells from pre-existing endothelium was suggested when Dil-conjugated acetylated low-density lipoprotein (Dil-Ac-LDL)-marked blood cells were retrieved in avian embryos of which the vascular endothelial surface had been labeled by intravenous injection of Dil-Ac-LDL (Jaffredo et al., 1998). In the mouse, candidate endothelial cells sorted by VE-cadherin expression from the embryonic aorta-gonad-mesonephros (AGM) region formed blood cells in culture (Nishikawa et al., 1998).

We herein addressed whether vascular endothelial cells in the human yolk sac, AGM and other prenatal blood-forming tissues are endowed with hematopoietic ability. To this end, we sorted endothelial and hematopoietic progenitor cells on their common expression of CD34 and CD31 but absence of CD45 at the surface of the former, a combination that allows to discriminate accurately these two cell lineages in the human embryo (Tavian et al., 1996; Charbord et al., 1996; Cortés et al., 1999).

We report that, when cultured in the presence of stromal cells that support human multilineage hematopoiesis, strictly

selected human embryonic and fetal endothelial cells produce blood cells. This was the case for endothelial cells sorted from the human yolk sac and embryonic aorta region but also, less expectedly, from the embryonic liver and fetal bone marrow. This indicates, for the first time, that blood cells born to the endothelium may play a key role in hematopoiesis incipience in the human embryo and fetus.

## MATERIALS AND METHODS

### Human tissues

Human embryonic and fetal tissues were obtained following voluntary, spontaneous or therapeutic abortions. The former were either induced with the RU486 anti-progestative compound or performed either by ultrasound-guided gentle aspiration or by regular curettage and aspiration. Informed consent to the use of the embryo in research was obtained from the patient, and tissue collection and use was approved by both our national (CCNE) and institutional (COPE) ethics committees. Developmental age was estimated, depending on the stage range, on the basis of several anatomic criteria: number of somite pairs, foot length, limb bud shape and eye pigmentation, according to Carnegie stages (O'Rahilly and Müller, 1987). Resected human umbilical cords were obtained after normal, full-term deliveries.

### Cell preparation

The yolk sac, para-aortic splanchnopleura/aorta-gonad-mesonephros (PSP/AGM) region, fetal aorta, liver, long bones, thymus, spleen, lungs, pancreas, heart and umbilical cord were excised sterilely, using microsurgery instruments and a dissecting microscope, in phosphate-buffered saline (PBS) containing 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Cergy-Pontoise, France). Tissues were dissociated for 1 hour at 37°C in Iscove's modified Dulbecco medium (IMDM; Gibco BRL) containing 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT), 0.1% type I/II/IV collagenase and type VIII hyaluronidase (Sigma-Aldrich, St Quentin Fallavier, France). Tissues were then disrupted mechanically through 18-, 23- and 26-gauge needles successively. Cell clumps were removed on a 50 µm nylon mesh (BioTechnofix, Bagnoux, France). Cells were washed and counted, after red blood cell lysis in 0.1% acetic acid. Cell viability was determined by 0.2% Trypan Blue exclusion (Gibco BRL). When needed, mononuclear cells were isolated on a Ficoll-Hypaque centrifugation gradient (Pharmacia, Orsay, France) and washed three times in PBS.

Endothelial cells were isolated from umbilical cord veins by enzyme digestion as described previously (Jaffe et al., 1973).

### Cell analysis and sorting by flow cytometry

Monoclonal antibodies to CD45 (PE-IgG1, clone J33), CD34 (FITC-IgG1, clone 581), CD33 (PE-IgG1, clone D3HL60-251), CD15 (FITC-IgM, clone 80H5), CD56 (PE-IgG1, clone N901-NKH1) and CD19 (PE-IgG1, clone J4.119) were purchased from Immunotech (Marseille, France). Monoclonal antibody to CD31 (FITC-IgG1, clone WM-59) was purchased from DAKO (Trappes, France). Biotinylated type 1 *Ulex europaeus* agglutinin (UEA-1) was provided by Vector Laboratories (Burlingame, CA).

For endothelial cell sorting, cell suspensions were incubated for 30 minutes on ice with either FITC-anti-CD34 or FITC-anti-CD31 and PE-anti-CD45 antibodies diluted in PBS 0.2% bovine serum albumin (BSA). Labeled cells were washed in PBS 0.2% BSA, resuspended in complete medium, then selected populations were sorted on a FACS Vantage SE fluorescence-activated cell sorter (Becton-Dickinson, Le Pont de Claix, France). Sorted cells were re-analyzed in all instances. In some experiments, cells sorted on differential expression of CD45 were incubated next, first with biotinylated UEA-1 and then with

APC-conjugated streptavidin (Becton-Dickinson). Background staining was evaluated using isotype-matched control antibodies or APC-conjugated streptavidin without biotinylated UEA-1 added first.

Hematopoietic cells developed in culture were harvested by non-enzymatic treatment, washed and labeled with fluorochrome-conjugated antibodies to differentiation markers as described above. Propidium iodide (PI) was added prior to acquisition in order to gate dead cells out. Cells were analyzed on a FACScan flow cytometer (Becton-Dickinson), using the CellQuest software.

### Cell culture

#### Culture on MS-5 stromal cells

Freshly sorted cells were cultivated on pre-established monolayers of the MS-5 mouse bone marrow stromal cell line (Itoh et al., 1989), which supports long-term myeloid and B-lymphoid human hematopoiesis (Issaad et al., 1993; Berardi et al., 1997; Robin et al., 1999; Tavian et al., 2001). Co-cultures were performed as described previously (Tavian et al., 2001). The frequency of cells forming hematopoietic colonies was determined by means of Poisson statistics as described previously (Ploemacher et al., 1989; Moore et al., 1997) with slight modifications. Serial half-dilutions were performed from 1000 to 15 cells per well, with 10 replicate wells seeded per cell dilution. Colonies of hematopoietic cells developing on or underneath the MS-5 stromal cells were scored from week 1 to week 5 under an ICM-405 phase-contrast inverted microscope (Carl Zeiss, Le Pecq, France). The number of cells seeded per well was plotted against the logarithm of the percentage of negative wells. As calculated from the line of best fit, the cell number at log 37% negative wells is the frequency of blood-forming cells in the starting population. Pictures of colonies were made using the AxioVision software (Carl Zeiss). At 21 days, some of the wells where significant proliferation had occurred were selected and adherent and non-adherent cells were collected using a non-enzymatic dissociation solution (Sigma-Aldrich) and labeled with monoclonal antibodies (see above).

#### Colony-forming cell assays in semi-solid medium

Freshly sorted cells were plated in duplicate in 35 mm plastic culture dishes at  $10^3$  cells/ml in 1 ml semi-solid growth medium that consisted of 1% methylcellulose (Sigma-Aldrich) in IMDM (Gibco BRL), 30% heat-inactivated FCS (Hyclone Laboratories), 1% BSA, (Sigma-Aldrich),  $10^{-4}$  M 2-mercaptoethanol (Sigma-Aldrich), 2 U/ml erythropoietin (Epo, Janssen-Cilag, Baar, Switzerland), 2 ng/ml IL3, 100 U/ml IL1, 1 ng/ml IL6, 100 U/ml GM-CSF, 100 ng/ml G-CSF (PeproTech) and 10 ng/ml SCF (R&D Systems). Cultures were incubated for 14 days at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Colonies ( $\geq 50$  cells) were counted on an inverted microscope.

#### Endothelial cell culture

Endothelial cells sorted by FACS from embryonic or fetal tissues or isolated from umbilical cord veins as described above were seeded in endothelial cell growth medium 2 (EGM2) for microvascular cells (Clonetics Corporation, San Diego, CA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml Fungizone (Gibco BRL). FACS-sorted endothelial cells and human umbilical vein endothelial cells (HUVEC) were cultured on multiwell plates (Becton-Dickinson) or on Lab-Tek culture chambers (Nalge Nunc International, Rochester, NY), coated with 0.2% gelatin (Sigma-Aldrich), at a density of  $6 \times 10^3$  cells/cm<sup>2</sup>, and grown to confluence at 37°C in 5% CO<sub>2</sub> in 5-7 days. Image acquisition was done on the inverted phase-contrast microscope using the AxioVision software (Carl Zeiss).

#### RNA reverse transcription and PCR amplification

Total RNAs were prepared from sorted cells using TRIzol (Gibco BRL), according to manufacturer's instructions (Chomczynski and Sacchi, 1987). Briefly,  $10^3$  cells were lysed in 200 µl TRIzol. After addition of 40 µl chloroform and centrifugation at 12,000 g for 15

minutes at 4°C, the aqueous phase containing RNA was recovered, RNA was precipitated by adding 100 µl isopropanol and the pellet washed in 70% cold ethanol. RNA was resuspended in 26 µl sterile distilled water.

Reverse transcription and PCR amplification were carried out as follows: cDNA was synthesized from 13 µl RNA in a reaction volume of 20 µl, using 10 mmol/l DTT (Gibco BRL), 200 µmol/l of each dNTP (New England Biolabs, Beverly, MA), 0.05 U pd(N)<sub>6</sub> random hexamers (Amersham Pharmacia Biotech, Saclay, France), 0.2 U RQ1 RNase-free DNase (Promega, Charbonnières, France), 302 U RNase inhibitor (Roche Diagnostics, Meylan, France) and 100 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL). Duplicates of each reaction were performed without reverse transcriptase. PCR amplifications were carried out in a final volume of 20 µl, using 200 µmol/l of each dNTP (New England Biolabs), 1 µmol/l of the reverse and forward primers, 1.5 mmol/l MgCl<sub>2</sub> (Roche Diagnostics), 2 µl cDNA, and 2.5 U of Taq DNA polymerase (Roche Diagnostics). As an internal standard, amplification of β-actin cDNA was performed on each cDNA sample. Amplification was conducted in a PTC-100™ Thermal Cycler (MJ Research, Waltham, MA) operating for 2 minutes at 94°C, followed by 40 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C. Final extension was carried out for 10 minutes at 72°C.

Oligonucleotide primers were synthesized by Gibco BRL according to sequences previously described for human CD45 (Streuli et al., 1987) and β-actin (Ponte et al., 1984).

Forward and reverse primers were, respectively, P1 and P2 for β-actin (GenBank Accession Number, X00351), P3 and P4 for CD45 (GenBank Accession Number, Y00638).

P1, 5'-AACCGCGAGAAGATGACCCAG-3'; P2, 5'-TGCGCTC-AGGAGGAGCAATGA-3'

P3, 5'-TGTACTGCTCCTGATAAGACA-3'; P4, 5'-ATCGCAATT-CCTTATGCGACTC-3'

All these primers were located on both sides of an intron to discriminate between messenger RNA and genomic amplification products.

### Immunohistochemistry

Tissues were fixed overnight at 4°C in PBS 4% paraformaldehyde (Sigma-Aldrich), rinsed twice in PBS, then twice in PBS/15% sucrose (Sigma-Aldrich) for at least 24 hours. Tissues were then embedded in PBS 15% sucrose, 7.5% gelatin (Sigma-Aldrich), frozen at -70°C and stored at -20°C. Frozen sections (5 µm) were cut, stored at -20°C until use, then thawed and hydrated in PBS. All following steps were carried out at room temperature. First, endogenous peroxidases were inhibited for 20 minutes in PBS containing 0.2% hydrogen peroxide (Sigma-Aldrich). Sections were then washed in PBS and nonspecific binding sites were blocked with PBS/5% FCS during 1 hour. Sections were first exposed to the primary antibody for 1 hour in PBS/5% FCS. Sections were then washed with PBS and incubated for 1 hour, first with biotinylated goat anti-mouse Ig antibody (Immunotech) and, subsequently, with peroxidase-labeled streptavidin (Immunotech).

Peroxidase activity was revealed with 0.025% 3,3'-diaminobenzidine (Sigma-Aldrich) in PBS containing 0.03% hydrogen peroxide (Sigma-Aldrich). Slides were counterstained with Gill's Hematoxylin (Sigma-Aldrich) and mounted in XAM neutral medium (BDH Laboratory Supplies, Poole, UK). All observations and photomicrographs were made on an Optiphot 2 microscope (Nikon, Champigny-sur-Marne, France).

Monoclonal antibodies to CD34 (IgG1, clone Qbend-10) and CD45 (IgG1, clone Hle-1) were purchased from Immunotech and Becton-Dickinson Biosciences, respectively. Monoclonal antibody to VE-cadherin (IgG2a, clone BV9) was a gift from Dr P. Huber (CEA, Grenoble, France).

### Immunocytochemistry

HUVEC or FACS-sorted endothelial cells were grown on plastic

culture chambers as described above. All following steps were carried out at room temperature. Medium was removed and cells were rinsed in PBS, fixed in PBS, 3.7% paraformaldehyde (Sigma-Aldrich) for 20 minutes, and rinsed again twice in PBS. Free aldehydes were quenched for 15 minutes in PBS, 0.1 M glycine (Sigma-Aldrich) and cells were washed twice in PBS. For cell-surface labeling, washes and incubations were carried out in PBS containing 0.2% BSA. Cells were incubated with a primary antibody to CD31 (IgG1, clone JC/70A, DAKO) or with biotinylated UEA-1 (Vector Laboratories) for 30 minutes, washed three times for 5 minutes, then incubated for 30 minutes with a secondary Alexa Fluor 594-conjugated goat anti-mouse IgG antibody (Molecular Probes) or with Alexa Fluor 594-conjugated streptavidin (Molecular Probes). For intracellular labeling, washes, permeabilization and incubations were carried out in PBS containing 0.2% BSA and 0.05% saponin (Sigma-Aldrich). Cells were first permeabilized for 20 minutes and subsequently incubated, for 30 minutes, with rabbit antibody to von Willebrand factor (vWF; Dako). Cells were then washed three times for 5 minutes and incubated, for 30 minutes, with Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (Molecular Probes). Finally, cells were washed twice for 5 minutes in PBS/0.2% BSA or PBS/0.2% BSA/0.05% saponin, and twice for 5 minutes in PBS, then, growth chambers were removed and slides were mounted in 12% Mowiol 4-88 (HOECHST; Calbiochem, La Jolla, CA), 33% glycerol (Sigma-Aldrich), 120 mM Tris-HCl (pH 8.5) and 2.5% 1,4-diazabicyclo [2.2.2] octane (Dabco; Sigma-Aldrich) to reduce fading.

Isotype-matched antibody controls, or Alexa Fluor 594-conjugated streptavidin without biotinylated UEA-1 added first, were used to ensure the specificity of staining.

Fluorescent staining was observed on a DMR/HCS fluorescence microscope (Leica, Rueil-Malmaison, France) and images were acquired using the Leica Lida software.

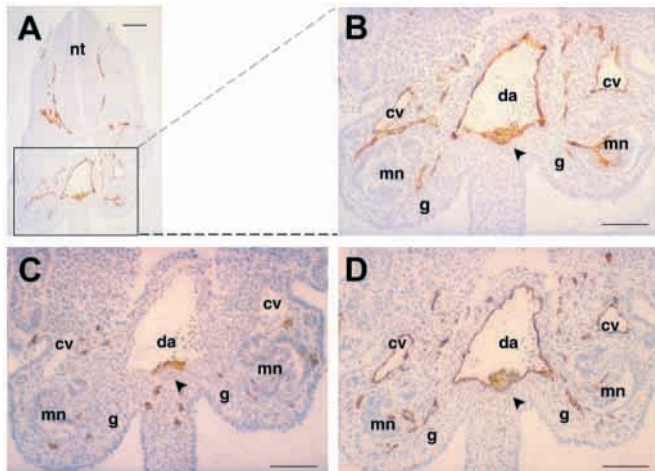
## RESULTS

The experiments we report aim to assay the blood-forming potential in culture of vascular endothelial cells present in human embryonic and fetal tissues. As a prerequisite to accurate cell sorting by FACS, the antigenic profile that typifies endothelial cells during early human ontogeny was reexamined. Special care was taken to define marker combinations that allow to discriminate endothelial cells from hematopoietic cells.

### Tracing endothelial and hematopoietic cells in the 3- to 6-week-old human embryo

Immunohistochemistry was used first to show that all vascular endothelial cells lining vessels of all diameters in the 3- to 6-week-old embryo express CD34 (Fig. 1A,B) and CD31 (Fig. 1D) along identical patterns. CD45 expression was restricted to hematopoietic cells, which were disseminated in very low numbers within tissues, albeit somehow more densely in the subaortic mesenchyme (Fig. 1C). CD45+ cells were very rarely present inside blood vessels (not shown), which contained almost exclusively erythrocytes. None of these hematopoietic cells seen scattered throughout embryonic tissues was ever found positive for CD34 expression. By contrast, CD34+ CD31+ CD45+ hematopoietic cells were many, from day 28, clustered on ventral endothelium within the dorsal aorta (Fig. 1), as amply documented before (Tavian et al., 1996; Tavian et al., 1999).

This description therefore confirmed that all vascular endothelial cells in the human embryo express CD34 and



**Fig. 1.** Immunolocalization of endothelial and hematopoietic cells in the AGM region. (A) Transverse section through a 31-day human embryo immunostained for CD34. The boxed area is the AGM region, including the aorta, gonads and mesonephros. (B–D) Higher magnifications of the same AGM region stained for CD34, CD45 and CD31, respectively. (B) Anti-CD34 stains the endothelium lining the dorsal aorta (da), cardinal veins (cv) and capillaries around the mesonephros (mn). The arrowhead indicates the hematopoietic CD34<sup>+</sup> cell progenitors associated with the ventral endothelium of the aorta. (C) CD45 is expressed on all hematopoietic cells present in the embryo, including the aortic stem cells (arrowhead), but is absent from endothelial cells. Rare CD45<sup>+</sup> hematopoietic cells scattered in the mesoderm underlying the dorsal aorta are CD34-negative. (D) CD31 stains both endothelial cells and adherent intra-aortic hematopoietic cells. Scale bars: 100  $\mu$ m; nt, neural tube; g, gonad.

CD31, and can be readily distinguished from hematopoietic progenitors, as only the latter express CD45.

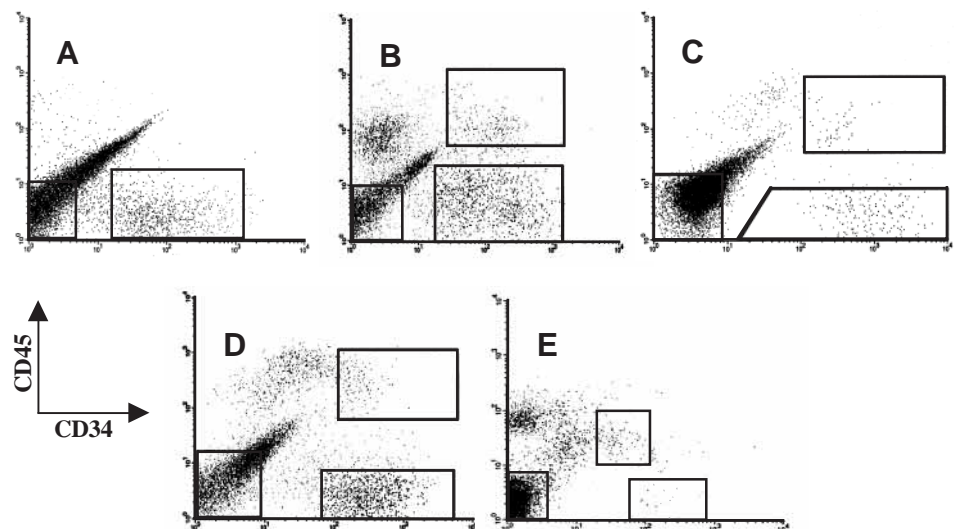
The presence of VE-cadherin, another marker of human vascular endothelial cells, was also assessed on embryo sections. The VE-cadherin adhesion molecule was present at the luminal aspect of vascular endothelium at all stages tested, but was not expressed by aorta-associated hematopoietic cell clusters (not shown), suggesting that this marker could also be suitable for sorting human endothelial cells by flow cytometry.

### Flow-cytometry analysis and sorting of vascular endothelial and hematopoietic cells

Embryonic and fetal tissues were dissociated with collagenase and further processed for flow cytometry analysis and sorting. Blood-forming tissues used were the yolk sac, embryonic aorta region, embryonic and fetal liver, and fetal bone marrow, thymus and spleen. Non-hematopoietic tissues included fetal aorta, lung, pancreas, heart and umbilical cord. Cell suspensions double-stained with antibodies to CD34 and CD45 all contained a clear-cut population of CD34<sup>+</sup> CD45<sup>-</sup> cells (Fig. 2A–E). In the experiment shown, CD34<sup>+</sup> CD45<sup>-</sup> cells accounted for 9.42% and 8.12% of the total cells in the yolk sac and liver, which are richly vascularized tissues (Fig. 2B,D), but represented only 2.25% and 1.14% of AGM and bone marrow cells (Fig. 2C,E). Hematopoietic CD34<sup>+</sup> CD45<sup>+</sup> cells were also present, as expected, in the yolk sac, embryonic aorta region, liver and bone marrow (Fig. 2B–E), but were virtually absent from non-blood-forming tissues (Fig. 2A). Using an antibody to CD31, instead of CD34, yielded identical results as of percentages of CD31<sup>+</sup> CD45<sup>-</sup> cells (not shown).

CD34<sup>+</sup> CD45<sup>-</sup> and CD34<sup>+</sup> CD45<sup>+</sup> cells, i.e. endothelial and hematopoietic cells, were next sorted inside the gates defined above. Purity of sorted cells always exceeded 98%, as seen on immediate FACS reanalysis of cells sorted from the embryonic liver (Fig. 3B,C). It was also confirmed by three-color analysis that all sorted CD34<sup>+</sup> CD45<sup>-</sup> cells, whatever the tissue of origin, express the UEA-1 ligand (Fig. 3E), an endothelial cell marker (Holthofer et al., 1982), whereas no CD34<sup>+</sup> CD45<sup>+</sup> cell does (Fig. 3D).

Absence of CD45<sup>+</sup> cells within sorted endothelial cells was also verified by semi-quantitative RT-PCR analysis, performed on a fraction of each sorted cell subset, and which never revealed the presence of detectable CD45 transcripts inside the population of CD34<sup>+</sup> CD45<sup>-</sup> cells (Fig. 4). To further confirm the absence of contaminating hematopoietic cells within sorted endothelial cells, sorted CD34<sup>+</sup> CD45<sup>-</sup> and CD34<sup>+</sup> CD45<sup>+</sup> cells were immediately seeded in semi-solid medium in the presence of hematopoiesis-stimulating growth factors. In these conditions no clonogenic hematopoietic progenitor was ever detected among CD34<sup>+</sup> CD45<sup>-</sup> cells, whereas those were



**Fig. 2.** Differential expression of CD34 and CD45 in human embryonic and fetal tissues. Dissociated cells were double-stained with FITC-anti-CD34 and PE-anti-CD45 MoAbs and analyzed by flow cytometry. Distinct CD34<sup>+</sup> CD45<sup>+</sup> and CD34<sup>+</sup> CD45<sup>-</sup> cell subsets were observed in the 26-day yolk sac (B), 32-day AGM region (C), 32-day embryonic liver (D) and 24-week fetal bone marrow (E). In 52-day lungs, only CD34<sup>+</sup> CD45<sup>-</sup> cells were present (A).

expectedly abundant within the counterpart CD34<sup>+</sup> CD45<sup>+</sup> cell subset (Table 1).

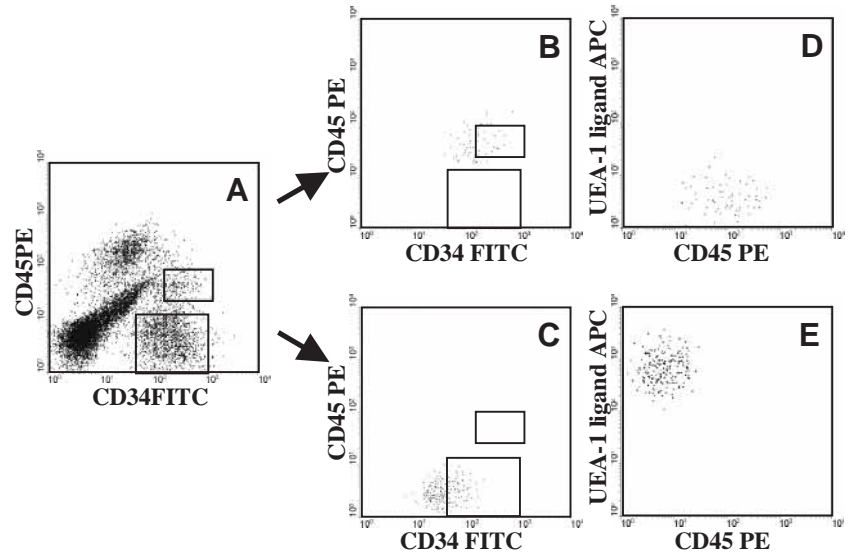
### Endothelial development in culture of embryonic CD34<sup>+</sup> CD45<sup>-</sup> cells

The ability of CD34<sup>+</sup> CD45<sup>-</sup> cells isolated from embryonic tissues to grow as endothelial cells was tested by culturing these, immediately after the sort, in endothelial cell growth medium at a density of  $6 \times 10^3$  cells/cm<sup>2</sup>. In such conditions, CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from either the yolk sac, embryonic aorta or embryonic liver adhered shortly after culture incipience and formed monolayers of stellate, adherent cells that came to confluence in about 7 days and exhibited typical endothelial shape, when compared with endothelial cells grown from the umbilical vein at term (Fig. 5A-D).

Cells grown in that setting were characterized, after 10 days of culture, by immunocytochemistry. In all instances, adherent cells developed from CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from either the yolk sac, embryonic aorta or embryonic liver were free of detectable CD45<sup>+</sup> cells (not shown) but uniformly expressed at their surface von Willebrand factor, UEA-1 ligand and CD31 when compared with endothelial cells grown from the umbilical vein at term (Fig. 5E-P).

### Blood-forming potential in culture of CD34<sup>+</sup> CD45<sup>-</sup> or CD31<sup>+</sup> CD45<sup>-</sup> endothelial cells sorted from hematopoietic tissues

Embryonic and fetal CD34<sup>+</sup> CD45<sup>-</sup> cells, which were sorted free of contaminating CD45<sup>+</sup> hematopoietic cells from blood-forming tissues as described above, were next cultured in the presence of the MS-5 cell line, a mouse bone marrow stroma that supports the multilineage, long-term development of human hematopoietic stem cells and has been used before to



**Fig. 3.** CD34<sup>+</sup> CD45<sup>+</sup> and CD34<sup>+</sup> CD45<sup>-</sup> cell sorting from the 40-day embryonic liver and re-analysis by flow cytometry. Dissociated cells were double-stained with FITC-anti-CD34 and PE-anti-CD45 MoAbs. CD34<sup>+</sup> CD45<sup>+</sup> and CD34<sup>+</sup> CD45<sup>-</sup> cell subsets were sorted inside the gates (A) and immediately re-analyzed (B,C). A fraction of each sorted cell subset was then incubated, first, with biotinylated UEA-1 and then with APC-conjugated streptavidin. All sorted CD34<sup>+</sup> CD45<sup>-</sup> cells were stained by UEA-1 (E), whereas none of the CD34<sup>+</sup> CD45<sup>+</sup> cells was (D).

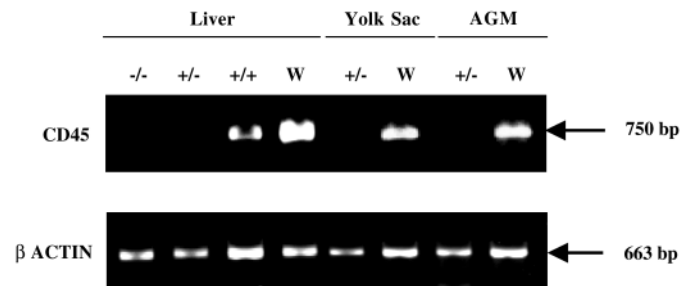
assess the blood-forming potential of human intra- and extra-embryonic tissues (Tavian et al., 2001). Counterpart CD34<sup>+</sup> CD45<sup>+</sup> cells sorted in parallel in each experiment were seeded in the same conditions. Both CD34<sup>+</sup> CD45<sup>+</sup> and CD34<sup>+</sup> CD45<sup>-</sup> cell populations sorted from the same tissue and co-cultured in bulk with MS-5 cells developed similarly into colonies of rounded, refringent, stroma-adherent typical hematopoietic cells (Fig. 6), visible from day 3 up to day 35 of co-culture. Both populations also produced cobblestone-like colonies of

**Table 1.** CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from embryonic blood-forming tissues are devoid of clonogenic hematopoietic progenitors

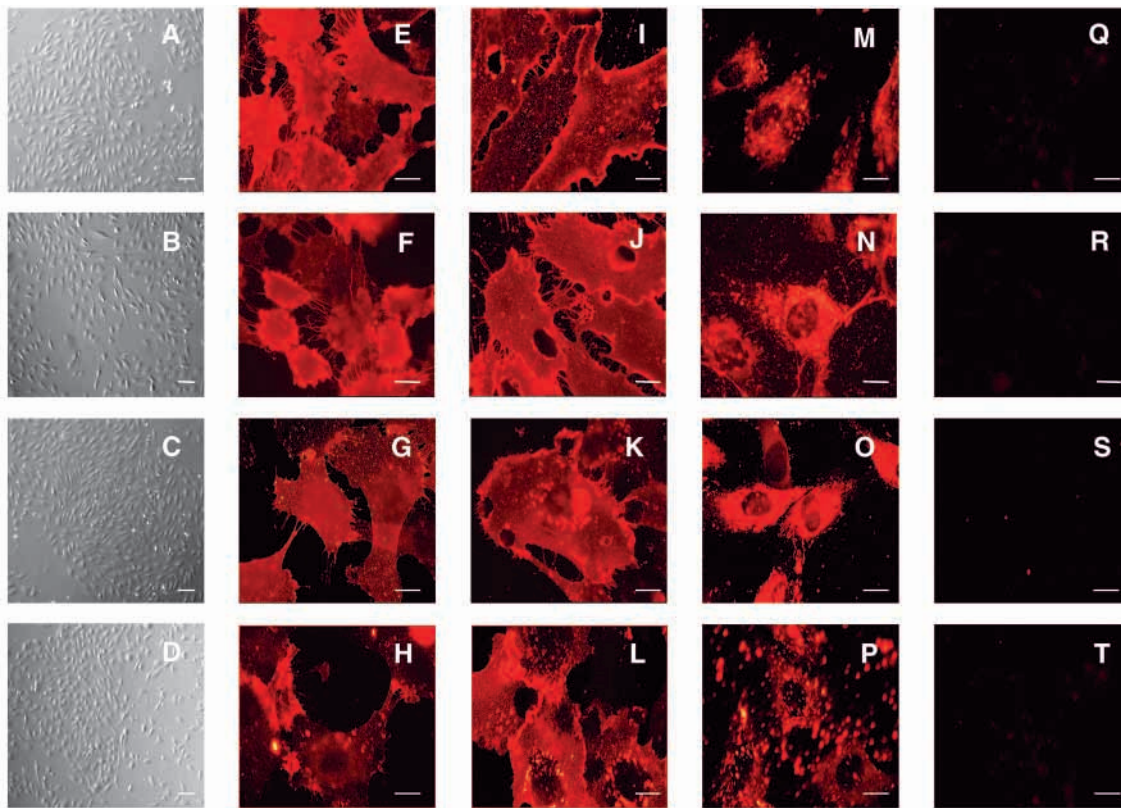
Colony	Yolk sac (32 days)	
	CD34 <sup>+</sup> CD45 <sup>-</sup> 1000 cells	CD34 <sup>+</sup> CD45 <sup>+</sup> 1000 cells
BFU-E	0	200±15
CFU-GM	0	27.5±2.5
CFU-GEMM	0	2.5±0.5
Colony	AGM (32 days)	
	CD34 <sup>+</sup> CD45 <sup>-</sup> 1000 cells	CD34 <sup>+</sup> CD45 <sup>+</sup> 1000 cells
BFU-E	0	106.5±8.5
CFU-GM	0	18.5±4.5
CFU-GEMM	0	3.5±0.5

CD34<sup>+</sup> CD45<sup>+</sup> and CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from the yolk sac and AGM were plated in semi-solid medium in the presence of growth factors. CFU-GM-, CFU-GEMM- and BFU-E-derived colonies were counted at 14 days of culture.

Each value is the mean±s.d. of duplicate experiments.

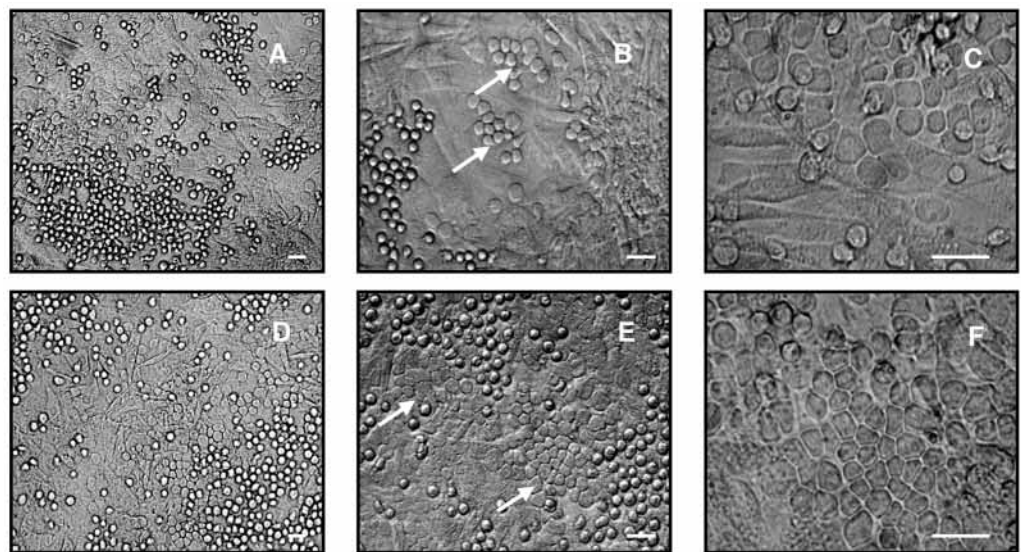


**Fig. 4.** RT-PCR analysis of CD45 expression within CD34<sup>+</sup> CD45<sup>+</sup> (+/+), CD34<sup>+</sup> CD45<sup>-</sup> (+/-) and CD34<sup>+</sup> CD45<sup>-</sup> (-/-) cell subsets sorted from the human embryo. mRNA was extracted from CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from the 32-day yolk sac and 32-day AGM and from CD34<sup>+</sup> CD45<sup>+</sup>, CD34<sup>+</sup> CD45<sup>-</sup> and CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from the 40-day embryonic liver. mRNA was also extracted from the whole (W) yolk sac, AGM and embryonic liver. mRNAs were then retro-transcribed into cDNAs and CD45 cDNAs were amplified by PCR. Amplified products were separated on a 1.5% agarose gel and visualized after BET staining. Product size was checked by running molecular weight markers. Signals obtained for beta-actin cDNA amplification were used as references to normalize differences between cDNA samples.



**Fig. 5.** CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from embryonic tissues develop into endothelial cells in culture. Endothelial cells isolated from umbilical cord veins and CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from the 32-day yolk sac, 32-day AGM and 44-day embryonic liver were grown in EGM2 medium. Cells were observed directly by phase-contrast microscopy (A-D) or, after immunostaining, by fluorescence microscopy (E-T). Fixed cells were stained indirectly with biotinylated UEA-1 (I-L), with an antibody to CD31 (E-H) or with an antibody to vWF, after permeabilization (M-P). After 7 days of culture, confluent layers of cells with typical endothelial shape were obtained from CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from the yolk sac (A), AGM (B) and embryonic liver (C), when compared with HUVEC (D). Endothelial cells developed from CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from the yolk sac (E,I,M), AGM (F,J,N) or embryonic liver (G,K,O), when compared with HUVEC (H,L,P), all express CD31, UEA-1 ligand and vWF. vWF isotype controls on endothelial cells developed in culture from CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from the yolk sac (Q), AGM (R), embryonic liver (S) and from the umbilical vein (T). Scale bars: 50 µm in A-D; 5 µm in E-T.

**Fig. 6.** Colony formation on MS-5 stroma by 24-week fetal bone marrow hematopoietic and endothelial cells. CD34<sup>+</sup> CD45<sup>+</sup> (A-C) and CD34<sup>+</sup> CD45<sup>-</sup> (D-F) cells sorted from the fetal bone marrow gave rise to colonies of packed rounded hematopoietic cells on MS-5 cells from day 3 up to day 35 of co-culture. Phase-dark cobblestone-shaped colonies also developed underneath the stroma (arrows) from day 7 to day 35 of co-culture. Scale bars: 20 µm.



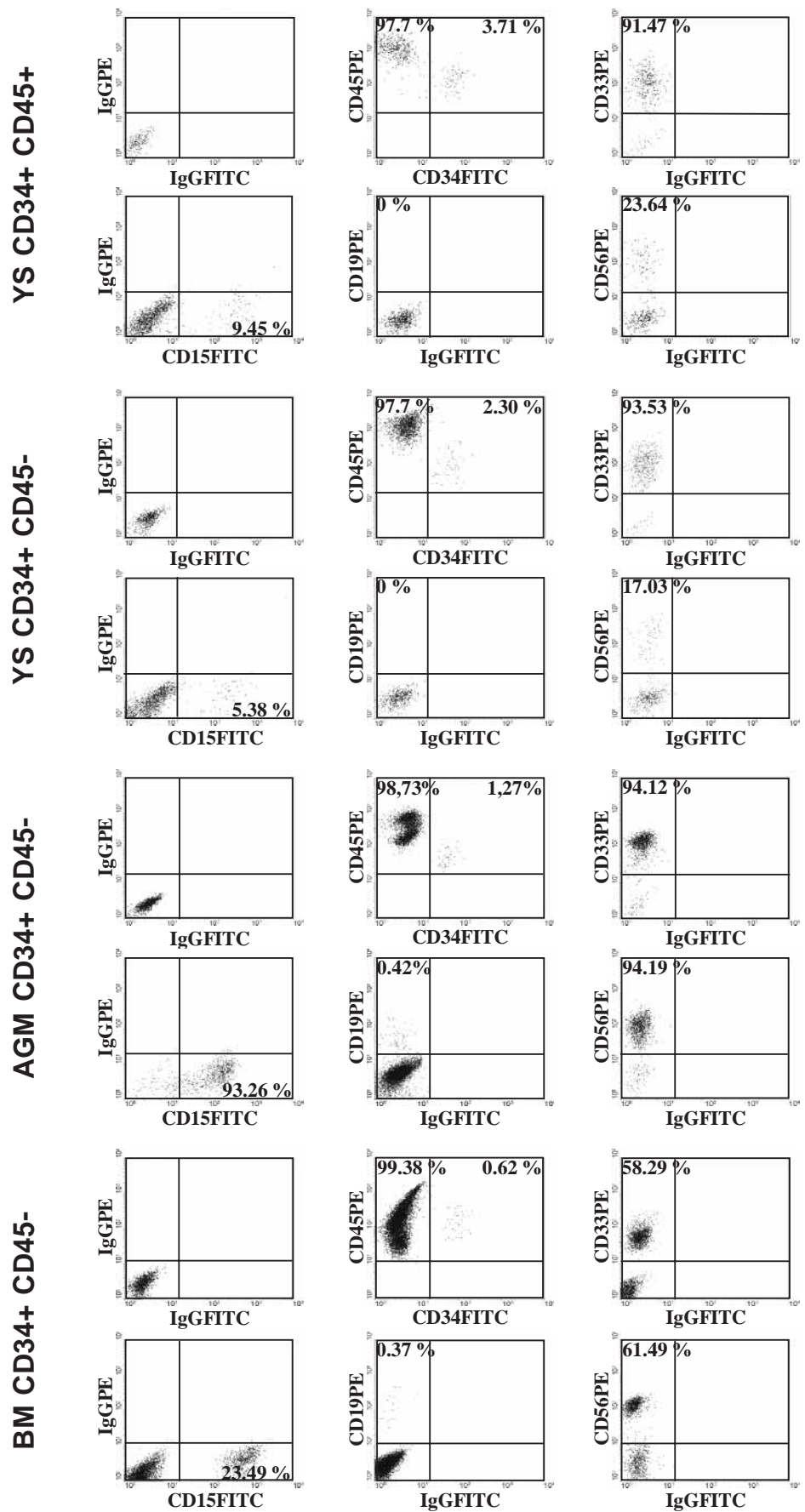
hematopoietic cells underneath the stroma, from day 7 to day 35 of co-culture, as is expected from late [day 7 cobblestone area-forming cell (CAFC)] and primitive (day-35 CAFC)

hematopoietic progenitors cultured in these conditions (Fig. 6). After 21 days, the whole content of MS-5/CD34<sup>+</sup> CD45<sup>+</sup> and MS-5/CD34<sup>+</sup> CD45<sup>-</sup> co-cultures was harvested by non-

**Fig. 7.** Endothelial and hematopoietic cells sorted from embryonic and fetal hematopoietic tissues yield identical blood cell progenies on MS-5 stroma. 26-day-old yolk sac CD34<sup>+</sup> CD45<sup>+</sup> and CD34<sup>+</sup> CD45<sup>-</sup> cells and 28-day AGM or 16-week fetal bone marrow CD34<sup>+</sup> CD45<sup>-</sup> cells were grown on MS-5 stromal cells. After 21 days, the whole content of co-cultures was harvested and double-stained with FITC-anti-CD34 and PE-anti-CD45 antibodies or simple-stained with PE-anti-CD33, FITC-anti-CD15, PE-anti-CD19 and PE-anti-CD56 antibodies. Immunostaining was analysed by flow cytometry. Hematopoietic cells were gated on forward and side scatter. YS, yolk sac; BM, bone marrow.

enzymatic digestion and pipetting, stained with antibodies and analyzed by flow cytometry. In both co-culture experiments, CD34<sup>+</sup> cells were still present and a large population of CD45<sup>+</sup> hematopoietic cells had differentiated, spanning myeloid (CD33<sup>+</sup>, CD15<sup>+</sup>), natural killer (CD56<sup>+</sup>) and lymphoid (CD19<sup>+</sup>) cell lineages (Fig. 7). In that setting, hemogenic CD34<sup>+</sup> CD45<sup>-</sup> cells were identified in the yolk sac, embryonic aorta-gonad-mesonephros region, embryonic and fetal liver and fetal bone marrow (Table 2). As observed before on whole tissues (Tavian et al., 2001), yolk sac endothelial cells produced myeloid and NK cells only whereas AGM and bone marrow endothelial cells yielded in addition B lymphocytes (Fig. 7). Conversely, CD34<sup>+</sup> CD45<sup>-</sup> cells sorted in the same conditions from the fetal aorta, thymus, spleen, lung, pancreas, heart or umbilical cord or from the umbilical vein at term, as well as cultured HUVEC did not produce hematopoietic cells in the presence of MS-5 stromal cells (Table 2).

Similar experiments were performed with endothelial cells sorted on expression of CD31 but absence of CD45 at their surface from the yolk sac, AGM and embryonic liver and from non-hematopoietic tissues such as the heart and umbilical cord. Like CD34<sup>+</sup> CD45<sup>-</sup> cells, CD31<sup>+</sup> CD45<sup>-</sup> cells co-cultured in bulk with MS-5 cells also produced colonies of stroma-adherent hematopoietic cells and cobblestone areas (not shown). Conversely, CD31<sup>+</sup> CD45<sup>-</sup> cells sorted from the umbilical



**Table 2. Hemogenic activity on MS-5 stroma of CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from hematopoietic and non-hematopoietic embryonic and fetal tissues**

Tissue of origin	Positive/total experiments	Stages
Yolk sac	12/13	26-40D
PSP/AGM	0/5	23-27D
	11/11	28-44D
Embryonic and fetal liver	15/23	27-56D
	0/5	59D-20W
Fetal bone marrow	1/3	11-24W
Thymus	0/1	12W+6D
Pancreas	0/1	12W+6D
Umbilical cord	0/2	28-40D
Heart	0/5	32-63D
Spleen	0/1	12W+6D
Lung	0/1	52D
Fetal aorta	0/1	66D
HUVEC	0/1	Newborn

Sorted cells were grown in bulk on MS-5 stromal cells. Colonies of hematopoietic cells were monitored from week 1 to week 5 of co-culture. D, days; W, weeks.

cord and heart did not produce hematopoietic colonies on MS-5 stromal cells (not shown).

**The frequency of hemogenic endothelial cells in blood-forming organs varies as a function of developmental age**

CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from the yolk sac, embryonic aorta, embryonic and fetal liver, and fetal bone marrow taken at successive stages of ontogeny were next cultured in limiting

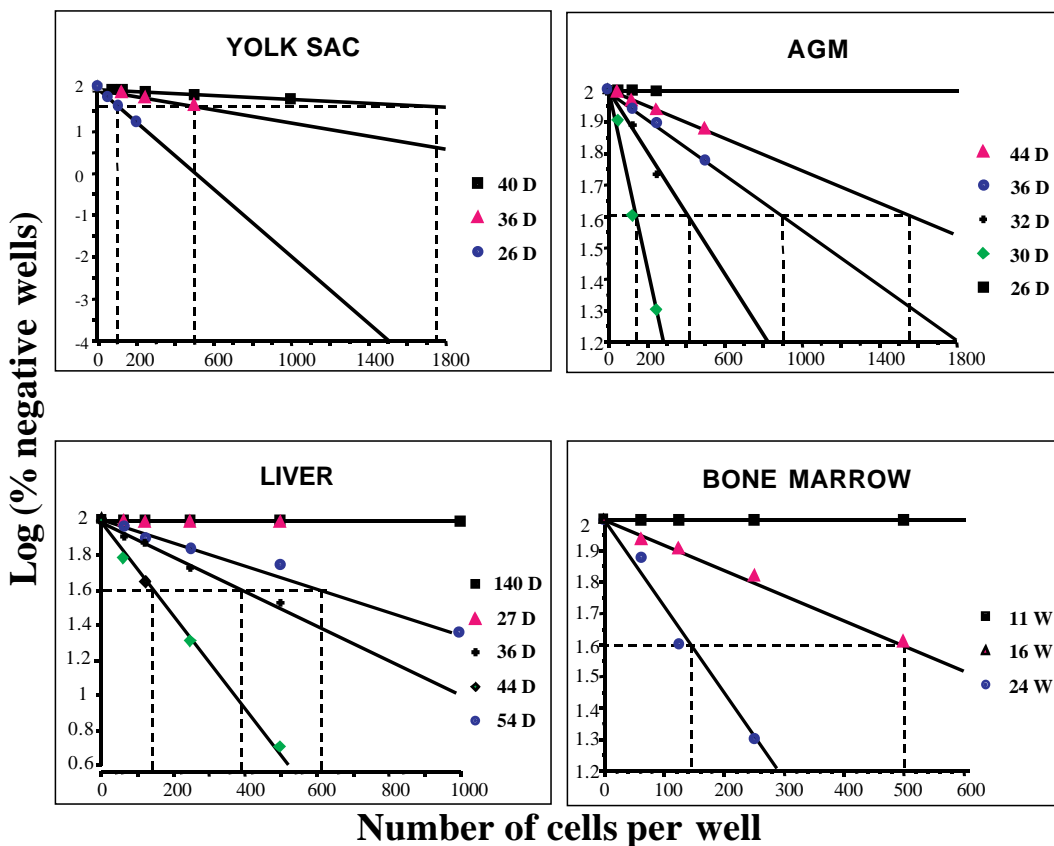
dilution conditions in the presence of MS-5 stromal cells, in order to evaluate the frequency of hemogenic endothelial cells in these organs. Fifteen to 1000 sorted cells were seeded in multiple wells on MS-5 stroma and linear regression analysis was used to determine the frequency of blood-forming cells within a given population of sorted CD34<sup>+</sup> CD45<sup>-</sup> endothelial cells.

Limiting dilution analysis confirmed the existence of hematopoietic endothelial cells within embryonic and fetal blood-forming tissues, as seen before on bulk cultures. However, dramatic differences were revealed in the frequency of these cells in the course of human ontogeny.

In the yolk sac, hematopoietic potential was detected among sorted CD34<sup>+</sup> CD45<sup>-</sup> cells at 26 days, the first stage tested, at a frequency of about 1/100. This frequency then declined progressively to become lowest at 40 days, the latest stage analyzed (Fig. 8).

In the dorsal aorta territory, where HSC emerge from day 28 (Tavian et al., 1999; Tavian et al., 2001), no hematopoietic potential was detected among sorted CD34<sup>+</sup> CD45<sup>-</sup> cells from 23 to 27 days of development. Blood-forming endothelial cells were present in the AGM from day 28. Their frequency culminated at about 1/150 cells at day 30, then dropped to become undetectable at day 44 (Fig. 8). In the liver rudiment, where incipient hematopoiesis is seen from day 30 (Tavian et al., 1999), no hematopoietic potential was detected among sorted CD34<sup>+</sup> CD45<sup>-</sup> cells at day 27. The frequency of hemogenic hepatic endothelial cells reached 1/390 at 36 days to culminate at 1/140 at 44 days. This frequency declined rapidly thereafter to 1/610 at 54 days. Blood-forming endothelial cells were virtually absent in the 4.5-month-old fetal liver (Fig. 8).

**Fig. 8.** Frequency of blood-forming CD34<sup>+</sup> CD45<sup>-</sup> endothelial cells in embryonic and fetal hematopoietic tissues. CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from the yolk sac, AGM, embryonic liver and fetal bone marrow were grown on MS-5 stromal cells in limiting dilution conditions. Hematopoietic cell colonies developing on or underneath MS-5 stromal cells were scored at week 5 under a phase-contrast inverted microscope. The number of cells seeded per well was plotted against the logarithm of the percentage of negative wells. As calculated from the line of best fit, the cell number at log 37% negative wells is the frequency of hemogenic cells in the starting population. D, days; W, weeks.





Similar experiments were performed on the developing bone marrow. Medullary hematopoiesis starts during the eleventh week of human development (Charbord et al., 1996). Although the vascular bed is already well developed at that stage in the marrow rudiment, endothelial cells sorted therefrom did not exhibit any hematopoietic potential (Fig. 8). By contrast, blood-forming medullary endothelial cells were detected at 16 weeks, and reached a frequency of about 1 in 140 at 6 months of gestation (Fig. 8), the latest stage tested in the present study.

Owing to the small size of the embryonic and fetal tissues analyzed and to the relatively low frequency of endothelial cells therein, sorted cells were too few to be recounted. Hence, numbers of cells seeded are those indicated by the flow cytometer, which are likely to be significantly overestimated.

## DISCUSSION

Primary generation of the life-long operating hematopoietic stem cells is believed to be circumscribed to the early stages of vertebrate ontogeny. Engagement of mesoderm into blood cell formation occurs first outside the embryo, in the yolk sac, and, slightly later, in the para-aortic splanchnopleura and derived AGM region. Both events were clearly shown in mammals to be autonomous (Medvinsky and Dzierzak, 1996; de Bruijn et al., 2000; Cumano et al., 2001; Tavian et al., 2001) and in amphibians, commitment to the formation of either ventral or dorsal blood islands – the equivalent of extra- and intra-embryonic hematopoietic territories – could be traced to distinct blastomeres as early as the morula stage (Ciau-Uitz et al., 2000).

The existence of hemangioblasts (Murray, 1932) at the origin of both endothelial cells and hematopoietic cells in the yolk sac has been supported by the results of experiments performed on quail embryos (Eichmann et al., 1997) and mouse ES cell-derived embryoid bodies (Choi et al., 1998). Whether an hemangioblast divides into mutually exclusive progenies of vascular and blood cells is not known. Alternative (or additional) differentiation intermediates may exist in the yolk sac, such as the long-hypothesized blood cell derivation from differentiated endothelial cells that was deduced from the observation of living chick blastoderms (Sabin, 1920). Indeed, the first cells that differentiate from hemangioblastic mesodermal clusters in the avian yolk sac are endothelial cells, and not hematopoietic cells (Peault et al., 1988), leaving the possibility open that at least some of the latter are derived from the endothelium.

Hematopoietic cells were even more suggestively observed to sprout from the vessel wall in the ventral region of the preumbilical embryonic aorta, where these timely emerge adjacent to pre-existing endothelial cells (Garcia-Porrero et al., 1995; Tavian et al., 1996; Tavian et al., 1999; Labastie et al., 1998). Semi-thin section histology revealed indeed that endothelial cells are profoundly disorganized, or even no more present, underneath intra-aortic hematopoietic cell clusters, which may reflect the hemogenic differentiation of local vascular endothelium (Tavian et al., 1999).

In order to determine whether vascular endothelium is indeed at the origin of hematopoietic cells in the human intra- and extra-embryonic sites where these emerge, we sought to assay in culture the blood-forming potential of endothelial cells

sorted from these territories. CD34 is expressed by vascular endothelial cells (Fina et al., 1990) and by hematopoietic progenitors and stem cells (reviewed by Holyoake et al., 1994), whereas CD45 is exclusively expressed by hematopoietic cells, including HSC (Craig et al., 1994; Trowbridge and Thomas, 1994). We did previously observe, and confirm here, that the differential expression of CD34 (and, similarly, CD31) and CD45 allows for accurate discrimination of intra-aortic hematopoietic cell clusters from neighboring endothelial cells in the human embryo (Tavian et al., 1996; Tavian et al., 1999).

We herein show that both yolk sac and AGM endothelial cells sorted as CD34<sup>+</sup> CD45<sup>-</sup> cells or CD31<sup>+</sup> CD45<sup>-</sup> cells yield abundant multilineage blood cell progeny when cultured in the presence of MS-5 stromal cells, like counterpart CD34<sup>+</sup> (or CD31<sup>+</sup>) CD45<sup>+</sup> hematopoietic progenitors. Similar to what was observed before on the whole tissues (Tavian et al., 2001), yolk sac endothelial cells gave rise to myeloid cells and NK cells, whereas AGM endothelial cells yielded, in addition, B lymphocytes. The frequency of hemogenic endothelial cells in the yolk sac and AGM was closely correlated with the blood-forming status of these tissues as a function of developmental age. That frequency in the AGM was highest around day 30 and declined dramatically after day 36, thus following the curve of definitive, intra-embryonic hematopoiesis emergence drawn after morphologic and functional observations (Tavian et al., 1999; Tavian et al., 2001). The highest frequency calculated for these cells, about 1/120, may look modest but it must be reminded that in these experiments the total AGM was analyzed, which includes (in addition to the aorta) the cardinal veins and a large number of capillary vessels (see Fig. 1), while only the ventral aspect of the aorta supports HSC emergence (Tavian et al., 2001). Hence, the actual frequency of blood-forming endothelial cells in the aorta alone, which was too small in size to be used for cell sorting, is likely to be much higher.

Virtually no hematopoietic endothelial cells were detected in the AGM before the 28-day stage, which marks the onset of human intra-embryonic hematopoiesis. Yet, we have found recently that the whole human PSp/AGM taken as early as day 21 of development produces hematopoietic cells in the presence of MS-5 stromal cells (Tavian et al., 2001). This apparent discrepancy may be explained by the fact that endothelial cells must receive some inductive signal from surrounding tissues in order to become hematopoietic. Hence, pre-hematopoietic endothelial cells sorted free of adjacent mesoderm and endoderm would not be able to sustain blood cell formation. The same may apply to the liver rudiment since at 27 days, i.e. 3 days before hepatic hematopoiesis incipience, sorted hepatic endothelial cells were devoid of blood-forming ability. Alternatively, hemogenic endothelial cells within the embryonic aorta may derive from angio-hematopoietic progenitors that migrate secondarily from the para-aortic splanchnopleura into the already formed aortic wall, which would explain why endothelial cells sorted from the pre-hematopoietic aorta exhibit no blood-forming activity. In agreement with this assumption, preliminary experiments have suggested that from 21 to 27 days of development, blood-forming activity in the PSp/AGM territory is confined within non-endothelial, CD34<sup>-</sup> CD45<sup>-</sup> cells (M. Tavian, unpublished). Such a population of candidate angio-hematopoietic progenitors expressing KDR, the human FLK1 receptor, but

negative for CD34 expression has been identified earlier in the PSp and subaortic mesenchyme (Cortés et al., 1999).

Hematopoietic vascular endothelial cells were found similarly in the yolk sac, the frequency of which reaches about 1 in 100 cells in the fourth week and also decreases dramatically as development proceeds, lending credit to observations made a long time ago by Sabin (Sabin, 1920). It was not possible in the present study, though, to examine the yolk sac at the time of hematopoiesis incipience, which takes place at the uneasily accessible 18-day stage of human development (Bloom and Bartelmez, 1940). At 26 days, the earliest analyzed stage reported here, yolk sac blood vessels are terminally differentiated and very few blood islands, and even less hemangioblastic cell clusters, if any, are still present. Therefore the endothelial hematopoiesis we observed at that stage may reflect a finishing activity that was quantitatively much more intense in the preceding week of development.

The possibility that the observations reported herein are due to the presence of contaminating hematopoietic progenitors among sorted endothelial cells could be ruled out because: (1) reanalyzed sorted cells were free of CD45<sup>+</sup> cells; (2) no CD45 mRNAs were ever detected by PCR within sorted CD45<sup>-</sup> cells; and (3) no clonogenic hematopoietic progenitors were detected either among sorted CD34<sup>+</sup> CD45<sup>-</sup> cells cultured in methylcellulose. Hence, contaminating hematopoietic cells, if at all present, should be extremely rare, which could not account for the frequency of hemogenic endothelial cells encountered in some tissues at some stages of development. It must be also stressed that no hematopoietic potential was ever detected among CD34<sup>+</sup> CD45<sup>-</sup> cells sorted from fetal thymus and spleen, from non-hematopoietic tissues such as the fetal aorta, heart, pancreas, lung or umbilical cord, from the umbilical vein at term, or among cultured HUVEC.

We should insist on the fact that the CD34<sup>+</sup> CD31<sup>+</sup> CD45<sup>-</sup> combination was chosen as an indicator of vascular endothelial cells after other phenotypes did not prove reliable for accurate cell sorting. Notably, anti-VE-cadherin staining, which satisfactorily, although dimly, delineated vascular endothelium on tissue sections, failed to circumscribe a sharp subpopulation of cells on flow cytometry analysis (not shown). Hence, it was decided to avoid using VE-cadherin for endothelial cell sorting.

The existence of blood-forming vascular endothelium thus seems to be a feature of early sites of HSC emergence in the human yolk sac and embryo and our results confirm the filiation between endothelial and hematopoietic cells that was proposed to exist in the avian and mouse AGM (Jaffredo et al., 1998; Nishikawa et al., 1998). However, we surprisingly also observed the presence of hematopoietic endothelial cells, albeit at lower frequency, within primary hematopoietic organs, i.e. the embryonic liver and fetal bone marrow. The mouse liver rudiment taken before the 28-somite stage and transplanted into a syngenic host does not support hematopoiesis, which led to assume that hepatic hematopoiesis originates in circulating embryonic progenitors of extrinsic origin (Johnson and Moore, 1975). Similarly, it was shown in chicken embryo parabionts (Metcalf and Moore, 1971) and quail/chicken embryonic chimeras (Le Douarin et al., 1975) that blood-borne stem cells are at the origin of medullary hematopoiesis. Migration of hematopoietic cells from the fetal liver to the bone marrow was suggested on direct marker gene transfer into the former (Clapp et al., 1995). Yet, these results do not rule out the participation

of fixed compartments of the hepatic and marrow stroma to hematopoietic development, nor do they exclude the possibility that the liver and marrow rudiments are colonized by angio-hematopoietic stem cells that may also contribute hemogenic vascular cells. In that respect, a filiation between endothelial and hematopoietic cells in the adult human was indirectly suggested through observations made in individuals with chronic myeloid leukemia, in whom vascular endothelial cells were observed to contain BCR/ABL fusion gene transcripts, previously assumed to be restricted to the leukemic cells (Gunsilius et al., 2000). Indeed, our preliminary results indicate that a subpopulation of endothelial cells in the bone marrow of the healthy adult is also endowed with hematopoietic potential (E. Oberlin and M. Souyri, personal communication).

Dissecting the structure of the *SCL* gene, which is essential for the normal development of blood and blood vessels, Sanchez et al. (Sanchez et al., 1999) have identified a 3' enhancer that drives the expression in transgenic mice of a reporter gene in both the endothelium and blood cell subsets in diverse sites of embryonic hematopoiesis, including the yolk sac and PSp/AGM. This enhancer also directed expression to adult HSCs, but expression in adult endothelial cells was not examined. These results suggest the existence of endothelial cells, but may also reflect the activity of primitive angio-hematopoietic stem cells. Indeed, our whole work relies on the assumption, which has not been questioned so far, that the CD34<sup>+</sup> CD45<sup>-</sup> antigen expression typifies only endothelial cells. However, we cannot formally exclude the possibility that, at least at the earliest developmental stages, a very rare subset of ancestral hemangioblasts en route to hematopoiesis, but not yet expressing CD45, could be co-purified with endothelial cells and participate in the blood cell production we have observed on MS-5 stromal cells (Traver and Zon, 2002). Transgenic mouse models in which the whole progeny of vascular endothelium is permanently tagged will lead to the deciphering of the full potential in normal development and postnatal life of a vascular cell population for which a role in cardiomyocyte formation has also been suggested elsewhere (Condorelli et al., 2001).

We are indebted to Dr I. Dagousset and I. Gonzales and to Prof. P. Blot for their help with human embryo procurement. We also acknowledge the assistance of Drs J. Martinovic and J. Tantau and of Prof. C. Nessman in providing fetal tissues. We thank Dr K. Hussein for his help with cell culture, Dr I. Casal and V. Schiavon for assistance with FACS sorting, and J. Chagraoui for providing technical advice. E. O. and M. T. are the recipients of fellowships from the Human Frontier Science Program and Fondation de France.

## REFERENCES

- Berardi, A. C., Meffre, E., Pflumio, F., Katz, A., Vainchenker, W., Schiff, C. and Coulombel, L. (1997). Individual CD34<sup>+</sup>CD38<sup>low</sup>CD19<sup>-</sup>CD10<sup>-</sup> progenitor cells from human cord blood generate B lymphocytes and granulocytes. *Blood* **89**, 3554-3564.
- Bloom, B. and Bartelmez, G. W. (1940). Hematopoiesis in young human embryos. *Am. J. Anat.* **67**, 21-53.
- Charbord, P., Taviani, M., Humeau, L. and Peault, B. (1996). Early ontogeny of the human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment. *Blood* **87**, 4109-4119.
- Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. and Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725-732.

- Chomczynski, P. and Sacchi, N.** (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.
- Ciau-Uitz, A., Walmsley, M. and Patient, R.** (2000). Distinct origins of adult and embryonic blood in *Xenopus*. *Cell* **102**, 787-796.
- Clapp, D. W., Freie, B., Lee, W. H. and Zhang, Y. Y.** (1995). Molecular evidence that in situ-transduced fetal liver hematopoietic stem/progenitor cells give rise to medullary hematopoiesis in adult rats. *Blood* **86**, 2113-2122.
- Condorelli, G., Borello, U., de Angelis, L., Latronico, M., Sirabella, D., Coletta, M., Galli, R., Balconi, G., Follenzi, A., Frati, G. et al.** (2001). Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration. *Proc. Natl. Acad. Sci. USA* **98**, 10733-10738.
- Cortés, F., Debacker, C., Peault, B. and Labastie, M. C.** (1999). Differential expression of KDR/VEGFR-2 and CD34 during mesoderm development of the early human embryo. *Mech. Dev.* **83**, 161-164.
- Craig, W., Poppema, S., Little, M. T., Dragowska, W. and Lansdorp, P. M.** (1994). CD45 isoform expression on human haemopoietic cells at different stages of development. *Br. J. Haematol.* **88**, 24-30.
- Cumano, A. and Godin, I.** (2001). Pluripotent hematopoietic stem cell development during embryogenesis. *Curr. Opin. Immunol.* **13**, 166-171.
- Cumano, A., Ferraz, J. C., Klaine, M., di Santo, J. P. and Godin, I.** (2001). Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity* **15**, 477-485.
- de Bruijn, M. F., Speck, N. A., Peeters, M. C. and Dzierzak, E.** (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J.* **19**, 2465-2474.
- Dieterlen-Lièvre, F.** (1994). Hemopoiesis during avian ontogeny. *Poultry Sci. Rev.* **5**, 273-305.
- Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Breant, C. and le Douarin, N. M.** (1997). Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc. Natl. Acad. Sci. USA* **94**, 5141-5146.
- Fina, L., Molgaard, H. V., Robertson, D., Bradley, N. J., Monaghan, P., Delia, D., Sutherland, D. R., Baker, M. A. and Greaves, M. F.** (1990). Expression of the CD34 gene in vascular endothelial cells. *Blood* **75**, 2417-2426.
- Garcia-Porrero, J. A., Godin, I. E. and Dieterlen-Lievre, F.** (1995). Potential intraembryonic hemogenic sites at pre-liver stages in the mouse. *Anat. Embryol.* **192**, 425-435.
- Gunsilius, E., Duba, H. C., Petzer, A. L., Kahler, C. M., Grunewald, K., Stockhammer, G., Gabl, C., Dirnhofer, S., Clausen, J. and Gasl, G.** (2000). Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. *Lancet* **355**, 1688-1691.
- Holthofer, H., Virtanen, I., Kariniemi, A. L., Hormia, M., Linder, E. and Miettinen, A.** (1982). Ulex europaeus I lectin as a marker for vascular endothelium in human tissues. *Lab. Invest.* **47**, 60-66.
- Holyoake, T. L. and Alcorn, M. J.** (1994). CD34+ positive haemopoietic cells: biology and clinical applications. *Blood Rev.* **8**, 113-124.
- Issaad, C., Croisille, L., Katz, A., Vainchenker, W. and Coulombel, L.** (1993). A murine stromal cell line allows the proliferation of very primitive human CD34+ cells. *Blood* **81**, 2916-2924.
- Itoh, K., Tezuka, H., Sakoda, H., Konno, M., Nagata, K., Uchiyama, T., Uchino, H. and Mori, K. J.** (1989). Reproducible establishment of hemopoietic supportive stromal cell lines from murine bone marrow. *Exp. Hematol.* **17**, 145-153.
- Jaffe, E. A., Nachman, R. L., Becker, C. G. and Minick, C. R.** (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* **52**, 2745-2756.
- Jaffredo, T., Gautier, R., Eichmann, A. and Dieterlen-Lievre, F.** (1998). Intraortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development* **125**, 4575-4583.
- Johnson, G. R. and Moore, M. A.** (1975). Role of stem cell migration in initiation of mouse foetal liver haemopoiesis. *Nature* **258**, 726-728.
- Labastie, M. C., Cortes, F., Romeo, P. H., Dulac, C. and Peault, B.** (1998). Molecular identity of hematopoietic precursor cells emerging in the human embryo. *Blood* **92**, 3624-3635.
- Le Douarin, N. M., Houssaint, E., Jotereau, F. V. and Belo, M.** (1975). Origin of hemopoietic stem cells in embryonic bursa of Fabricius and bone marrow studied through interspecific chimeras. *Proc. Natl. Acad. Sci. USA* **72**, 2701-2705.
- Ling, K. W. and Dzierzak, E.** (2002). Ontogeny and genetics of the hemato/lymphopoietic system. *Curr. Opin. Immunol.* **14**, 186-191.
- Medvinsky, A. and Dzierzak, E.** (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897-906.
- Metcalf, D. and Moore, M. A. S.** (1971). Hematopoietic cells. In *North-Holland Research Monographs Frontiers of Biology - Volume 24* (ed. A. Neuberger and E. L. Tatum), pp. 1-550. Amsterdam, London: North Holland Publishing.
- Moore, K. A., Ema, H. and Lemischka, I. R.** (1997). In vitro maintenance of highly purified, transplantable hematopoietic stem cells. *Blood* **89**, 4337-4347.
- Murray, P. D. F.** (1932). The development in vitro of blood of the early chick embryo. *Proc. R. Soc. London* **111**, 497-521.
- Nishikawa, S. I., Nishikawa, S., Kawamoto, H., Yoshida, H., Kizumoto, M., Kataoka, H. and Katsura, Y.** (1998). In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunity* **8**, 761-769.
- North, T., Gu, T. L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marin-Padilla, M. and Speck, N. A.** (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* **126**, 2563-2575.
- O'Rahilly, R. and Müller, F.** (1987). *Development Stages in Human Embryos*. Washington: Carnegie Institution of Washington.
- Peault, B., Coltey, M. and le Douarin, N. M.** (1988). Ontogenic emergence of a quail leukocyte/endothelium cell surface antigen. *Cell Differ.* **23**, 165-174.
- Ploemacher, R. E., van der Sluijs, J. P., Voerman, J. S. and Brons, N. H.** (1989). An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. *Blood* **74**, 2755-2763.
- Ponte, P., Ng, S. Y., Engel, J., Gunning, P. and Kedes, L.** (1984). Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. *Nucleic Acids Res.* **12**, 1687-1696.
- Robin, C., Pflumio, F., Vainchenker, W. and Coulombel, L.** (1999). Identification of lymphomyeloid primitive progenitor cells in fresh human cord blood and in the marrow of nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice transplanted with human CD34(+) cord blood cells. *J. Exp. Med.* **189**, 1601-1610.
- Sabin, F.** (1920). Studies on the origin of blood vessels and of red blood corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Contrib. Embryol. Carnegie Inst. of Washington* **9**, 214-262.
- Sanchez, M., Gottgens, B., Sinclair, A. M., Stanley, M., Begley, C. G., Hunter, S. and Green, A. R.** (1999). An SCL 3' enhancer targets developing endothelium together with embryonic and adult haematopoietic progenitors. *Development* **126**, 3891-3904.
- Streuli, M., Hall, L. R., Saga, Y., Schlossman, S. F. and Saito, H.** (1987). Differential usage of three exons generates at least five different mRNAs encoding human leukocyte common antigens. *J. Exp. Med.* **166**, 1548-1566.
- Tavian, M., Coulombel, L., Luton, D., Clemente, H. S., Dieterlen-Lievre, F. and Peault, B.** (1996). Aorta-associated CD34+ hematopoietic cells in the early human embryo. *Blood* **87**, 67-72.
- Tavian, M., Hallais, M. F. and Peault, B.** (1999). Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo. *Development* **126**, 793-803.
- Tavian, M., Robin, C., Coulombel, L. and Peault, B.** (2001). The human embryo, but not its yolk sac, generates lympho-myeloid stem cells: mapping multipotent hematopoietic cell fate in intraembryonic mesoderm. *Immunity* **15**, 487-495.
- Traver, D. and Zon, L. I.** (2002). Walking the walk: migration and other common themes in blood and vascular development. *Cell* **108**, 731-734.
- Trowbridge, I. S. and Thomas, M. L.** (1994). CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu. Rev. Immunol.* **12**, 85-116.
- Watt, S. M., Butler, L. H., Tavian, M., Buhring, H. J., Rappold, L., Simmons, P. J., Zannettino, A. C., Buck, D., Fuchs, A., Doyonnas, R. et al.** (2000). Functionally defined CD164 epitopes are expressed on CD34(+) cells throughout ontogeny but display distinct distribution patterns in adult hematopoietic and nonhematopoietic tissues. *Blood* **95**, 3113-3124.
- Wood, H. B., May, G., Healy, L., Enver, T. and Morriss-Kay, G. M.** (1997). CD34 expression patterns during early mouse development are related to modes of blood vessel formation and reveal additional sites of hematopoiesis. *Blood* **90**, 2300-2311.