

# Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny

Thierry Jaffredo\*, Rodolphe Gautier, Anne Eichmann and Françoise Dieterlen-Lièvre

Institut d'Embryologie Cellulaire et Moléculaire du CNRS et du Collège de France; 49 bis, Avenue de la Belle Gabrielle, 94736 Nogent-sur-Marne Cedex, France

\*Author for correspondence (e-mail: jaffredo@infobiogen.fr)

Accepted 10 September; published on WWW 20 October 1998

## SUMMARY

We have investigated the developmental relationship of the hemopoietic and endothelial lineages in the floor of the chicken aorta, a site of hemopoietic progenitor emergence in the embryo proper. We show that, prior to the onset of hemopoiesis, the aortic endothelium uniformly expresses the endothelium-specific membrane receptor VEGF-R2. The onset of hemopoiesis can be determined by detecting the common leukocyte antigen CD45. VEGF-R2 and CD45 are expressed in complementary fashion, namely the hemopoietic cluster-bearing floor of the aorta is CD45<sup>+</sup>/VEGF-R2<sup>-</sup>, while the rest of the aortic endothelium is CD45<sup>-</sup>/VEGF-R2<sup>+</sup>.

To determine if the hemopoietic clusters are derived from endothelial cells, we tagged the E2 endothelial tree from the inside with low-density lipoproteins (LDL) coupled to DiI. 24 hours later, hemopoietic clusters were labelled by LDL. Since no CD45<sup>+</sup> cells were inserted among endothelial cells at the time of vascular labelling, hemopoietic clusters must be concluded to derive from precursors with an endothelial phenotype.

Key words: Avian embryo, Hematopoietic progenitor, Endothelial cell, VEGF-R2, CD45, LDL

## INTRODUCTION

The endothelial network and the hemopoietic system develop in parallel and are strikingly intertwined in the yolk sac blood islands and in the embryo's aorta. In the present report, we examine the possible filiation between hemopoietic cells (HC) and endothelial cells (EC). We focus on intraaortic hemopoietic clusters, which produce cells involved in the colonization of definitive hemopoietic organ rudiments and give rise to definitive hemopoietic stem cells (HSC). These clusters have been demonstrated to develop *in situ* in 'yolk sac chimeras' (Martin, 1972), i.e. quail embryos grafted onto a chicken host yolk sac (Dieterlen-Lièvre and Martin, 1981). Red cells of intraembryonic origin appeared in the blood (Beaupain et al., 1979) and the thymic rudiments were colonized by HSC of intraembryonic origin (Martin et al., 1978). The production of multipotential progenitors by the aortic region was demonstrated in clonal cultures, and no progenitors developed from the rest of the embryonic body (Cormier et al., 1986; Cormier and Dieterlen-Lièvre, 1988; Cormier, 1993). In the mouse, the homologous region was demonstrated to produce progenitors with lymphohemopoietic potential, when separated from the yolk sac prior to the establishment of circulation. In contrast, progenitors from the yolk sac had only myeloid potential (Cumano et al., 1996; for review see; Dieterlen-Lièvre, 1997; Dzierzak et al., 1998).

Intraaortic clusters have been reported in a vast range of

species from shark to human. They display a striking polarization, being inserted in the floor of the aorta as two parallel longitudinal ridges or as a single central row (Dantschakoff, 1907; Maximow, 1909; Minot, 1910; Emmel, 1916; Jordan, 1917, 1918; Dieterlen-Lièvre, 1975; Smith and Glomsky, 1982; Garcia-Porrero et al., 1995; Tavian et al., 1996; Wood et al., 1997). Despite this stereotyped appearance, little is known about the origin of the intraaortic clusters in relationship to the endothelium of the aorta.

Based on their close spatial and temporal relationships, HC and EC have been proposed to originate from a common precursor, designated as the hemangioblast (Sabin, 1917; Murray, 1932; Wagner, 1980). The two lineages possess several features that support the idea of a common origin. In birds, they both express the MB1/QH1 antigen (Labastie et al., 1986; Pardanaud et al., 1987), the newly reported thrombomucin MEP 21 (Mc Nagny et al., 1997) and the transcription factor SCL/tal (Drake et al., 1997). In mammals, the two lineages express CD34 (Young et al., 1995; Tavian et al., 1996; Wood et al., 1997), CD31 (Baldwin et al., 1994) and SCL-tal (Hwang et al., 1993; Kallianpur et al., 1994). Furthermore, several gene deletions affect both lineages (Shalaby et al., 1995, 1997; Robb et al., 1995; Shivdasani et al., 1995; Ferrara et al., 1996).

In the present study, the emergence of HC in the aortic floor was explored by double-staining for an EC (Quek-1/VEGF-R2) and an HC (CD45) antigen. Quek-1 (Eichmann et al.,

1993, 1996) is the avian homologue of flk-1/KDR/VEGF-R2 (Matthews et al., 1991; Terman et al., 1991), one of the seven endothelial-specific growth factor receptors with tyrosine kinase (for review see Mustonen and Alitalo, 1995). Targeted gene inactivation demonstrated that several of these receptors are essential for the development of the vascular system (Dumont et al., 1994; Fong et al., 1995; Puri et al., 1995; Sato et al., 1995); each is probably involved at a particular step of EC differentiation. VEGF-R2 is a major regulator of vasculogenesis and angiogenesis; it appears to be EC-specific (Eichmann et al., 1993; Millauer et al., 1993; Yamaguchi et al., 1993).

In mouse, chick and quail embryos, VEGF-R2 is present as early as the primitive streak stage (Millauer et al., 1993; Eichmann et al., 1993) in a region containing precursors for EC and HC and is the earliest marker identified to date. It was found on angioblasts and EC at very early developmental stages, and in the allantois, yolk sac and on all the EC of the embryo proper at least until 15 dpc for the mouse (Millauer et al., 1993; Yamaguchi et al., 1993; Dumont et al., 1995) and E9 for birds (Eichmann et al., 1993; Couly et al., 1995; Wilting et al., 1997), persisting in subsets of EC afterwards (Wilting et al., 1997). Quek-1<sup>+</sup> cells sorted out from the chick gastrula give rise to both hemopoietic and endothelial progenitors, which develop into HC or EC colonies (Eichmann et al., 1997).

The pan leukocyte antigen CD45 is a transmembrane glycoprotein with phosphotyrosine phosphatase activity expressed exclusively by cells of the hemopoietic lineage (for a review see Thomas, 1989; Trowbridge and Thomas, 1994) from an early stage of ontogeny (Tavian et al., 1996). In the chick, several monoclonal antibodies (mAbs) have been obtained that detect the avian homologue of CD45 with intrinsic phosphotyrosine phosphatase activity (Paramithiotis et al., 1991).

The processes triggering intraaortic HC formation are still unknown. To investigate the developmental relationships between EC and intraaortic clusters, we analyzed the expression patterns of VEGF-R2 and CD45, and we carried out a dynamic study, by tagging the endothelial tree at a time when no hemopoietic clusters are present. Cells in the endothelial tree were traced with DiI-conjugated acetylated low-density lipoproteins (Ac LDL-DiI) which can be visualized as early as 2-3 hours after injection.

This approach indicates that intraaortic clusters are seeded by progenitors derived from the aortic endothelium. At E3-4, CD45 was found to replace VEGF-R2 on some of the cells lining the floor of the aorta so that, in this location, 'true' EC seemed absent. Labelling revealed a developmental link between EC and intraaortic clusters. Furthermore a subpopulation of CD45<sup>+</sup> cells was shown to ingress into the dorsal mesentery.

## MATERIALS AND METHODS

### Inoculations

Brown Leghorn (JA 57 strain) chick embryos and Japanese quail (*Coturnix coturnix japonica*) were staged according to Hamburger and Hamilton (HH) (1951). HH13-14 (19 to 22 somites) chick embryos were inoculated in the heart. Briefly, the embryo was visualized by injecting a India ink solution (Pelikan) 1/1 in PBS into

the subgerminal cavity. A small opening was made into the body wall at the level of the heart and LDL solution (0.3-0.5 µl; Molecular probes, Eugene, USA) was inoculated with a borosilicated glass needle pulled in a micropipette puller into a tip of approximately 30 µm, small enough not to damage the heart muscle wall. The liquid, gently flushed into the circulation, distributed to the whole vascular tree.

### Cytology

The embryos were processed for LDL histology, CD45 or/and Quek-1/VEGF-R2 immunostaining. Embryos were transferred in a solution containing phosphate buffer 0.12 M (PB)/saccharose 15% (Merck) for 24-48 hours, impregnated in PB/15% saccharose/7.5% gelatin for 1 hour at 37°C, then embedded at room temperature. Blocks were frozen in isopentane (Prolabo) at -65°C for 1 minute and transferred to -80°C until cryostat sectioning. 8-10 µm serial sections were deposited on Superfrost plus slides (Menzel Gläser Germany) which were stored at -80°C until use.

### Antibodies

QH1 is a monoclonal antibody of the IgM subclass recognising quail endothelial and hemopoietic cells except mature erythrocytes (Pardanaud et al., 1987). Monoclonal anti-chick CD45 (IgG2a subclass; Jeurissen et al., 1988) was supplied by ID-DLO (The Netherlands). It was found to have no affinity for quail hemopoietic cells. The anti-Quek-1 (IgG1 subclass) is a monoclonal antibody recognizing the avian VEGF-R2 (Eichmann et al., 1997). It has affinity for EC in both chick and quail species.

### Immunohistochemistry

CD45 and Quek-1 were revealed using the Tyramide Signal Amplification system (TSA, NEN Life Science) according to the manufacturer's recommendations. Primary antibodies were applied overnight at 4°C in a humid atmosphere alone or together, followed by secondary antibodies (Southern Biotechnology), respectively, a sheep anti-mouse IgG2a-HRP-coupled for CD45 or a sheep anti-mouse IgG1 biotinyl-coupled for Quek-1. For double fluorescent detection, after the two primary antibodies, non-specific interactions were blocked for 30 minutes with TNB (Tris-buffered saline-containing 0.5% NEN Life Science Blocking reagent). The Quek-1-specific secondary antibody was then added first 1/100 in TNB for 1 hour. After 3 rinses in TNT (Tris-HCl 0.1 M pH 7.5, NaCl 0.15 M, Tween 20 0.05%), streptavidine-HRP 1/500 in TNT was applied for 30 minutes followed by biotinylated tyramide 1/50 in amplification buffer for 10 minutes and Streptavidin Cy3 (red, Amersham) 1/1000 in TNT for 30 minutes. Excess HRP was inactivated by 3.3% H<sub>2</sub>O<sub>2</sub> (Merck) in PBS for 45 minutes between the two amplification steps. CD45 was revealed in the same manner with a sheep anti-mouse IgG2A-HRP 1/100 in TNB for 1 hour, followed by biotinylated tyramide 1/50 for 10 minutes and then Streptavidin Cy2 (green, Amersham) 1/1000 in TNT for 30 minutes. The slides were mounted in Glycergel (Dako) and observed in a Nikon AF microscope equipped with epifluorescence and appropriate filters.

When chromogenic substrates were used rather than fluorochromes, the detection technique was modified as follow. The two secondary antibodies were applied simultaneously for 1 hour. Tyramide-FITC 1/100 in amplification buffer was applied for 30 minutes to detect CD45. Quek-1 was detected with streptavidine-HRP followed by 2-Diamino Benzidine (10 mg tablets, Sigma) in PBS. At the end of the procedure, CD45 was revealed with anti-fluorescein-alkaline phosphatase (Amersham) 1/100 in TNT for 1 hour detected with the Sigma Fast red-Naphtol ASMX kit (Sigma). Detailed protocols are available upon request.

QH1 immunohistochemistry on quail embryo semithin sections was performed as described in Pardanaud et al. (1996).

### Semithin and ultrathin sections, electron microscopy

The truncal regions of HH19 chick embryos were trimmed and fixed in 2.5% glutaraldehyde/2% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.6 at 4°C for 1.5 hour. They were washed and stored in cacodylate buffer/8% sucrose for 15-18 hours at 4°C and post fixed with 1% OsO<sub>4</sub> in cacodylate buffer at 4°C for 1.5 hours. Tissues were dehydrated in graded ethanol, embedded in Araldite and cut on a Reichert ultramicrotome equipped with a glass or diamond knife. Sections were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope JEM 1200. Semithin sections (1 μm) were also examined with a light microscope after QH1 immunostaining and/or toluidine blue counterstaining.

## RESULTS

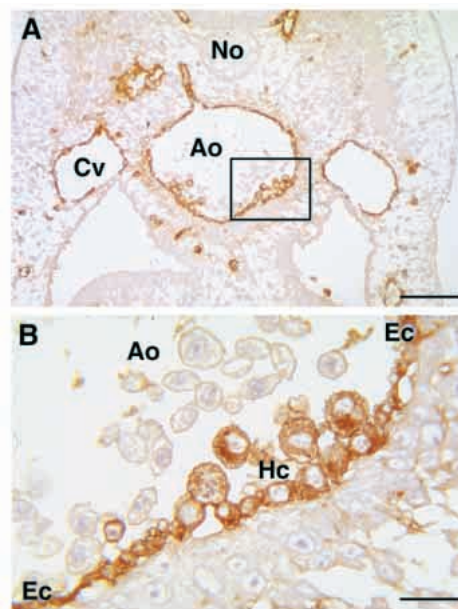
Although intraaortic clusters are prominent in the chick (Dantschakoff, 1907; Sabin, 1917; Dieterlen-Lièvre, 1975) and in the quail embryo (Dieterlen-Lièvre et al., 1977), neither their developmental timing nor their anatomical distribution have been precisely described before. We set out to identify HC and EC through their antigenic phenotypes. QH1, which has been widely used to study vascular development in quail-chicken chimeras as it recognizes both quail HC and EC, was used to map the extent of intraaortic clusters during the switch from the flat endothelial morphology to the puffed morphology of hemopoietic cells and the distribution of the clusters within the quail vascular tree. Anti-VEGF-R2/anti-CD45 double staining, which made it possible to distinguish the developmental relationship between the endothelial tree and HC in the chicken species, was used to analyze the two cell types.

### Developmental mapping of intraaortic clusters in birds

In E2 embryos with 19 to 22 pairs of somites (HH 13-14), the aortic endothelium consisted entirely of VEGF-R2-positive flat cells. Intraaortic clusters were not detected until the two aortic anlagen began to fuse. The first HC appeared between HH 16 and 17 (early E3) at the cephalic most levels of the floor of the dorsal aorta. In HH 18-20 chick and quail embryos, the clusters extended from branchial arches to the pre-umbilical vessels. They developed as two longitudinal symmetric ridges according to a rostrocaudal gradient, larger aggregates being detected rostrally while fewer and smaller ones were present in caudalmost regions. These ridges were located on the ventrolateral aspects of the aorta ('the floor of the aorta', Fig. 1A). These budding cells were not usually observed on the median line. Cells in the clusters were usually variable in size and shape and were markedly basophilic with a prominent nucleoli. While no flat EC could be seen at the position of the clusters, typical aortic endothelium appeared to merge laterally with the cluster-bearing areas (Fig. 1B). In the junction zones, cells bulged towards the aortic lumen. The mesoderm appeared denser ventral to the aortic floor while it was less dense dorsal to the roof. From stage 21-22, the HC production decreased and the aortic floor reverted to its EC appearance.

### Complementary expressions of VEGF-R2 and CD45

Standard immunocytological protocols appeared unable to detect Quek-1/VEGF-R2 and CD45 in situ: the signal was either very weak or absent. We modified the NEN Tyramide Signal Amplification technique (see Materials and methods) to



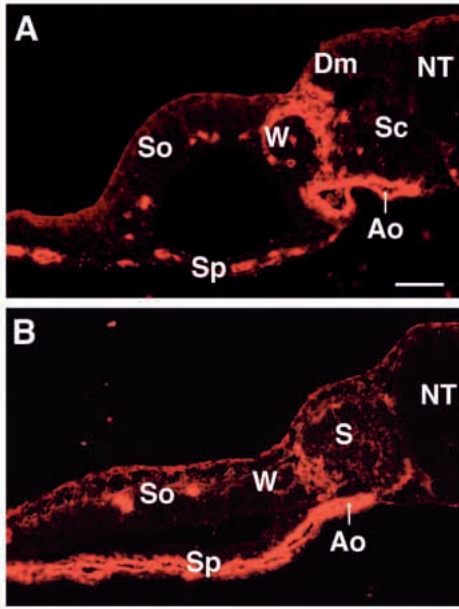
**Fig. 1.** Intraaortic clusters in the E3 quail embryo. Semithin cross section at the truncal level, QH1 immunostaining. (A) The clusters are symmetric groups of QH1<sup>+</sup> cells that appear to bud from the floor of the aorta (Ao). No clusters are present in cardinal veins (Cv) or in any other vessel. Longitudinally, the clusters build up two long ridges. No, notochord. Bar= 75 μm. (B) Enlarged inset showing the progressive transition between flat endothelial (Ec) and round hemopoietic (Hc) cells. Bar, 25 μm.

be able to stain both CD45 and Quek-1/VEGF-R2 simultaneously without ambiguity.

At E2 and until the beginning of E3 (HH17), the whole chicken endothelial tree was lined by typical VEGF-R2<sup>+</sup> cells. No CD45<sup>+</sup> cells were present in the embryo until stage HH14-15 (Fig. 2A,B). Precisely at this stage, numerous scattered CD45<sup>+</sup> cells first appeared in the yolk sac and in the blood, particularly in the cardiac cavity and the ducts of Cuvier (Fig. 3). These cells, then appeared to exit from the blood through the endothelium and rapidly invaded the whole embryo, beginning with the aortic arches. These numerous cells were stained so intensely by CD45 that they did not need tyramide amplification to be detected, in contrast to cells in the intraaortic clusters. In distribution, time and mode of appearance, these CD45<sup>+++</sup> cells correspond exactly to the cells identified by Cuadros et al. (1992) as monocyte-macrophages and shown in chicken embryos grafted on a quail yolk sac to originate from the yolk sac (Cuadros et al., 1992).

Another CD45<sup>+</sup> population appeared 15 hours later at stages 17-18 HH, after the two aortic anlagen had fused. Two features clearly distinguished these cells from the CD45<sup>+++</sup> macrophage-monocyte group; firstly, instead of being distributed all over the embryo, they were inserted in the aortic floor in small groups; secondly, the CD45 expression that they displayed was weaker by several orders of magnitude. Together with the chimerae (Dieterlen-Lièvre and Martin, 1981) and clonal culture data (Cormier et al., 1986), the restricted localization of these cells and their CD45 weak affinity allow their identification as the first intraembryonic hemopoietic cells. Usually these CD45<sup>+</sup> cells bulged into the aortic lumen



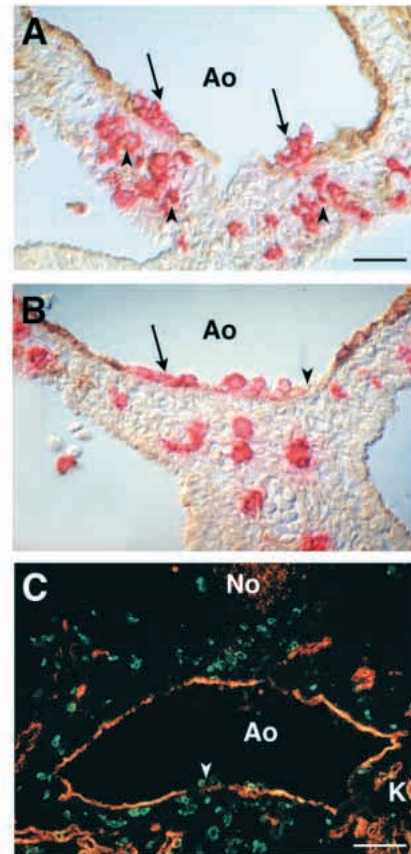
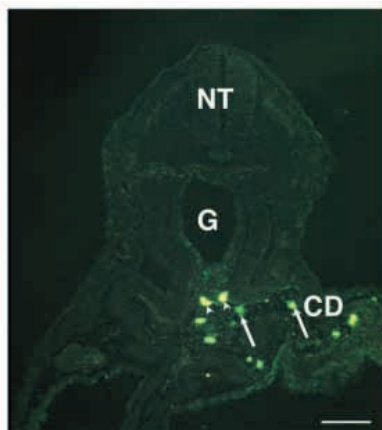


**Fig. 2.** VEGF-R2 expression at truncal level in a 22-somite chick embryo (E2). Quek1 antibody, tyramide amplification, Cy3 fluorescence. Cross sections. Bar, 125  $\mu$ m. (A) 10<sup>th</sup> somite level. The aorta (Ao) is entirely VEGF-R2<sup>+</sup> (arrow). VEGF-R2<sup>+</sup> cells are present in the lateral part of the dermomyotome (Dm) and surround the Wolffian duct (W). VEGF-R2<sup>+</sup> cells are more abundant in the splanchnopleural (Sp) than in the somatopleural (So) mesoderm. Dm, dermomyotome; NT, Neural tube; Sc, Sclerotome. (B) 17<sup>th</sup> somite level. Angioblasts are present at the basolateral edge of the somite (S) near the Wolffian duct. At this level the vascular network is densely arranged in the splanchnopleural mesoderm.

in the vicinity of flat VEGF-R2<sup>+</sup> endothelial-like cells. VEGF-R2 was not expressed by cells expressing CD45.

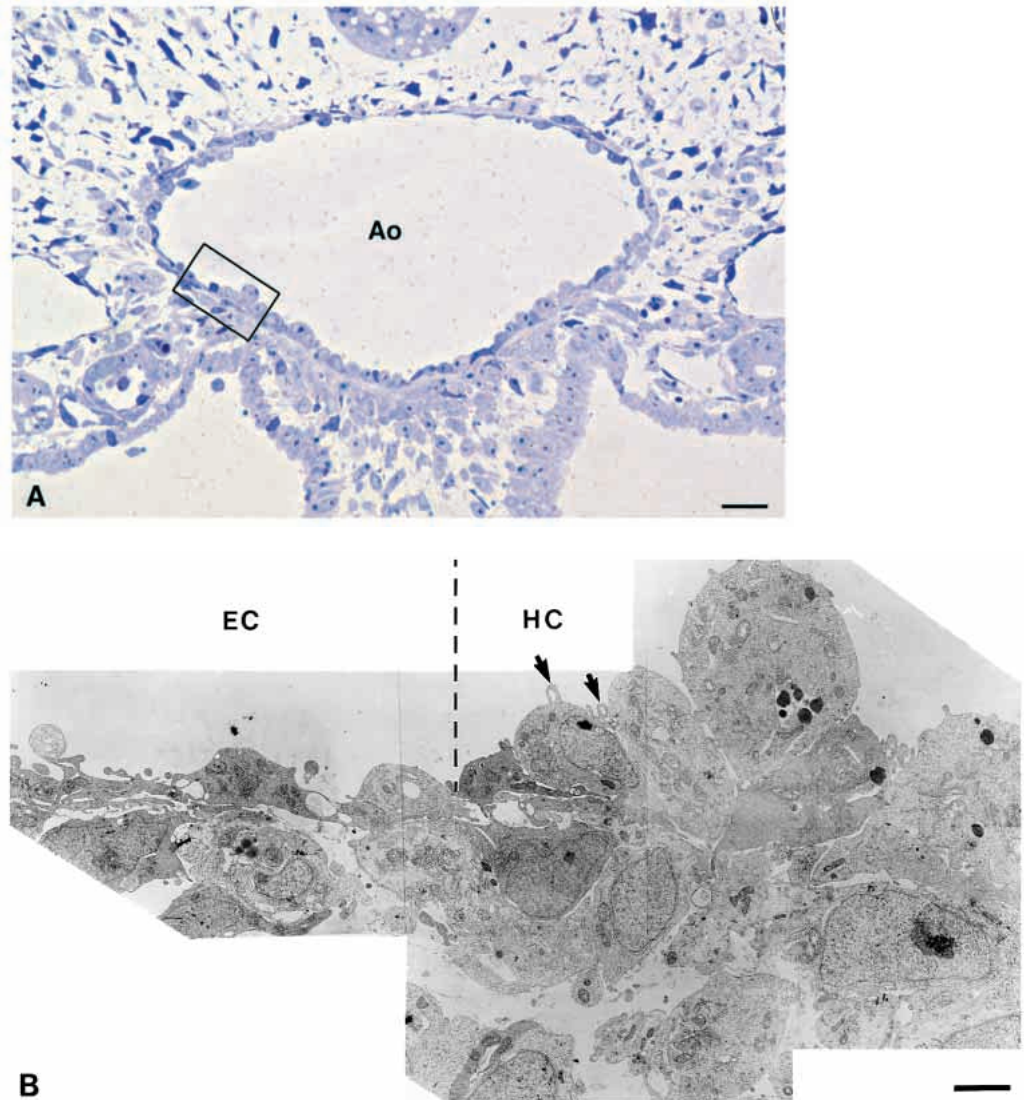
From stage 19 HH, these HC were found all along the aorta from the level of the branchial arches to that of the vitelline veins. Different steps could be recognized in the emergence of these clusters. At the upper trunk level, the CD45<sup>+</sup> clusters were large and did not express VEGF-R2, which was completely missing from the cluster-bearing stretches of aortic floor. VEGF-R2 staining was found immediately adjacent to CD45<sup>+</sup> stretches (Fig. 4A). Many single round CD45<sup>+</sup> cells were also present in the dorsal mesentery immediately

**Fig. 3.** Appearance of CD45<sup>+++</sup> cells in the embryo proper (HH15, 26-somite chick embryo). No tyramide amplification. Foregut level. A few of these cells are detected in the duct of Cuvier (CD, arrows). Two particularly bright cells have crossed the vessel wall into the gut (G) mesenchyme (arrowheads). NT, notochord. Bar, 80  $\mu$ m.



**Fig. 4.** VEGF-R2/CD45 double staining. Tyramide amplification. (A,B) HRP detection of VEGF-R2 (brown), alkaline phosphatase detection of CD45 (red). (C) Fluorochrome detection of VEGF-R2 (orange) and CD45 (green). (A) E3 chick embryo (HH19), brachial level. CD45<sup>+</sup>/VEGF-R2<sup>-</sup> clusters occupy the aortic floor (arrows). Red cells ventral to the clusters in the dorsal mesentery may be either CD45<sup>+</sup> or CD45<sup>+++</sup>, but cannot be distinguished after tyramide amplification (arrowheads). Ao, Aorta. Bar, 125  $\mu$ m. (B) E3 chick embryo (HH19). Stomach level where the clusters are less prominent. Flat endothelial-shaped VEGF-R2<sup>-</sup>/CD45<sup>+</sup> cells (arrow) are wedged between typical EC and typical HC. Some cells do not express either marker (arrowhead). (C) E4 Chick embryo, mid-trunk level. The whole aortic endothelium, including the floor, expresses VEGF-R2 (orange). A few CD45<sup>+</sup> intraluminal cells (green, arrowhead) overlay the VEGF-R2<sup>+</sup> endothelial cells. Ao, Aorta; K, kidney; No, notochord. Bar, 65  $\mu$ m

underlying the intraaortic clusters. At the cephalicmost levels (branchial arches level), few intraaortic cells were present but large intramesenteric groups of CD45<sup>+</sup> cells were detected (Fig. 4A). More caudally, the size of the intraaortic clusters decreased, CD45<sup>+</sup> and VEGF-R2<sup>+</sup> cells being intermixed in the aortic floor. At this level, the aortic floor was a mosaic between EC and HC. In these regions, CD45 staining was low and adjacent cells expressed VEGF-R2 as dots on the cell surface. Cells negative for VEGF-R2 and CD45 as well as cells with a bulging phenotype but positive for VEGF-R2 were sometimes detected (data not shown). In caudalmost parts where HC were beginning to appear, single VEGF-R2<sup>-</sup> cells were present in the endothelium in addition to cells expressing CD45 at low levels (Fig. 4B). Intramesenteric cells became rarer and rarer



**Fig. 5** Ultrastructure of the aortic endothelium at the level of the clusters. HH19 chick embryo. (A) Semithin cross section at the mid-trunk level. Toluidine blue staining. The two symmetric hemopoietic ridges (less prominent on a semi thin section than on thin section) occupy the whole floor of the aorta. The inset frames the transition between EC and hemopoietic cluster. Bar, 65  $\mu$ m. Ao, Aorta. (B) Inset in A at ultrastructural level. The dotted line indicates the transition between EC and cluster cells. In the cluster proper, cells are large and round. Close to the cluster, cells are enlarged but display the filopodia characteristic for EC (arrows). Bar, 2  $\mu$ m.

caudalwards and disappeared below the posterior intestinal portal. At this level, the aortic endothelium was completely positive for VEGF-R2.

In E4 embryos, the aortic endothelium was entirely VEGF-R2<sup>+</sup> again. CD45<sup>+</sup> HC clusters, occasionally observed in the aortic lumen, now overlaid EC (Fig. 4C).

#### Ultrastructure of the 'hemopoietic aorta'

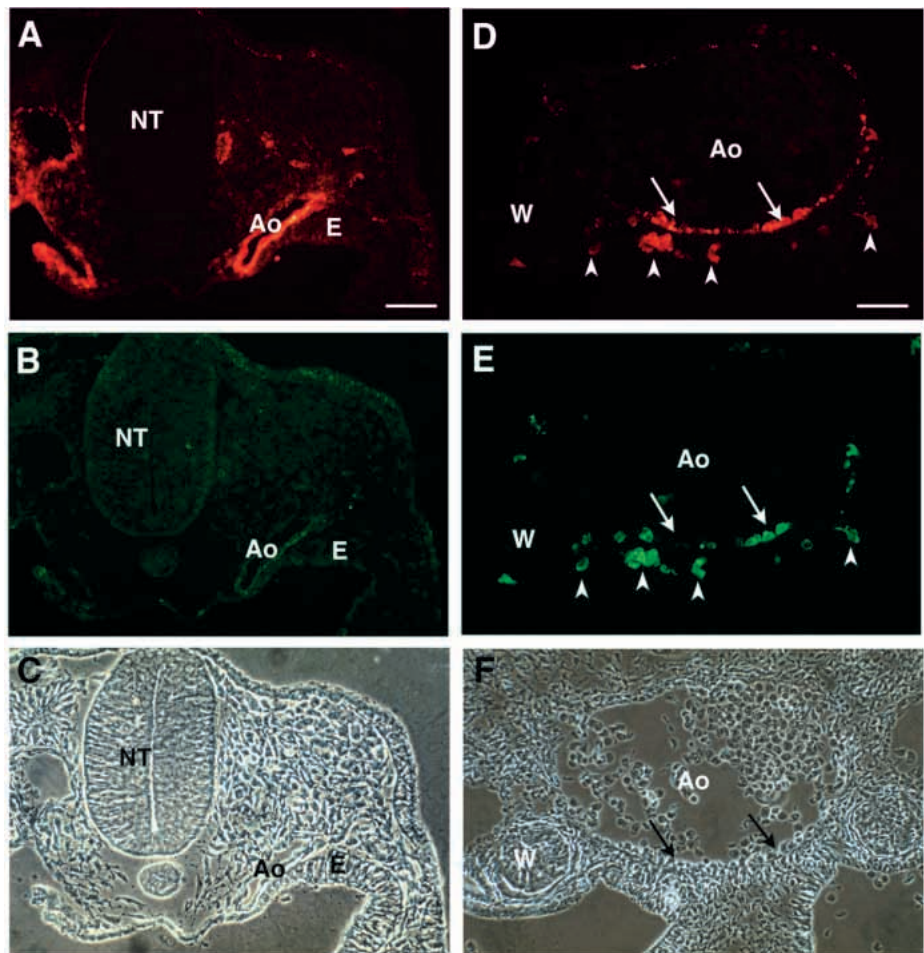
Since endothelium changes so abruptly into clusters, we investigated whether the transition was reflected in the ultrastructural organization of the cells. Unexpectedly the cells in the two regions are very much alike, apart from their shape (Fig. 5A,B). Both are characterized by abundant ribosomes and scarce endoplasmic reticulum. In both cell types, the ribosomal content varies, so that some cells appear much more electron dense than others (Fig. 5B). This is true for the flat endothelial cells of the roof of the aorta (not shown) and for the budding cells of the floor. Both cell types display active membrane blebbing. There appears to be no continuous basement membrane surrounding either the endothelial or the cluster cells. The nuclei of both cell types possess prominent nucleoli,

more filamentous in the case of cluster cells. EC are endowed with long filopodia projecting into the aortic lumen, which are particularly numerous in the cells adjoining the clusters (Fig. 5B). In contrast cluster cells emit filopodia that are directed towards the mesentery. Finally EC and cluster cells are joined by tight junctions.

#### LDL tracing of EC derivatives

AcLDL is a specific marker of endothelial cells and macrophages that relies on the receptor-mediated endocytotic capacities of these two cell types. HH13 chick embryos were inoculated with AcLDL into the heart, then left to develop for various times (six embryos for 3 to 5 hours, four for 15-18 hours and two for 20-24 hours). 4 hours after inoculation, the whole vascular tree was LDL-labelled; the two aortic endothelia as well as the segmental artery network were brightly stained (Fig. 6A). No CD45<sup>+</sup> cells were detected among LDL<sup>+</sup> cells in the aortae at that stage (Fig. 6B). 18 hours after inoculation, the two aortae had fused and the first signs of intraaortic hemopoiesis were detected. The whole vascular tree was AcLDL positive. CD45<sup>+</sup> cluster cells budding into the aortic lumen were vividly





**Fig. 6.** Tracing of aortic endothelium derivatives labelled by Ac-LDL-DiI injection in HH13 chick embryo. (A,D) LDL; (B,E) CD45 with tyramide amplification; (C,F) phase contrast. (A-C) 3 hours after inoculation. Bar, 150  $\mu$ m. AcLDL-DiI (red) has been endocytosed by endothelial cells (A). No CD45<sup>+</sup> cells in the aorta on day 2 (B). (D-F) 20 hours after inoculation. AcLDL-DiI has been taken up by CD45<sup>-</sup> endothelial cells of the aorta and CD45<sup>+</sup> budding cells (arrows) as well as CD45<sup>+</sup> cells in the dorsal mesentery (arrowheads). Ao, Aorta; E, endoderm; NT, Neural tube, W, Wolffian duct. Bars, 150  $\mu$ m

labelled by LDL (Fig. 6D,E). A small population of LDL<sup>+</sup>/CD45<sup>+</sup> cells was detected in the dorsal mesentery beneath the intraaortic clusters (Fig. 6D,E). These cells decreased in numbers in a rostrocaudal gradient from the aortic arches to the umbilical level, and few if any were detected in the caudalmost regions. These LDL<sup>+</sup>/CD45<sup>+</sup> cells were located immediately under the endothelial layer and the deeper cells extended cellular processes that contacted the luminal cell layer. On the contrary, some cells from the CD45<sup>+++</sup> population were also labelled by AcLDL, in the blood as early as HH 14-15 (in embryos injected at HH13) and thereafter in tissues all over the embryos, their number remaining small and constant. Because of their high CD45 expression, their time of appearance and scattered distribution, these cells are easily identified as belonging to the monocyte-macrophage population; we conclude that they are cells from the yolk that became labelled while migrating in the blood. It should be noted that only a small fraction of this CD45<sup>+++</sup> monocyte-macrophage population endocytosed AcLDL in our experiments.

## DISCUSSION

Until now, the development of the blood system has been mainly analyzed in quail embryos and in quail-chick chimeras relying on the detection of QH1<sup>+</sup> quail cells, distinguished as

HC or EC because of their round or elongated shape. It was thus difficult to assert whether isolated QH1<sup>+</sup> cells are angioblasts that have not linked up with others or whether they are either hemopoietic progenitors or dendritic cells. The latter are macrophage-like cells, originating from the yolk sac, that belong to the monocyte-macrophage lineage (Cuadros et al., 1992).

The double detection of an endothelial antigen and a hemopoietic antigen established the hemopoietic nature of the intraaortic clusters. This finding agrees with a similar identification in mammals where these clusters express CD45, have no affinity for the endothelial-specific lectin Ulex and are positive for CD34 and CD31, which label both lineages (Tavian et al., 1996; Wood et al., 1997; Garcia-Porrero et al., 1998).

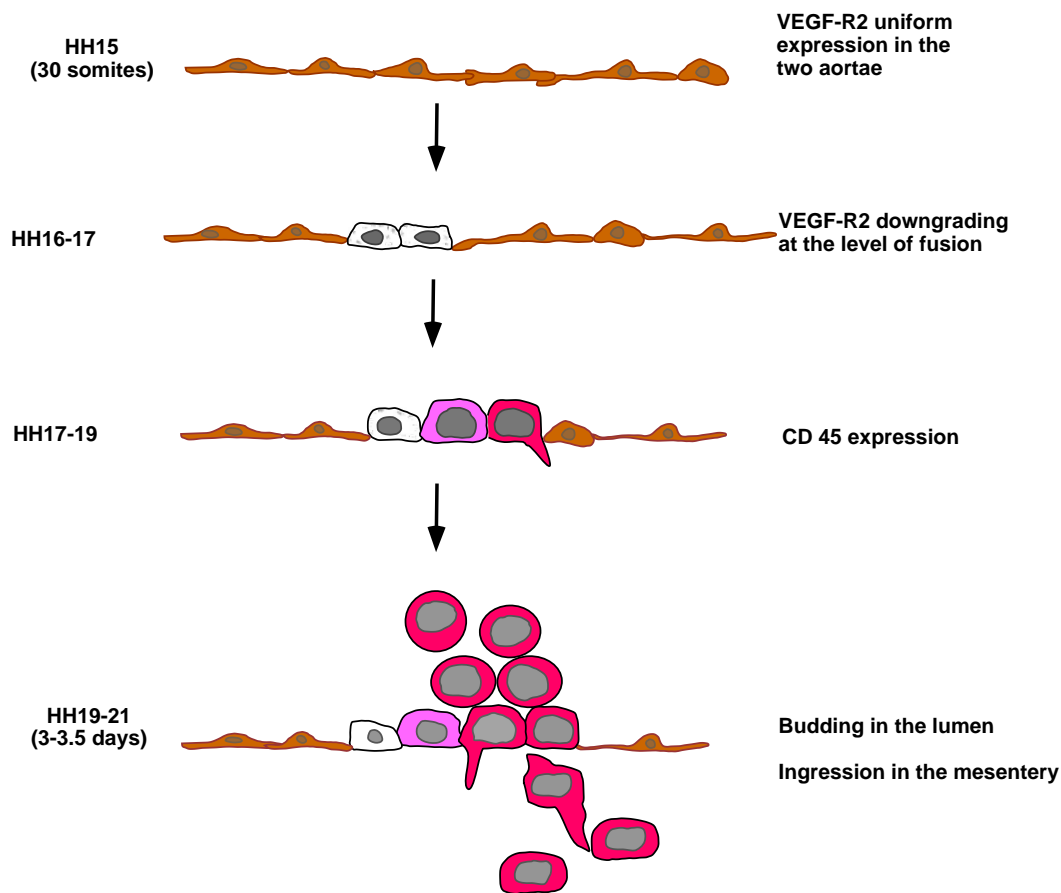
The developmental relationship between the two lineages was traced in our work by labelling the E2 vascular tree, which at that time is entirely lined by typical endothelial cells, with Ac-LDL which were seen to remain within the blood vessels and have a short half life. Thus only endothelial, and not subjacent cells, had access to the label. Once Ac-LDL were injected into the blood flow, cells became labelled within 2-3 hours, long before the appearance of the first intraembryonic hemopoietic progenitors. 1 day later, LDL<sup>+</sup> clusters materialized in the floor of the aorta. The whole CD45<sup>+</sup> hemopoietic clusters as well as single CD45<sup>+</sup> cells in the dorsal mesentery were labelled.

Double CD45/VEGF-R2 staining demonstrated that cells in the aorta always displayed either an endothelial (VEGF-R2<sup>+</sup>) antigenic phenotype or a hemopoietic phenotype (CD45<sup>+</sup>), never both. This finding agrees strikingly with the results of Eichmann et al. (1997) obtained from clonal cultures: VEGF-R2<sup>+</sup> cells sorted out from the early blastodisc and seeded in a permissive semisolid medium gave rise to both endothelial and hemopoietic colonies but never to mixed colonies. By tracing the progeny of cells labelled in situ, our present approach demonstrates the emergence of hemopoietic cells within stretches of a previously continuous endothelium. It appears that the commitment to the hemopoietic fate in the embryo involves an abrupt commutation and the extinction of a previous cell identity, testified by the disappearance of a specific surface antigen and the appearance of another.

It is clear from the yolk sac chimerae data (Dieterlen-Lièvre and Martin, 1981; see also Introduction) that HSC emerge de novo in the aortic floor. It is striking that, during this particular process, cells that become committed to hemopoiesis derive from cells that had several features of bona fide EC. In particular, VEGF-R2 and CD45 expressions were always dissociated, and a decrease in VEGF-R2 expressions was observed in the aortic floor before and during HC emergence. The switch from VEGF-R2 to CD45 coincided precisely with the initiation of budding by cells of the aortic floor. A few cells at the frontier between flat and budding cells were devoid of both markers, which suggests that VEGF-R2 was lost before CD45 became expressed. When the clusters were present, the

floor of the aorta appeared not constituted by EC proper but by a patchwork of EC and HC stretches arranged side by side. This anatomical segregation of the two phenotypes, confirmed at the ultrastructural level, matches the impossibility of obtaining mixed colonies in vitro (Eichmann et al., 1997). In the mouse, *flk-1* (EC-specific) and *c-kit* (expressed by HC and not by EC) were recently reported to be mutually exclusive at the level of the intraaortic clusters (Yoshida et al., 1998). Furthermore lymphohemopoietic cells could be obtained from VE-cadherin<sup>+</sup> CD45<sup>-</sup> sorted cells (Nishikawa et al., 1998). Finally using a clonal approach with embryonic stem cells, Choi et al. (1998) reported the existence of blast colonies able to generate cells displaying either EC or HC characteristics.

Cell tracing concurs with immunocytological analysis, by demonstrating the direct cell filiation between EC and HC. Perhaps in this particular process of intraaortic hemopoiesis, the elusive hemangioblast may be the rare cell that is losing VEGF-R2 (Fig. 7). Discriminating information has been recently acquired with the study of SCL/TAL1, currently considered as the hemangioblast-specific transcription factor. SCL/TAL1<sup>-/-</sup> mice lack both HC and EC in the yolk sac and die at E 9.5 (Robb et al., 1995; Shivdasani et al., 1995). HC, but not EC, were significantly rescued by a construct containing the SCL gene under the control of the hemopoietic-specific GATA1 promoter (Visvader et al., 1998). In the model proposed by these authors, hemopoietic differentiation occurs when SCL/TAL1 is expressed while the endothelial pathway is activated when VEGF-R2 is expressed.



**Fig. 7.** Schematic representation of the emergence of hemopoietic cells from the aortic endothelium. VEGF-R2 expression in brown, CD45 in red. Cells with low levels of CD45 presumably entering the hemopoietic pathway are shown in pink.

There is another developmental process that raises the problem of the hemangioblast: this is the colonization of the bone marrow, which may be accomplished either by two distinct precursors for the hemopoietic and endothelial lineages or alternately by hemangioblasts, in the embryo (Caprioli et al., 1998) and maybe even in the adult (Asahara et al., 1997).

To conclude, we have addressed the hemangioblast enigma in a situation where the emergence of HC is conspicuously polarized. Our findings indicate a switch from an EC phenotype to the HC phenotype. Interestingly other data from our group have suggested the existence of two distinct lineages, a dorsal somitic lineage strictly endothelial and a ventral splanchnopleural lineage with the dual potential for angiopoiesis and hemopoiesis (Pardanaud et al., 1996). Another piece of work from our group (L. Pardanaud et al., unpublished) describes how these two fates could be flipped by a short contact of mesodermal subsets, selected for their distinct potential, with endoderm or ectoderm and alternatively by treatment with growth factors.

We thank C. Batejat for his contribution to the immunocytological aspects of this work. We are grateful to C. Corbel for helpful suggestions and constant support throughout this work, M.-F. Hallais for her expertise in semithin and ultrathin sectioning, M.-F. Meunier for manuscript preparation, F. Viala and F. Baujean for excellent photographic assistance. This work was supported by the Centre National de la Recherche Scientifique and by AFM grant no. 587132.

## REFERENCES

- Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G. and Isner, J. M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**, 964-967.
- Baldwin, H., Shen, H., Yan, H., Delisser, H., Chung, A., Mickanin, C., Trask, T., Kirschbaum, N., Newman, P., Albelda, S. et al. (1994). Platelet endothelial cell adhesion molecule-1 (PE-CAM-1/CD31): alternatively spliced, functionally distinct isoforms expressed during mammalian cardiovascular development. *Development* **120**, 2539-2553.
- Beaupain, D., Martin, C. and Dieterlen-Lièvre F. (1979). Are developmental hemoglobin changes related to the origin of stem cells and site of erythropoiesis? *Blood* **53**, 212-225.
- Caprioli, A., Jaffredo, T., Gautier, R., Dubourg, C. and Dieterlen-Lièvre, F. (1998). Blood-borne seeding by hematopoietic and endothelial precursors from the allantois. *Proc. Natl. Acad. Sci. USA* **95**, 1641-1646.
- 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.
- Cormier, F. (1993). Avian pluripotent haemopoietic progenitor cells: detection and enrichment from the para-aortic region of the early embryo. *J. Cell Sci.* **105**, 661-666.
- Cormier, F. and Dieterlen-Lièvre, F. (1988). The wall of the chick embryo aorta harbours M-CFC, G-CFC, GM-CFC and BFU-E. *Development* **102**, 279-85.
- Cormier, F., de Paz, P. and Dieterlen-Lièvre, F. (1986). In vitro detection of cells with monocytic potentiality in the wall of the chick embryo aorta. *Dev. Biol.* **118**, 167-75.
- Couly, G., Coltey, P., Eichman, A. and Le Douarin N. M. (1995). The angiogenic potentials of the cephalic mesoderm and the origin of brain and head blood vessels. *Mech. Dev.* **53**, 97-112.
- Cuadros, M. A., Coltey, P., Nieto, M. C. and Martin, C. (1992). Demonstration of a phagocytic cell system belonging to the hemopoietic lineage and originating from the yolk sac in the early avian embryo. *Development* **115**, 157-168.
- Cumano, A., Dieterlen-Lièvre, F. and Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell* **86**, 907-916.
- Dantschakoff, V. (1907). Über das erste Auftreten der Blut-Elemente in Hühnerembryo. *Folia Haematol* **4**, 159-166.
- Dieterlen-Lièvre, F. (1975). On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. *J. Embryol. Exp. Morph.* **33**, 607-619.
- Dieterlen-Lièvre F. (1997). Intraembryonic hematopoietic stem cells. *In: Hematology/Oncology clinics of North America.* (ed. L. Zon). Vol. **11**, 1149-1171. W.B. Saunders Company.
- Dieterlen-Lièvre, F. and Martin, C. (1981). Diffuse intraembryonic hemopoiesis in normal and chimeric avian development. *Dev. Biol.* **88**, 180-191.
- Dieterlen-Lièvre, F., Martin, C., Beaupain, D. (1977). Origin of erythropoietic and lymphopoietic stem cells in the avian embryo, studied in interspecific chimaeras. *Folia. Biol. (Praha).* **23**, 373-375.
- Drake, C. J., Brandt, S. J., Trusk, T. C. and Little, C. D. (1997). TAL1/SCL is expressed in endothelial progenitor cells/angioblasts and defines a dorsal-to-ventral gradient of vasculogenesis. *Dev. Biol.* **192**, 17-30.
- Dumont D. L., Gradwohl, G., Fong, G. H., Puri, M. C., Gertsenstein, M., Auerbach, A. and Breitman, M. L. (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* **8**, 1897-1909.
- Dumont, D. J., Fong, G. H., Puri, M. C., Gradwohl, G., Alitalo, K. and Breitman, M. L. (1995). Vascularization of the mouse embryo: A study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev. Dynam.* **203**, 80-92.
- Dzierzak, E., Medvinsky, A. and de Bruijn, M. (1998). Qualitative and quantitative aspects of hematopoietic cell development in the mammalian embryo. *Immunology Today* **19**, 228-236.
- Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Bréant, C. and Le Douarin, N. (1997). Ligand-dependant 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.
- Eichmann, A., Marcelle, C., Bréant, C. and Le Douarin, N. M. (1993). Two molecules related to the VEGF receptor are expressed in early endothelial cells during avian embryonic development. *Mech. Dev.* **42**, 33-48.
- Eichmann, A., Marcelle, C., Bréant, C. and Le Douarin, N. (1996). Molecular cloning of Quek1 and 2, two quail vascular endothelial growth factor (VEGF) receptor-like molecules. *Genes Dev.* **174**, 3-8.
- Emmel, V.E. (1916). The cell clusters in the dorsal aorta of mammalian embryos. *Am. J. Anat.* **19**, 401-421.
- Ferrara N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439-442.
- Fong, G. H., Rossant, J., Gertsenstein, M. and Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376**, 66-70.
- García-Porrero, J. A., Godin, I. E. and Dieterlen-Lièvre, F. (1995). Potential intraembryonic hemogenic sites at pre-liver stages in the mouse. *Anat. Embryol.* **192**, 425-435.
- García-Porrero, R., Manaia, A., Jimeno, J., Lasky, L., Dieterlen-Lièvre, F. and Godin, I. (1998). Antigenic profiles of endothelial and hemopoietic lineages in murine intraembryonic hemogenic sites. *Dev. Comp. Immunol.* **22**, 303-319.
- Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.
- Hwang, L.-Y., Siegelman, M., Davis, L., Oppenheimer-Marks, N. and Baer, R. (1993). Expression of the TAL1 proto-oncogene in cultured endothelial cells and blood vessels of the spleen. *Oncogene* **8**, 3043-3046.
- Jeurissen, S. H. M., Janse, E. M., Ekino, S., Nieuwenhuis, P., Koch, G. and De Boer, G. F. (1988). Monoclonal antibodies as probes for defining cellular subsets in the bone marrow, thymus, bursa of Fabricius, and spleen of the chicken. *Vet. Immunol. Immunopathol.* **19**, 225-229.
- Jordan, H. E. (1917). Aortic cell clusters in vertebrate embryos. *Proc. Natl. Acad. Sci. USA.* **3**: 149-157.
- Jordan, H. E. (1918). A study of a 7mm human embryo; with special reference to its peculiar spirally twisted form, and its large aortic cell-clusters. *Anat. Rec.* **14**, 479-492.
- Kallianpur, A., Jordan, J. E. and Brandt, S. J. (1994). The SCL/TAL-1 gene is expressed in progenitors of both the hematopoietic and vascular systems during embryogenesis. *Blood* **83**, 1200-1208.
- Labastie, M.-C., Poole, T. J., Péault, B. and Le Douarin, N. M. (1986).



- MB1, a quail leukocyte-endothelium antigen: partial characterization of the cell surface and secreted forms in cultured endothelial cells. *Proc. Natl. Acad. Sci. USA* **83**, 9016-9020.
- Martin, C.** (1972). Technique d'explantation in ovo de blastodermes d'embryons d'oiseaux. *C. R. Soc. Biol. Fr.* **116**, 283-285.
- Martin, C., Beaupain, D. and Dieterlen-Lièvre, F.** (1978). Developmental relationships between vitelline and intra-embryonic haemopoiesis studied in avian 'yolk sac chimaeras'. *Cell. Differ.* **7**, 115-130.
- Matthews, W., Jordan, C. T., Gavin, M., Jenkins, N. A., Copeland, N. G. and Lemischka, I. R.** (1991). A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit. *Biochemistry* **88**, 9026-9030.
- Maximow, A. A.** (1909). Untersuchungen über Blut und Bindegewebe. I. Die frühesten Entwicklungsstadien der Blut und Bindegewebezellen beim Säugtier-Embryo, bis zum Anfang der Blutbildung in der Leber. *Archiv für Mikroskopische Anatomie* **73**, 444-450.
- McNagny, K. M., Pettersson, I., Rossi, F., Flamme, I., Shevchenko, A., Mann, M. and Graf, T.** (1997). Thrombomucin, a novel cell surface protein that defines thrombocytes and multipotent hematopoietic progenitors. *J. Cell Biol.* **138**, 1395-1407.
- Millauer, B., Wizigmannvoos, S., Schnurch, H., Martinez, R., Möller, N. P. H., Risau, W. and Ullrich, A.** (1993). High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* **72**, 835-846.
- Minot, C. S.** (1910). Development of the blood, the vascular system and the spleen. In *Human Embryology*. (ed. Keibel and Mall, F.R.). Philadelphia: J. B. Lippincott Company.
- Murray, P. D. F.** (1932). The development 'in vitro' of blood of the early chick embryo. *Proc. Roy. Soc. C* **11**, 497-521.
- Mustonen, T. and Alitalo, K.** (1995). Endothelial receptor tyrosine kinases involved in angiogenesis. *J. Cell Biol.* **129**, 895-898.
- 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.
- Paramithiotis, E., Tkalec, L. and Ratcliffe, J.H.** (1991). High levels of CD45 are coordinately expressed with CD4 and CD8 on avian thymocytes. *J. Immunol.* **147**, 3710-3717.
- Pardanaud, L., Altmann, C., Kitos, P., Dieterlen-Lièvre, F. and Buck, C.A.** (1987). Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development* **100**, 339-49.
- Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L. M., Catala, M. and Dieterlen-Lièvre, F.** (1996). Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development* **122**, 1363-1371.
- Puri, M. C., Rossant, J., Alitalo, K., Bernstein, A. and Partanen, J.** (1995). The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO J.* **14**, 5884-5891.
- Robb, L., Lyons, I., Li, R., Hartley, L., Köntgen, F., Harvey, R. P., Metcalf, D. and Begley, C. G.** (1995). Absence of yolk sac hematopoiesis from mice with a targeted disruption of the SCL gene. *Proc. Natl. Acad. Sci. USA* **92**, 7075-7079.
- Sabin, F. R.** (1917). Origin and development of the primitive vessels of the chick and of the pig. *Contrib. Embryol. Carnegie Inst.* **226**, 61-124.
- Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Wolburg, H., Risau, W. and Qin, Y.** (1995). Distinct roles of the receptor tyrosine kinase Tie-1 and Tie-2 in blood vessel formation. *Nature* **376**, 70-74.
- Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. and Schuh, A. C.** (1995). Failure of blood-island formation and vasculogenesis in Flk-1 deficient mice. *Nature* **376**, 62-66.
- Shalaby, F., Ho, J., Stanford, W. L., Fisher, K. D., Schuh, A. C., Schwartz, L., Bernstein, A. and Rossant, J.** (1997). A requirement for flk-1 for primitive and definitive hematopoiesis and vasculogenesis. *Cell* **89**, 981-990.
- Shivdasani, R. A., Mayer, E. L. and Orkin, S. H.** (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* **373**, 432-434.
- Smith, R. A. and Glomski, C. A.** (1982). 'Hemogenic endothelium' of the embryonic aorta: does it exist? *Dev. Comp. Immunol.* **6**, 359-368.
- Tavian, M., Coulombel, L., Luton, D., San Clémente, H., Dieterlen-Lièvre, F. and Péault, B.** (1996). Aorta-associated CD34<sup>+</sup> hematopoietic cells in the early human embryo. *Blood* **87**, 67-72.
- Terman, B. I., Carrion, M. E., Kovacs, E., Rasmussen, B. A., Eddy, R. L. and Shows, T. B.** (1991). Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene* **6**, 1677-1683.
- Thomas, M. L.** (1989). The leukocyte common antigen family. *Annu. Rev. Immunol.* **7**, 339-342.
- 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.
- Visvader, J. E., Fujiwara, Y. and Orkin, S. H.** (1998). Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development. *Genes Dev.* **12**, 473-479.
- Wagner, R.C.** (1980). Endothelial cell embryology and growth. In *Advances Microcirc.* **9**, 45-75. Basel: Karger.
- Wiltling, J., Eichmann, A. and Christ, B.** (1997). Expression of the avian VEGF receptor homologues Quek1 and Quek2 in blood-vascular and lymphatic endothelial and non-endothelial cells during quail embryonic development. *Cell Tissue Res.* **288**, 207-233.
- Wood, H. B., May, G., Healy, L., Enver, T. and Morris-Kay, G.** (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.
- Yamaguchi, T. P., Dumont, D. J., Conlon, R. A., Breitman, M. L. and Rossant, J.** (1993). flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development* **118**, 489-498.
- Yoshida, H., Takakura, N., Hirashima, M., Kataoka, H., Tsuchida, K., Nishikawa, S. and Nishikawa, S.-H.** (1998). Hematopoietic tissues, as a playground of receptor tyrosine kinases of the PDGF-receptor family. *Dev. Comp. Immunol.* **22**, 321-332.
- Young, P. E., Baumhueter, S. and Lasky, L. A.** (1995). The sialomucin CD34 is expressed on hematopoietic cells and blood vessels during murine development. *Blood* **85**, 96-105.