Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages

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SUMMARY

Totipotent murine ES cells have an enormous potential for the study of cell specification. Here we demonstrate that ES cells can differentiate to hemopoietic cells through the proximal lateral mesoderm, merely upon culturing in type IV collagen-coated dishes. Separation of the Flk1+ mesoderm from other cell lineages was critical for hemopoietic cell differentiation, whereas formation of the embryoid body was not. Since the two-dimensionally spreading cells can be monitored easily in real time, this culture system will greatly facilitate the study of the mechanisms involved in the cell specification to mesoderm, endothelial, and hemopoietic cells. In the culture of ES cells, however, lineages and stages of differentiating cells can only be defined by their own characteristics. We showed that a combination of monoclonal antibodies against E-cadherin, Flk1/KDR, PDGF receptor α, VE-cadherin, CD45 and Ter119 was sufficient to define most intermediate stages during differentiation of ES cells to blood cells. Using this culture system and surface markers, we determined the following order for blood cell differentiation: ES cell (E-cadherin+Flk1−PDGFRα−), proximal lateral mesoderm (E-cadherin−Flk1+VE-cadherin−), progenitor with hemoangiogenic potential (Flk1+VE-cadherin+CD45−), hemopoietic progenitor (CD45+c-Kit+) and mature blood cells (c-Kit−CD45+ or Ter119+), though direct differentiation of blood cells from the Flk1+VE-cadherin− stage cannot be ruled out. Not only the VE-cadherin+CD45− population generated from ES cells but also those directly sorted from the yolk sac of 9.5 dpc embryos have a potential to give rise to hemopoietic cells. Progenitors with hemoangiogenic potential were identified in both the Flk1+VE-cadherin− and Flk1+VE-cadherin+ populations by the single cell deposition experiment. This line of evidence implicates Flk1+VE-cadherin+ cells as a diverging point of hemopoietic and endothelial cell lineages.

Key words: FLK1, VE-Cadherin, Hemangioblast, ES cell

INTRODUCTION

Embryogenesis is the process by which totipotent cells undergo progressive cell specification according to body plan. During this process, cell specification and body plan development proceed in an inseparably coordinated manner, so that it has been considered that normal cell specification cannot be recapitulated in the absence of body organization. This belief is typified in previous in vitro methods for the specification of embryonic stem (ES) cells into blood and endothelial cells. In a culture system established by Doetschman et al. (1985), ES cells are first cultured to establish the structure known as the embryoid body. Although the embryoid body is far less organized than the actual embryo, it is reasonable to think that it can partially mimic the spatial organization in the embryo. Several reports demonstrated blood cell generation within this structure (Doestchmann et al., 1985; Wiles and Keller, 1991; Schmitt et al., 1991; Burkert et al., 1991). Moreover, a recent study by Kabrun et al. (1997) demonstrated that Flk1+

mesoderm was induced in the embryoid body with a time schedule similar to that of actual embryo. In some studies, the microenvironment in the embryoid body for the specification to blood cells has been successfully substituted using feeder cell lines (Gutierrez-Ramos and Palacios, 1992; Nakano et al., 1994), but this is not the proof that the process occurs independently of the embryoid structure.

An aim of the present study was to challenge the notion that the 3-dimensional (3-D) structure in the embryoid body is requisite for blood cell differentiation, by exploring culture conditions in which ES cells differentiate into hemopoietic cells in the absence of the embryoid body formation or feeder cells. As the spatial information of the embryo is absent in the culture of ES cells, the lineage and stage of differentiation should be defined by the cells' own character. Thus, another aim of this study was to establish a panel of surface markers that can define and sort the cells at intermediate stages of differentiation to blood cells. With such surface markers, living cells at various intermediate stages could be sorted for

evaluation of their differentiation potential. The final goal of this study was to identify all intermediate stages during blood cell differentiation of ES cells, and specify the diverging point of hemopoietic and endothelial lineages.

Our result show that ES cells spreading on a 2-D plane can differentiate to FLK1$^+$ mesoderm and blood cells, suggesting that neither 3-D structures in the embryoid body nor feeder cells are required for this process. Progressive lineage analysis by repeated cell sorting and culture under this condition revealed two intermediate stages, FLK1$^+$VE-cadherin$^-$ and FLK1$^+$VE-cadherin$^+$, between ES cells and CD45$^+$ blood cells. Thus, FLK1$^+$VE-cadherin$^+$CD45$^-$ cells represent a population at the diverging point of hemopoietic and endothelial cell lineages.

MATERIALS AND METHODS

Preparation of single cell suspensions from embryos

Pregnant ICR mice were purchased from Shimizu Co. Ltd (Japan). We dissected the females at 7.4-9.5 days post coitum (dpc). To dissociate 7.5-8.5 dpc embryos, the deciduas were removed and embryos were dissected in α-modified minimum essential media (α-MEM, Gibco, NY) supplemented with 5% calf serum. After removing Reichert's membrane, the embryos were washed in phosphate buffered saline, incubated with the cell dissociation buffer (Gibco/BRL) for 30 minutes at 37°C, and dissociated by gentle pipetting. Yolk sacs of 9.5 dpc embryos were separated as described (Ogawa et al., 1993) and dissociated in the same way. Cell clumps were removed with nylon mesh and the dissociated cells were suspended in 1% bovine serum albumin containing Hanks' balanced salt solution (Gibco/BRL).

Antibodies, staining and sorting

The cells were stained by various combinations of mAbs. Antibodies used in this study are FITC-conjugated anti-E-cadherin mAb ECCD2 (Shirayoshi et al., 1986), FITC-, phycoerythrin (PE)- or biotin-conjugated anti-FLK1 mAb AVAS12 (Kataoka et al., 1997), biotin-conjugated anti-PDGFRα mAb APA5 (Takakura et al., 1996), biotin-conjugated anti-VE-cadherin mAb VECD1 (Matsuyoshi et al., 1997), and PE-conjugated anti-c-Kit mAb ACK2 (Nishikawa et al., 1991). The mAbs are prepared and labeled in our laboratory. FITC-anti-CD45 (common leukocyte antigen), biotin-anti-Ter119 (erythroid marker), biotin-anti-CD31 (PECAM), biotin-anti-CD34, FITC-labeled anti-Gr1 and biotin-labeled anti-Mac1 mAbs were purchased from Pharmingen (Pharmingen, CA, USA). For surface staining, cells were incubated with mixtures of labeled mAbs, followed by development with either PE-conjugated or apophycocyanin (APC)-conjugated streptavidine (Molecular probe, Oregon). The stained cells were analyzed with FACS Vantage^{TM} (Becton-Dickinson, MA). Dead cells stained by propidium-iodide were excluded from the analysis. Cell sorting was performed as described (Sudo et al., 1993).

Immunostaining of cultured cells on dishes by anti-CD45 mAb (Pharmingen), anti-GATA1 mAb (Santacruz) or anti-embryonic hemoglobin (Miwa et al., 1991) was performed as described (Kataoka et al., 1997).

Cell culture

CCE ES cells (a gift from Dr M. Evans; Robertson et al., 1986) were initially maintained on Mitomycin C (Kyowa-Hakko, Japan)-treated embryonic fibroblast layers in Dulbecco modified essential medium (DMEM; Gibco) containing 15% fetal calf serum (FCS; Whittaker, USA), 5,000 units/ml leukemia inhibitory factor (LIF; R&D systems) and 5×10^{-5} M 2 mercaptoethanol (2ME). Two weeks before the differentiation induction, one thousand ES cells were transferred to gelatin (Sigma, USA)-coated culture dishes to remove fibroblasts. 10^4

ES cells were then transferred to each well of type IV collagen-coated 6-well cluster dishes (Biocoat, Becton-Dickinson) and incubated in α-MEM supplemented with 10% FCS and 5×10^{-5} M 2ME. Although no exogenous growth factors were added, a more than 100-fold increase of cells was observed during a 4-day incubation. Cultured cells were harvested with cell dissociation buffer (Gibco) and analyzed for expression of surface markers. In preliminary experiments, dishes coated with gelatin, typeI collagen, or fibronectin were purchased from Becton-Dickinson and compared with the typeIV collagen-coated dish in the ability to support the differentiation of ES cells into FLK1$^+$ cells. For culturing FLK1$^+$VE-cadherin$^+$ cells, a mixture of recombinant growth factors containing vascular endothelial growth factor (VEGF), stem cell factor (SCF), interleukin 3 (IL-3), erythropoietin (Epo), and granulocyte growth factor (G-CSF) was used. Recombinant human VEGF, Epo and G-CSF were purchased from R&D Systems (Minneapolis, USA). Recombinant mouse IL-3 and SCF were prepared as described (Ogawa et al., 1991).

To measure the clonogenic activity of sorted cells, cells were cultured either in the semisolid medium for colony formation (Ogawa et al., 1991) or co-cultured with OP9 stromal cell line (Kodama et al., 1994).

For time lapse recording of the cultured cells, 2×10^4 ES cells were cultured in a 6 cm type IV collagen-coated dish (Becton-Dickinson), and placed on the stage of a microscope (Axiovert, Zeiss, Germany) equipped with an atmosphere controller (Zeiss). The atmosphere in the chamber was kept at 37°C, 5% CO$_2$ in air and 100% humidity. Time-lapse video recording was performed every 5 minutes for 4 days.

Single cell deposition assay for hemoangiogenic potential

An OP9 stromal cell layer was prepared in 96-well cluster dishes. Single cell deposition of sorted cells onto each well was carried out by the Clon-Cyt^{TM} system of FACS Vantage (Becton-Dickinson). The culture was incubated for 5 days in the presence of SCF, erythropoietin, IL-3 and G-CSF. Generation of clusters with round cells was screened under microscope. Formation of sheets with VE-cadherin$^+$ cells was determined by immunohistostaining of dishes with anti-VE-cadherin mAb. The well containing both round hemopoietic cells and a sheet-like cluster of VE-cadherin$^+$ cells was judged as containing the progenitor with hemoangiogenic potential.

RESULTS

Cell surface markers for ES cell differentiation to blood cells

Since E-cadherin expression was shown to be downregulated upon differentiation into mesoderm (Nose and Takeichi, 1986; Choi and Gumbiner, 1989; Burdsal et al., 1993), anti-E-cadherin monoclonal antibody (mAb) was used to distinguish mesoderm from non-mesodermal epithelial cells. Monoclonal antibodies against platelet-derived growth factor receptor (PDGFR) α and FLK-1/KDR were used as markers for paraxial (Mercola et al., 1990; Orr-Urtreger et al., 1992; Schatteman et al., 1992) and proximal lateral mesoderm (Yamaguchi et al., 1993; Millauer et al., 1993; Palis et al., 1995; Dumont et al., 1995), respectively, according to our results on the immunolocalization of these molecules in gastrulating embryos (Takakura et al., 1997; Kataoka et al., 1997). For detecting the stage near the diverging point of proximal lateral mesoderm into endothelial cells and hemopoietic cells, we employed mAbs to vascular endothelial (VE)-cadherin (Matsuyoshi et al., 1997), Ter119, and CD45.

To examine whether this panel of mAbs can define cells at intermediate differentiation stages, we dissociated cells of 7.4-7.8 dpc embryos and analyzed them by flow cytometry. As shown in Fig. 1A, most E-cadherin⁻ cells expressed either FLK-1 or PDGFRα, or both, confirming that the mesoderm differentiation is accompanied by downregulation of E-cadherin expression. Only a few VE-cadherin⁺ cells and CD45⁺ cells were detected at this stage of embryogenesis (Fig. 1A). Of note is that the proportion of VE-cadherin⁺ cells in FLK1⁺ cells increased from 0.5/9.4 (5%) to 2.2/4.7 (45%) within next 24 hours (Fig. 1B).

Mesoderm differentiation from ES cells without formation of the embryoid body

With the surface markers described above, we next investigated how far the specification of ES cells reaches in the absence of the embryoid body structure. In a series of preliminary experiments, we compared dishes coated with gelatin, fibronectin, type I collagen, or type IV collagen in terms of their ability to support ES cell differentiation into E-cadherin⁻ FLK-1⁺ cells, representing the proximal lateral mesoderm. Such cells were most efficiently generated in type IV collagen-coated dishes, though other matrices showed the activity to some extent. Based upon this result, type IV collagen-coated dishes were used throughout this study. No exogenous factors were included in the culture, while 10% fetal calf serum was added.

Fig. 2 presents the shift of surface marker expression during 4 days of ES cell culture under this condition. ES cells maintained with LIF in gelatin-coated dishes expressed high levels of E-cadherin but not FLK-1 or PDGFRα (Fig. 2A). After transferring them to type IV collagen-coated dishes and culturing without LIF, E-cadherin⁻ cells started to appear from day 3 (Fig. 2B), and this population increased 10-fold within the next 24 hours. These kinetics are very similar to that reported in the embryoid body (Kabrun et al., 1997). Among the E-cadherin⁻ population, heterogeneity in FLK-1 and PDGFRα expression was observed from the time of their appearance (Fig. 2B), suggesting that mesodermal subsets have separated at the earliest stage of mesoderm induction.

To confirm that mesoderm subsets are generated in the absence of the embryoid body formation, the culture process was time-lapse video recorded. All cultured cells in the frame continued to spread on the dish surface, while diversifying in cell morphology (data not shown), proving that the 3-D embryoid body structure is not requisite for differentiation of mesoderm cells.

Differentiation potential of FLK1⁺ cells on typeIV collagen

While FLK1⁺ mesoderm with a potential to give rise to blood cells (Kabrun et al., 1997; Eichmann et al., 1997) was induced from ES cells in the absence of the embryoid body formation, only few CD45⁺ or Ter119⁺ cells were generated even by prolonged incubation of ES cells on type IV collagen (Fig. 3A). Since Kessel and Fabian (1987) demonstrated that the chicken embryonic ectoderm inhibits the differentiation of mesoderm to erythrocytes, we examined whether the separation of E-cadherin⁻ FLK-1⁺ cells from other cells in the culture restore their potential to give rise to blood cells. Fig. 3A demonstrates that E-cadherin⁻FLK1⁺ cells gave rise to cells expressing either

VE-cadherin, Ter119, or CD45 within 3 days, and the proportion of the three surface phenotypes were comparable to those found in the coculture with OP9 feeder cells that were shown to support blood cell generation from ES cells (Nakano et al., 1994). By FACS analysis, four distinct surface phenotypes were identified in this culture: (1) FLK1⁻ cells of unknown lineage, (2) FLK-1⁺ VE-cadherin⁺ cells, (3) Ter119⁺ nucleated erythrocytes (Fig. 3B) expressing embryonic hemoglobin (data not shown), and (4) CD45⁺ hemopoietic cells containing both c-Kit positive immature stem cells (Fig. 3B) and c-Kit negative monocytes and polymorph nuclear cells (Fig. 3B). Taken together, the generation of blood cells from ES cells requires neither the embryoid body formation nor feeder cells, but separation of FLK1⁺ cells from other lineages upon their generation is critical for efficient induction of blood cells.
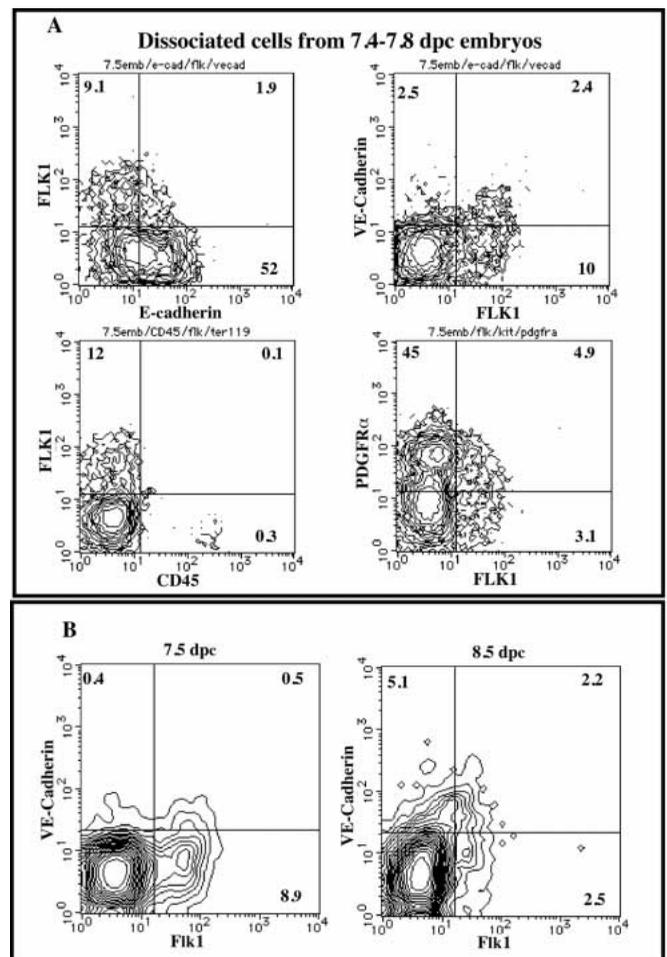

Fig. 1. Mesoderm subsets defined by a panel of surface markers. (A) Single cell suspensions were prepared from embryos at 7.4 to 7.8 dpc and the expression of FLK1, E-cadherin, PDGFRα, VE-cadherin and CD45 was analyzed. Ter119 expression was analyzed at the same time and 1.3% of the cells were positive (data not shown). It is difficult to rule out the possibility that CD45⁺ and Ter119⁺ cells could be contamination from maternal blood. (B) Expression of VE-cadherin and FLK1 in dissociated cells from 7.5 or 8.5 dpc embryos. PE-anti-FLK1 and FITC-anti-FLK1 were used to stain 7.5 and 8.5 dpc embryos, respectively. FITC-anti-FLK1 stained more faintly than PE-anti-FLK1. Note that the proportion of VE-cadherin⁺ cells increased rapidly.

VE-cadherin expression prior to blood cell generation

To further dissect the processes of blood cell generation from FLK1$^+$ mesoderm, we investigated the time-course of VE-cadherin-, CD45- and Ter119-expression. E-cadherin$^-$FLK1$^+$ cells were induced from ES cells on type IV collagen-coated dishes and sorted. At the time of cell sorting, FLK1$^+$ cells did not express CD34 nor VE-cadherin (Fig. 4A), while CD31 expression was already detected in both FLK1$^-$ and FLK1$^+$ cells. The sorted E-cadherin$^-$FLK1$^+$ cells were then cultured on type IV collagen and expression of FLK1, VE-cadherin, Ter119 and CD45 was analyzed (Fig. 4B). Within 24 hours after the culture, most FLK1$^+$ cells started to express VE-cadherin, whereas only a few CD45$^+$ or Ter119$^+$ cells were detected. This result indicates an order of differentiation from FLK1$^+$VE-cadherin$^-$ to FLK1$^+$VE-cadherin$^+$ cells.

Although the purity of FLK1$^+$ cells after the cell sorting was
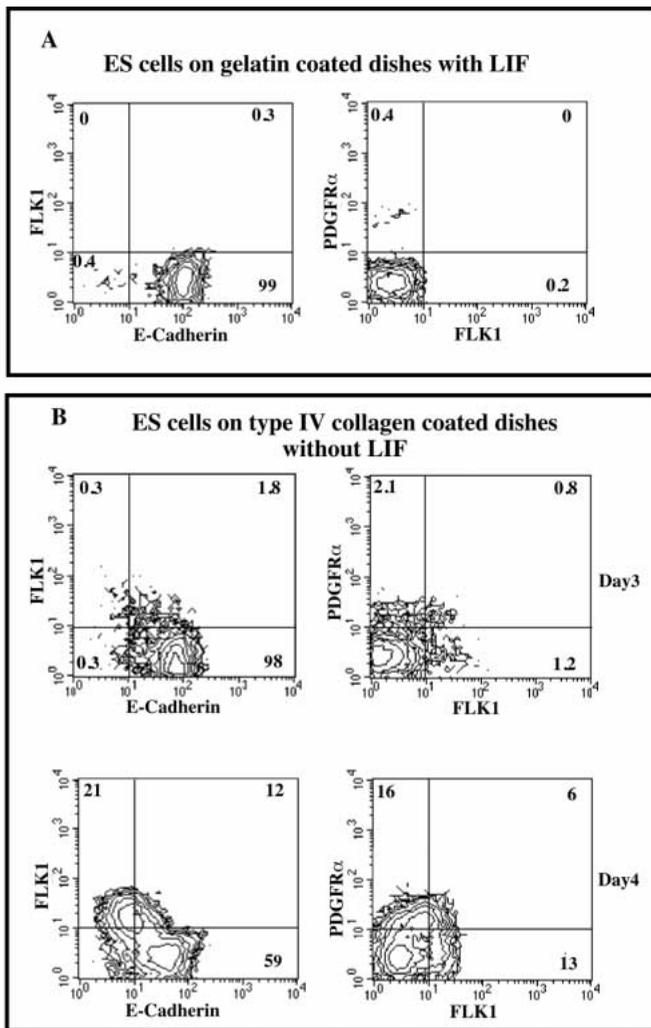
Fig. 2. Mesoderm induction from ES cells in the absence of embryoid body formation. CCE ES cells (a gift from M. Evans) that were cultured on gelatin-coated dishes with LIF were transferred to typeIV collagen-coated dishes (10^4 cells/3 cm dish) and cultured in the absence of LIF. Before (A) and 3 and 4 days after (B) the transfer, cells were harvested and expression of E-cadherin, FLK1, and PDGFRα was analyzed.

98% in this particular experiment, the percentage of FLK1$^-$ cells increased during the culture, attaining almost 50% as early as 2 days after culture. As sorted FLK1$^-$ cells could not proliferate rapidly under the same conditions (data not shown), the majority of FLK1$^-$ cells in this culture may be derived from FLK1$^+$ cells. This suggests that FLK1$^+$VE-cadherin$^-$ cells diverge to FLK1$^-$VE-cadherin$^-$ and FLK1$^+$VE-cadherin$^+$ populations at the earliest step of differentiation, and CD45$^+$ blood cells are generated later.

Hemogenic potential of VE-cadherin$^+$CD45$^-$ cells

The time course of the appearance of VE-cadherin$^+$ and CD45$^+$ cells strongly suggests that some CD45$^+$ blood cells are differentiated from VE-cadherin$^+$CD45$^-$ cells. To investigate this possibility, we sorted VE-cadherin$^+$CD45$^-$Ter119$^-$ cells from the culture of FLK1$^+$ cells.

Unlike FLK1$^+$ VE-cadherin$^-$ cells that proliferate in the absence of growth factors, VE-cadherin$^+$ cells did not proliferate without exogenous growth factors. Colony formation of CD45$^+$+ Ter119$^+$ mixed, VE-cadherin$^-$CD45$^-$ Ter119$^-$, and VE-cadherin$^+$CD45$^-$ Ter119$^-$ populations in response to the growth factor mixture containing SCF, VEGF, IL-3, erythropoietin, and G-CSF was 31±3.2, 0±0, and 48±5.6 per 15,000 input cells, respectively. On the other hand, none of these populations could generate colonies in the absence of the growth factor mixture. This result indicates that VE-cadherin$^+$CD45$^-$ as well as CD45$^+$ cells contain hemopoietic precursors, while VE-cadherin$^-$CD45$^-$ cells do not.

To further dissect the process of blood cell generation from VE-cadherin$^+$ cells, VE-cadherin$^+$CD45$^-$Ter119$^-$ cells were cultured on type IV collagen in the presence of SCF, VEGF, IL-3, Epo, and G-CSF. The cultured cells were harvested everyday to analyze surface phenotypes. As shown in Fig. 5, CD45$^+$ cells were generated from VE-cadherin$^+$CD45$^-$ cells within 24 hours. In contrast to the rapid induction of CD45$^+$ cells, Ter119$^+$ erythroblasts were detected 2 days after the initiation of culture. Ter119$^+$ cells increased extensively in the next 48 hours. Cytological examination demonstrated erythroblasts, megakaryocytes, macrophages, polymorphonuclear cells and mast cells in the culture (data not shown).

Hemogenic potential of VE-cadherin$^+$CD45$^-$ cells in the yolk sac of a 9.5 dpc embryo

The above results indicate that VE-cadherin$^+$CD45$^-$ cells are the direct precursor of CD45$^+$ hemopoietic cells. To investigate whether or not this is also true for VE-cadherin$^+$ cells in the embryo, we sorted VE-cadherin$^+$CD45$^-$ Ter119$^-$ cells from a 9.5 dpc yolk sac and assessed their potential to give rise to blood cells in response to the growth factor mixture. A more than 100-fold increase in cell recovery was induced during a 4 day incubation of VE-cadherin$^+$CD45$^-$Ter119$^-$ cells in typeIV collagen-coated dishes in the presence of the growth factor mixture. The majority of the cells recovered from the cultures were either CD45$^+$ or Ter119$^+$ (Fig. 6). Expression of Mac1 and Gr1 representing the myeloid cell lineage was also detected. This result indicates that VE-cadherin$^+$CD45$^-$Ter119$^-$ cells in early embryos do have hemogenic potential.

Hemoangiogenic potential of FLK1$^+$VE-cadherin$^-$ and FLK1$^+$VE-cadherin$^+$ cells

The results described above demonstrated a hemogenic

potential for VE-cadherin^{+} cells. Given that blood and endothelial lineages share a common precursor, it is important to evaluate the angiogenic potential of VE-cadherin^{+} cells. Despite our extensive attempts, however, we could not find feeder cell-free conditions that can support clonogenic proliferation of VE-cadherin^{+} cells. In contrast, when an OP9 stromal cell line was used for feeder cells, VE-cadherin^{+} cells proliferated from a single cell and formed a sheet of cells expressing VE-cadherin (Fig. 7D). In addition, clusters of round cells were also detected in culture with an OP9 feeder layer. Using the single cell deposition experiment, we found that virtually all wells containing such clusters of round cells contain cells stained either with anti-CD45, -GATA1 or -embryonic-hemoglobin Abs (Fig. 7A-C). Considering previous studies that VE-cadherin is expressed exclusively in endothelial cells (Breier et al., 1996; Matsuyoshi et al., 1997), it is plausible that formation of the VE-cadherin[+] sheet on OP9 feeder cells represents an activity of endothelial cells. Indeed, thus generated VE-cadherin[+] sheets incorporated LDL and were positive in FLK1 and CD34 (M. Hirashima et al., unpublished observation).

With this clonogenic culture condition, we analyzed the frequency of cells that can give rise to blood and endothelial cell lineages. FLK1^{+}VE-cadherin^{−} cells were sorted from the ES cell differentiation culture at day 4. FLK1^{+}VE-cadherin^{+} cells

Fig. 3. Induction of blood cells in typeIV collagen-coated dishes without embryoid body formation. (A) ES cells cultured in gelatin-coated dishes in the presence of LIF were transferred to type IV collagen-coated dishes (2×10^4 cells/10 cm dish). Only a few CD45^{+} or Ter119^{+} cells had been generated from ES cells on type IV collagen-coated dishes after a 7-day incubation (first column). Some cultures of ES cells in type IV collagen-coated dishes (10^4 cells/3 cm dish) were interrupted on day 4 when the number of E-cadherin[−]FLK1^{+} cells reached a peak. FLK1^{+} cells were sorted (96% pure in this particular experiment) and 10^5 cells were recultured either in type IV collagen-coated 3 cm dishes (second column) or in 3 cm dishes with an OP9 monolayer (third column). In both cultures, Ter119^{+} and CD45^{+} cells representing the blood cell lineage were generated, though the proportion of Ter119^{+} cells was higher in the culture with OP9. (B) CD45^{+}c-Kit^{−}, CD45^{+}c-Kit^{+} and Ter119^{+} fractions were sorted from the same culture, cytospinned onto slide glasses, and stained with May-Grünwald Giemsa.
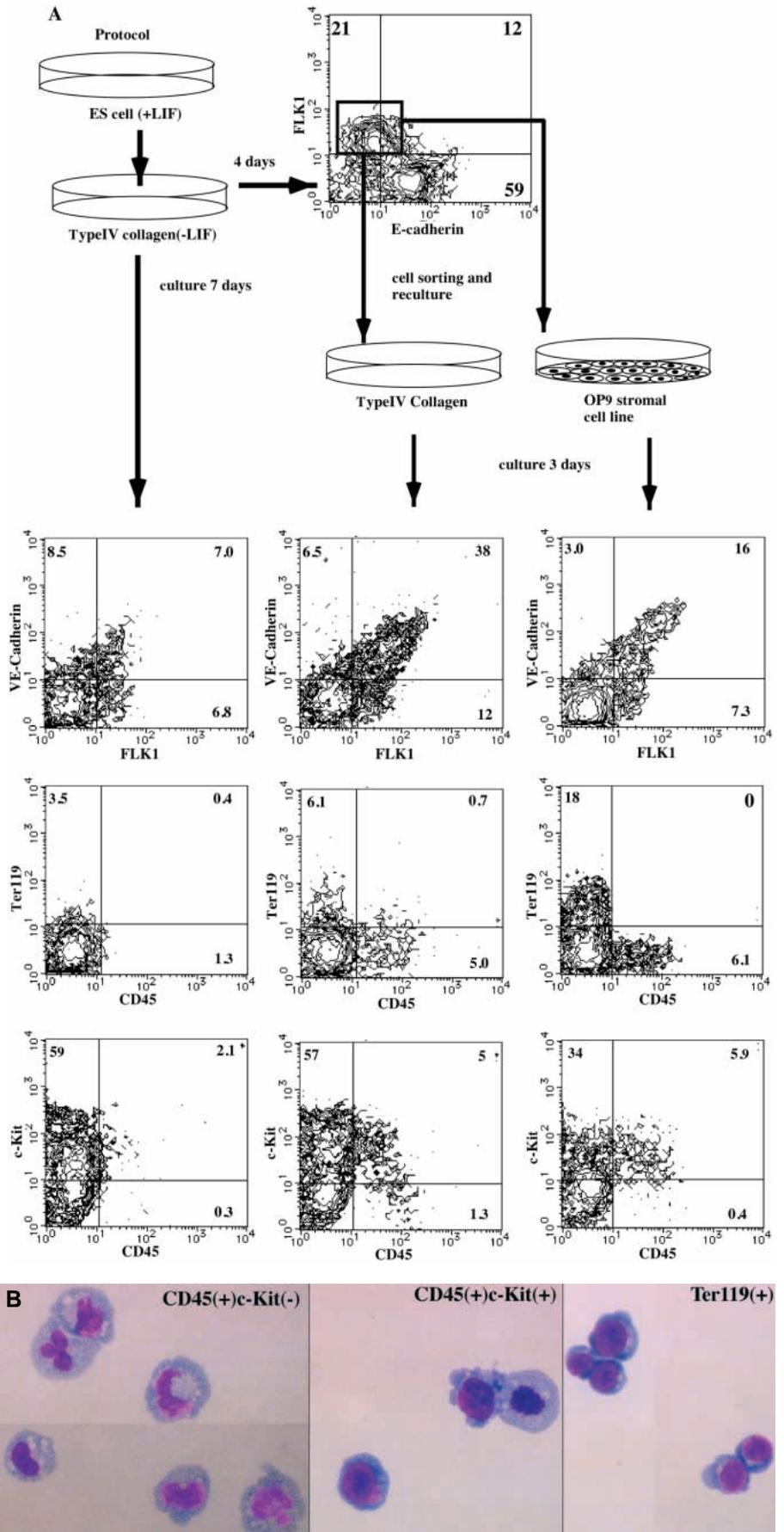
were sorted from the culture of FLK1^{+} cells at day 1. Single FLK1^{+}VE-cadherin[−] or FLK1^{+}VE-cadherin^{+} cells were deposited by FACS Vantage on a 96-well cluster dish containing OP9 feeder cells, erythropoietin, SCF, IL-3 and G-CSF. After a 5 day incubation, wells were screened for the presence of clusters of round cells (Fig. 7E-C). Positive wells were then immunostained with anti-VE-cadherin mAb and the presence of VE-cadherin[+] sheets were scored. In a parallel experiment, the frequency of clonogenic cells giving rise to a VE-cadherin[+] sheet were evaluated. Among 192 cells deposited, 23 FLK1^{+}VE-cadherin^{−} cells and 29 FLK1^{+}VE-cadherin^{+} cells gave rise to a VE-cadherin[+] sheet. In all positive wells, only a single sheet was detected. Among 384 FLK1^{+}VE-cadherin^{−} cells and 1,920 FLK1^{+}VE-cadherin^{+} cells, respectively, 25 and 104 cells gave rise to the cluster of round cells and 17 and 29 of them were also positive for the VE-cadherin[+] sheet formation (Table 1). These results clearly demonstrated the presence of hemoangiogenic progenitors in both FLK1^{+}VE-cadherin[−] and FLK1^{+}VE-cadherin[+] population.

DISCUSSION

Mesoderm and blood cell induction in the absence of embryoid body formation

More than a decade after Doetchman's paper describing the embryoid body-dependent method of inducing blood cells from ES cells (Doetschman et al., 1985), we eventually determined a condition in which a similar process is reproduced within the 2-D spreading cell mass. The most plausible reasons for our success are: (1) use of type IV collagen matrix, (2) use of a panel of cell surface markers that can define intermediate stages in the course of blood cell differentiation, and (3) the reculturing of sorted E-cadherin[−]FLK-1^{+} mesoderm cells upon their appearance. Thus, purification of the cells at

intermediate stages turned out to be useful not only for analyzing the differentiation process of a given cell lineage but also for improving culture conditions.
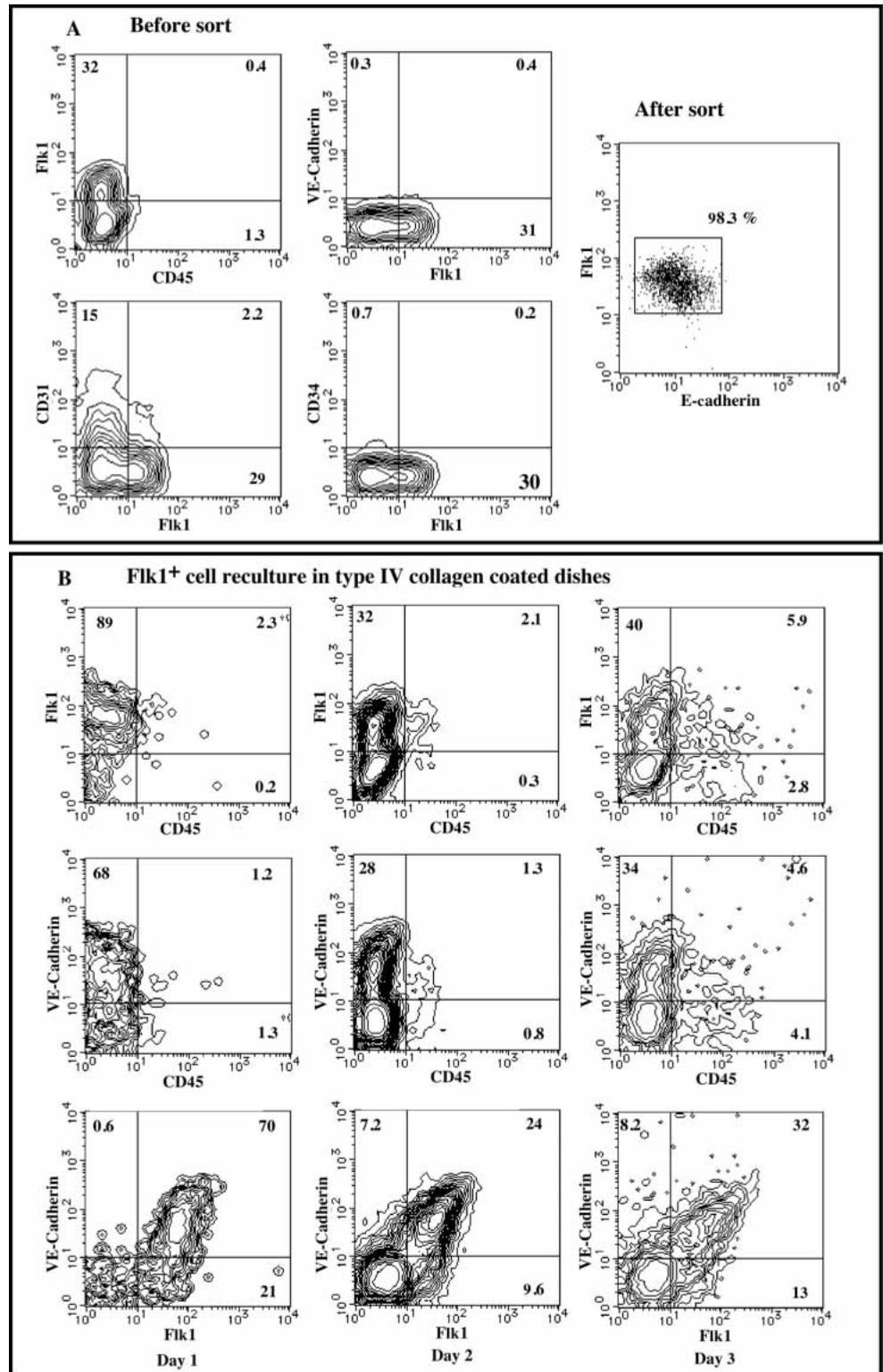
Fig. 4. Rapid induction of VE-cadherin in FLK1^{+}VE-cadherin^{−} cells. FLK1^{+} cells were sorted and 2×10^5 cells were cultured in type IV collagen-coated dishes. (A) On the day of cell sorting, FLK1^{+} cells did not express VE-cadherin nor CD34, though some expressed CD31. The purity of the FLK1^{+} cells used in this experiment is also presented. (B) At 1, 2 and 3 days after the culture of FLK1^{+} cells, cells were harvested and analyzed for expression of FLK1, VE-cadherin, and FLK1.
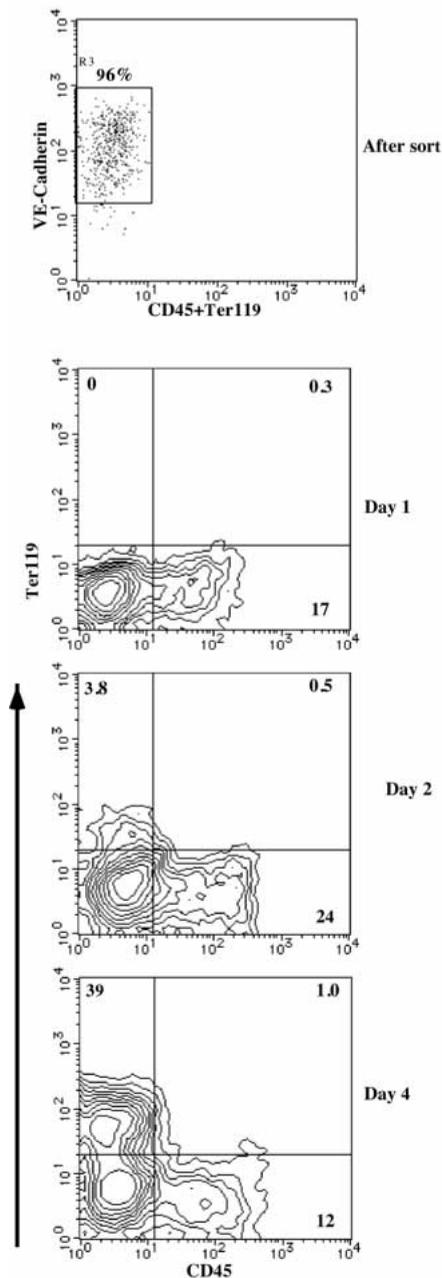
Fig. 5. Generation of blood cells from VE-cadherin$^+$CD45$^-$ cells. Cells harvested from the culture of FLK1$^+$ cells were stained with PE-anti-VE-cadherin and FITC-anti-CD45 and FITC-anti-Ter119. VE-cadherin$^+$CD45$^-$Ter119$^-$ cells were purified and 10^4 cells were cultured in the presence of VEGF, SCF, IL-3, Epo, and G-CSF. 1.2 million cells were recovered from the dish after day 4 of incubation. At 1, 2 and 4 days after culture, cells were harvested and analyzed for the expression of Ter119 and CD45.

In addition to their usefulness in the study of hemopoietic cell differentiation, the present findings are significant in that they indicate that the specification of ES cells to mesoderm and their further specification to blood cells do not require the embryoid body structure, feeder cells nor exogenous growth factors other than those present in the fetal calf serum. Indeed, comparable numbers of FLK1$^+$VE-cadherin$^-$ mesoderm are

Table 1. Clonogenic assay for hemoangiogenic potential of FLK1$^+$VE-cadherin$^-$ and FLK1$^+$VE-cadherin$^+$ cells

Cell source	Sheet formation	Round cell cluster formation (+sheet formation)
FLK1$^+$VE-cadherin$^-$	23/192 (12.0%)	25/384 (6.5%)
		(17/384) (4.7%)
FLK1$^+$VE-cadherin$^+$	29/192 (15%)	104/1,920 (5.4%)
		(29/1,920) (1.5%)

Either FLK1$^+$VE-cadherin$^-$ or FLK1$^+$VE-cadherin$^+$ cells were sorted and single cells of each fraction were inoculated into 96-well cluster dishes by the single cell deposition apparatus connected to FACS Vantage. Deposited cells were incubated in the presence of OP9 feeder cells, erythropoietin, SCF, IL-3 and G-CSF. Five days later, two dishes for each group were fixed and immunostained by the anti-VE-cadherin mAb to assess the frequency of cells that can give rise to the sheet-like cluster of VE-cadherin$^+$ cells (Fig. 7D). Rest dishes, 4 and 20 dishes, respectively, for FLK1$^+$VE-cadherin$^-$ and FLK1$^+$VE-cadherin$^+$ fractions, were screened for the presence of the cluster of round cells. Positive wells were stained by anti-VE-cadherin mAb to determine the co-generation of VE-cadherin$^+$ sheets.

induced in our culture and the kinetics of their appearance is almost identical to that with the embryoid body (Kabrun et al., 1997). This indicates that the cell-cell interactions required for this differentiation pathway proceed within 2-D placed cells as efficiently as in the embryoid body. In light of the latest notion of an organizer based process of mesoderm induction (reviewed by Zorn, 1997), it is intriguing how cell diversification required for mesoderm induction proceeds in this randomly placed 2-D cell mass.

It should be noted, however, that our culture conditions are less efficient in supporting embryonic processes than that in the embryoid body formation. For instance, tube formation of vascular endothelial cells which is observed in the embryoid body (Vittet et al., 1996) could not be seen in our culture conditions. Moreover, clonogenic proliferation of VE-cadherin$^+$ cells is supported by OP9 feeder cells but not by our feeder-free culture condition. Further attempts are thus required to make our culture conditions comparable to those in the embryoid body.

Nevertheless, real-time monitoring of the cell specification process is increasingly important to embryology, and our culture system provides a promising method for following ES cell specification. At present, time-lapse video recording of cells on dish surfaces can show only morphology, motility, mitosis, and cell death etc. The development of new techniques, such as real-time monitoring of gene expression and innovations in cell manipulation should enable us to perceive the orderly interactions of differentiating cells.
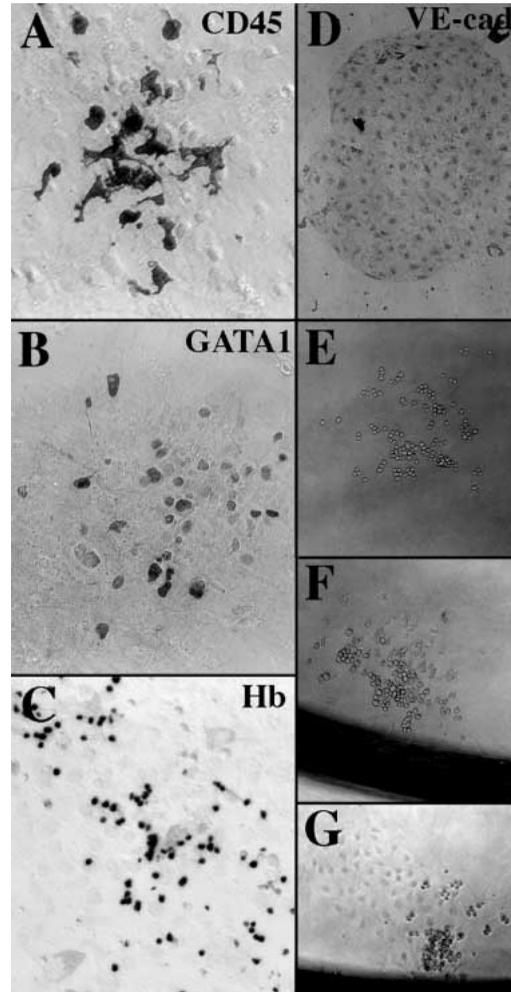
Pathway of blood cell differentiation defined by surface markers

Lineage analysis of the mouse embryo has been based upon the use of cell marking and subsequent identification of the marked cells in the embryo (Beddington and Lawson, 1990). On some occasions, living cells have been microdissected from the embryo to analyze their differentiation potential, but their lineage and staging have been determined only by their spatio-temporal position, if they remain alive (Strum and Tam, 1993). However, once cells are dissociated and plated in culture, the spatial information is lost, so that each cell has to be defined by its own characteristics. Sorting of multicolor
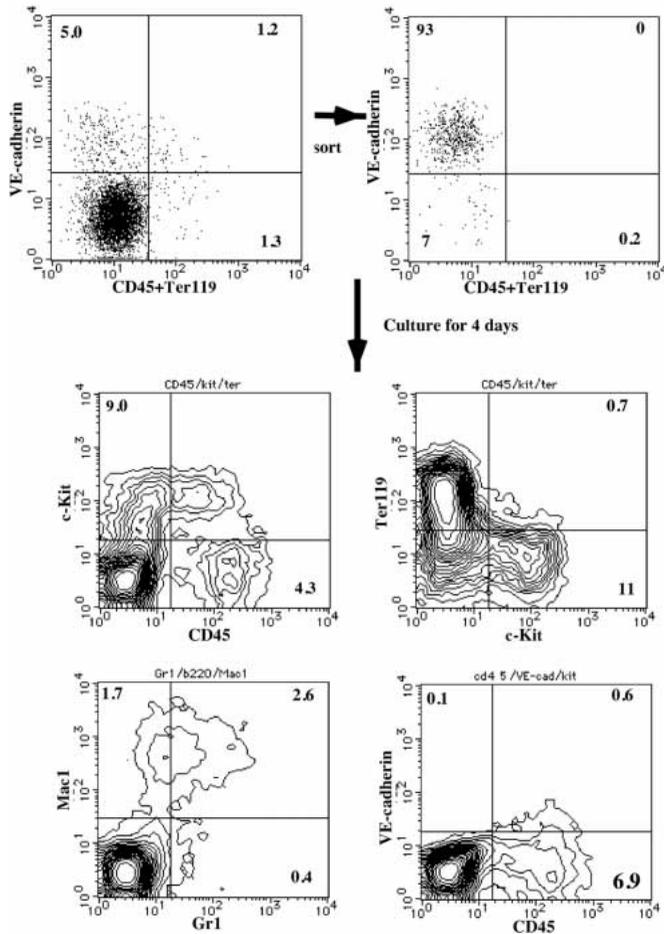
Fig. 6. Hemogenic potential of VE-cadherin⁺CD45⁻Ter119⁻ cells in the yolk sac. Yolk sacs of 9.5 dpc embryos were microdissected and dissociated to form a single cell suspension. The cells were stained with PE-anti-VE-cadherin and FITC-anti-CD45 and FITC-anti-Ter119. VE-cadherin⁺CD45⁻Ter119⁻ cells were purified and 10^4 cells were cultured in the presence of VEGF, SCF, IL-3, Epo, and G-CSF. Four days after the culture, the cells were harvested and analyzed for expressions of surface markers.

Fig. 7. Cells generated in the culture of FLK1⁺VE-cadherin⁺ cells. Single FLK1⁺VE-cadherin⁺ cells were inoculated into each well of 96-well cluster dishes with OP9 feeder cells. Five days after the culture, wells containing clusters of round cells were stained either with anti-CD45 (A), anti-GATA1 (B) or anti-embryonic hemoglobin (C). When a mixtures of all these antibodies was used for staining wells containing clusters of round cells (E-G), all of them were positive. In some wells, a sheet of cells that expressed VE-cadherin was detected by immunostaining. Results of these experiments are summarized in Table 1.

stained cells by FACS may be the most appropriate method for this purpose. As shown here, mAbs against E-cadherin, FLK1, VE-cadherin, c-Kit, CD45 and Ter119 are useful for elucidation of the process of blood cell differentiation from ES cells. With respect to this process, Kabrun et al. (1997) and Eichmann et al. (1997) succeeded in isolating an FLK1⁺ population which is competent to give rise to hemopoietic cells. In the present study, we further extended their observations and demonstrated that the FLK1⁺ stage can be divided into VE-cadherin-negative and -positive stages, and the latter is a more differentiated precursor of hemopoietic cells, though direct differentiation of blood cells from FLK1⁺ VE-cadherin⁻ cells cannot be ruled out. According to the results described here, the simplest differentiation pathway from ES cells to blood cells may be as follows: ES cell (E-cadherin⁺FLK1⁻), proximal lateral mesoderm (E-cadherin⁻ FLK1⁺PDGFRα⁺ or ⁻), progenitor with a hemoangiogenic potential (FLK1⁺VE-cadherin⁺) and hemopoietic precursor (CD45⁺c-Kit⁺). CD45⁺c-Kit⁺ cells then generate various

blood cell lineages including Ter119⁺ erythroblasts in response to hemopoietic growth factors. While this pathway was derived from our results on ES cell culture, our results on actual embryos also support this scheme. First, analysis of 7.5 and 8.5 dpc embryos demonstrated that the proportion of FLK1⁺VE-cadherin⁺ cells in the FLK1⁺ population increases as embryogenesis proceeds. Moreover, FLK1⁺VE-cadherin⁺ cells from the 9.5 dpc yolk sac gave rise to both myeloid and erythroid cells.

In addition to the stages in the differentiation pathway of blood cells, intermediate stages of other lineages were identified. In the culture of ES cells, four distinct surface phenotypes were identified. E-cadherin⁺ cells may include both immature and differentiated ectoderm and endoderm. According to our previous studies on the expression of

PDGFRα in the gastrulating embryo (Takakura et al., 1996; Kataoka et al., 1997), E-cadherin⁻ FLK1⁻PDGFRα⁺ cells may represent the paraxial mesoderm, though we have not tested their differentiation potential. While E-cadherin⁻ FLK1⁺ cells are further divided into PDGFRα-positive and - negative populations, both populations are able to give rise to CD45⁺ blood cells (S.-I. Nishikawa et al., unpublished observation). Hence, they are treated here as representing the same cell population, though our previous immunohistochemical studies suggested that FLK1 and PDGFRα double positive cells correspond to allantoic mesoderm (Kataoka et al., 1997). In the subsequent culture of E-cadherin⁻FLK1⁺ cells, two populations, FLK1⁻VE-cadherin⁻ and FLK1⁺VE-cadherin⁺ cells, were generated within 24 hours. If appropriate methods are available, it would be interesting to determine the actual fate of all those intermediate stages.

Hemangioblast or hemogenic endothelial cell

Although the hemangioblast as a bipotent precursor for blood and endothelial cell lineages is hypothesized (reviewed by Risau and Flamme, 1995), it has been difficult to prove the existence of the hemangioblast at single cell level. This is due to difficulties in: (1) sorting precursor cells that are distinct from early multipotent mesoderm, (2) clonogenic assay for multipotent precursors, and (3) proving fate restriction only to blood and endothelial lineages. Though the third problem remains unsolved in this study, surface markers described here and culture of single cells deposited onto OP9 feeder cells demonstrated the presence of clonogenic cells that can give rise to blood and endothelial cell lineages. Our criterion for endothelial cell generation is the formation of the sheet-like cluster of VE-cadherin⁺ cells. In this sheet, cells adhere to each other by the adherence junction where VE-cadherin is concentrated. This sheet incorporated LDL and was positive in FLK1 and CD31 expression (M. Hirashima et al., unpublished observation). Moreover, a previous study

demonstrated that VE-cadherin is expressed exclusively in endothelial cells (Breier et al., 1996; Matsuyoshi et al., 1997). Thus, although we could not induce such a morphogenic activity in VE-cadherin⁺ cells as tube- and/or network-formation, we believe that formation of the sheet of VE-cadherin⁺ cells on OP9 feeder cells represents an activity of endothelial cells.

Both FLK1⁺VE-cadherin⁻ and FLK1⁺VE-cadherin⁺ fractions gave rise to three types of colony containing: (1) a VE-cadherin⁺ sheet alone, (2) a cluster of round cells alone, and (3) both. That 15-20% of single cells cultured on OP9 feeder cells could generate visible cell clusters of either type suggests a high clonogenic efficiency for this culture condition. In fact, the colony assay in the semisolid medium was 100-fold less efficient than the culture with OP9. In the simple diagram of ES cell differentiation, FLK1⁺VE-cadherin⁻ and FLK1⁺VE-cadherin⁺ stages represent,
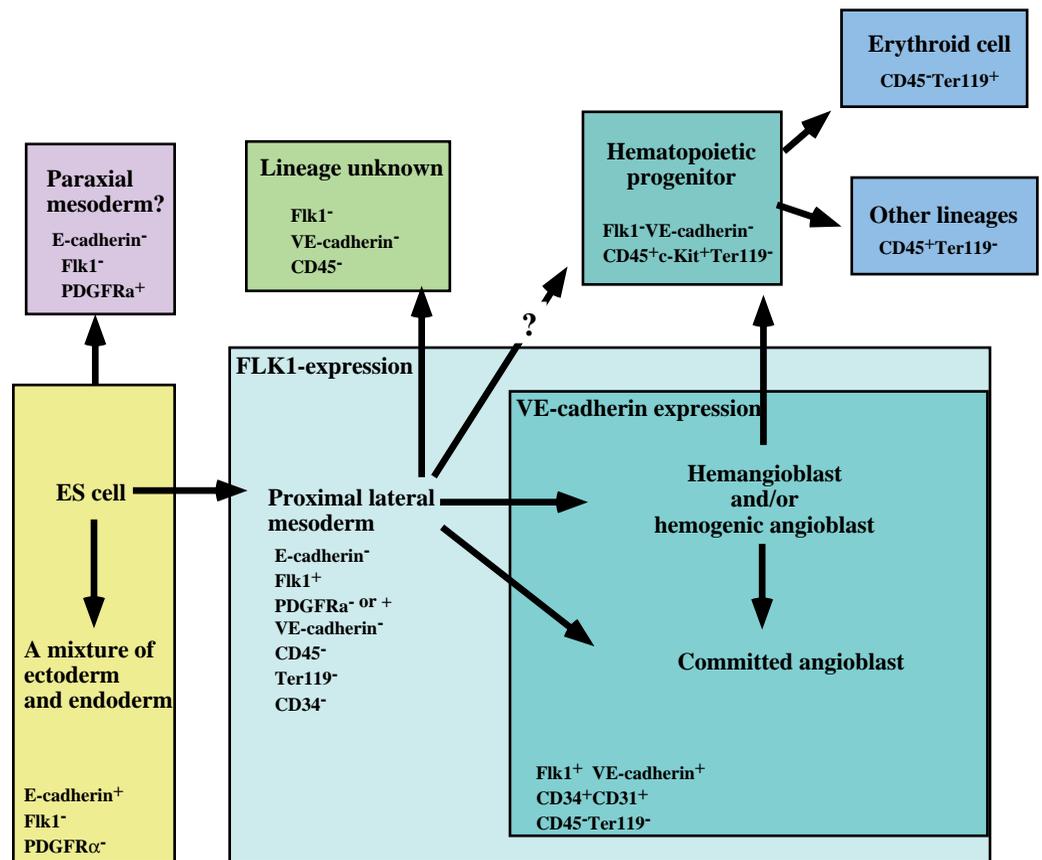
Fig. 8. A model of hemopoietic and endothelial development defined by a panel of surface markers. This diagram of ES cell differentiation to blood cells is based upon repeated cell sorting and subsequent culture of the sorted cells. All surface phenotypes that were detected in the culture of each intermediate stage are listed in addition to the differentiation pathway to blood cells. Although not listed here, a stage in which FLK1 and VE-cadherin were co-expressed with CD45 was consistently found between the VE-cadherin⁺CD45⁻ and VE-cadherin⁻CD45⁺ stages (see Fig. 5B). However, this co-expression disappeared quickly, probably reflecting a transient stage in which endothelial markers are downregulated along with the upregulation of hemopoietic cell markers. While CD45⁺c-Kit⁺ cells can give rise to both Ter119⁺ erythroid and CD45⁺ cells, the possibility that erythroid cells differentiate directly from VE-cadherin⁺CD45⁻ cells cannot be ruled out. Likewise, a direct differentiation pathway from the FLK1⁺VE-cadherin⁻ stage to the CD45⁺ or Ter119⁺ blood cell is difficult to be ruled out. ES cells and other E-cadherin⁺ epithelial cells cannot be distinguished by the surface markers used here. Likewise, hemoangiogenic progenitors and committed angioblasts are difficult to discriminate with the surface markers listed here.

respectively, mesoderm and further differentiated progenitors, both of which are considered to give rise to blood and endothelial lineages. As mesoderm in non-hemogenic sites should differentiate directly to VE-cadherin$^+$ angioblasts, it is conceivable that twice as many cells in each fraction generated the VE-cadherin$^+$ sheet compared to the cluster of round cells. Interestingly, some FLK1$^+$VE-cadherin$^-$ cells, which are supposed to represent the multipotent mesoderm, could generate only the cluster of round cells. This implies a pathway in which blood cells are generated directly from FLK1$^+$VE-cadherin$^-$ cells, though it is difficult to rule out the possibility that angioblasts were generated in the same cultures but they could not proliferate to form visible clusters. In either case, our single cell deposition experiment suggests that cells with a diverse set of differentiation and proliferation potentials are generated during differentiation of mesoderm to VE-cadherin$^+$ cells. Therefore the straightforward diagram of the blood cell differentiation pathway described in the preceding section should be modified so as to include some diverse pathways as shown in Fig. 8. As frequencies of multipotent progenitors in FLK1$^+$VE-cadherin$^-$ and FLK1$^+$VE-cadherin$^+$ fractions are 4.7 and 1.5%, respectively, there should be a constant decrease in potentiality during this differentiation step.

Though it is not clear whether the FLK1$^+$VE-cadherin$^-$ population represents only the multipotent mesoderm or includes the bipotent hemangioblast, our single cell deposition experiment identified hemoangiogenic progenitors in the VE-cadherin$^+$CD45$^-$Ter119$^-$ population. Of note in this context is that this surface phenotype has been considered to be specific to endothelial cells (Breier, 1996; Matsuyoshi, 1997). Hence, this notion should be corrected to say that VE-cadherin$^+$ cells include both hemangioblast and endothelial cells. Alternatively, VE-cadherin expression is specific to endothelial cells, but some endothelial cells in the hemogenic sites can give rise to blood cells. In fact, the presence of hemogenic endothelial cells has been implied by histological observations that round cells appear to bud from the endothelial wall (Smith and Glomski, 1982; Tavian et al., 1996). Whether hemogenic VE-cadherin$^+$ cells represent hemangioblast or hemogenic endothelial cells is dependent upon the function of VE-cadherin. If VE-cadherin expression is sufficient to confer the ability to form an organized endothelial layer, all VE-cadherin$^+$ cells should be regarded as endothelial cells. In agreement with this notion, a recent study by Navarro et al. (1995) demonstrated that even a truncated VE-cadherin that lacks the catenin binding domain can contribute to the homophilic adhesion. Hence, it is likely that during differentiation of FLK1$^+$ mesoderm, a diverse set of endothelial cells including hemogenic endothelial cells are generated. While some blood cells may differentiate without passing through the VE-cadherin$^+$ stage, most of them are generated from VE-cadherin$^+$ hemogenic endothelial cells. If, as suggested by previous studies (Delassus and Cumano, 1996; Medvinsky and Dzierzak, 1996), generation of blood cells still continues in the aorta-gonado-mesonephros region until such a late stage as 10 dpc, it is more plausible that hemogenic endothelial cells rather than hemangioblasts are the immediate precursor of blood cells.

REFERENCES

Beddington, R. S. P. and Lawson, K. A. (1990). Clonal analysis of cell lineages. In *Postimplantation Mammalian Embryos, a Practical Approach* (ed. A. J. Copp and D. L. Cockroft), pp. 267-316. IRL Press, Oxford, New York, Tokyo.

Breier, G., Breviario, F., Caveda, L., Berthier, R., Schnurch, H., Gotsch, U., Vestweber, D., Risau, W. and Dejana, E. (1996). Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. *Blood* **87**, 630-641.

Burkert, U., von Ruden, T. and Wagner, E. F. (1991). Early fetal hemopoietic development from in vitro differentiated embryonic stem cells. *New Biol.* **3**, 698-708.

Burdsal, C. A., Damsky, C. H. and Pedersen, R. A. (1993). The role of E-cadherin and integrins in mesoderm differentiation and migration at the mammalian primitive streak. *Development* **118**, 829-844.

Choi, Y. S. and Gumbiner, B. (1989). Expression of cell adhesion molecule E-cadherin in Xenopus embryos begins at gastrulation and predominates in the ectoderm. *J. Cell Biol.* **108**, 2449-2458.

Delassus, S. and Cumano, A. (1996). Circulation of hematopoietic progenitors in the mouse embryo. *Immunity* **4**, 97-106.

Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* **87**, 27-45.

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.

Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Bréant, C. and Le Douarin, N. (1997). Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc. Nat. Acad. Sci. USA* **94**, 5141-5146.

Gutierrez-Ramos, J. C. and Palacios, R. (1992). In vitro differentiation of embryonic stem cells into lymphocyte precursors able to generate T and B lymphocytes in vivo. *Proc. Nat. Acad. Sci. USA* **89**, 9171-9175.

Kabrun, N., Bühring, H. J., Coi, K., Ullrich, A., Risau, W. and Keller, G. (1997). FLK-1 expression defines a population of early embryonic hematopoietic precursors. *Development* **124**, 2039-2048.

Kataoka, H., Takakura, N., Nishikawa, S., Tsuchida, K., Kodama, H., Kunisada, T., Risau, W., Kita, T. and Nishikawa, S. I. (1997). Expression of PDGF receptor, c-Kit and FLK1 gene clustering in mouse chromosome 5 define distinct subsets of nascent mesoderm cells. *Dev. Growth Differ.* **39**, 729-740.

Kessel, J. and Fabian, B. (1987). Inhibitory and stimulatory influences on mesodermal erythropoiesis in the early chick blastoderm. *Development* **101**, 45-49.

Kodama, H., Nose, M., Niida, S., Nishikawa, S. and Nishikawa, S. I. (1994). Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. *Exp. Hematol.* **22**, 979-984.

Matsuyoshi, N., Toda, K., Horiguchi, Y., Tanaka, T., Nakagawa, S., Takeichi, M. and Imamura, S. (1997). In vivo evidence of the critical role of cadherin-5 in murine vascular integrity. *Proc. Assoc. Am. Physicians* **109**, 362-371.

Medvinsky, A. and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 896-906.

Mercola, M., Wang, C., Kelly, J., Brownlee, C., Jackson-Grusby, L., Stiles, C. and Bowen-Pope, D. (1990). Selective expression of PDGF A and its receptor during early mouse embryogenesis. *Dev. Biol.* **138**, 114-122.

Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N. P., 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.

Miwa, Y., Atsumi, T., Imai, N. and Ikawa, Y. (1991). Primitive erythropoiesis of mouse teratocarcinoma stem cells PCC3/A/1 in serum-free medium. *Development* **111**, 543-549.

Nakano, T., Kodama, H. and Honjo, T. (1994). Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* **265**, 1098-1101.

Navarro, P., Caveda, L., Breviario, F., Mandoteanu, I., Lampugnani, M.

G. and Dejana, E. (1995). Catenin-dependent and -independent functions of vascular endothelial cadherin. *J. Biol. Chem.* **270**, 30965-30972.

Nishikawa, S., Kusakabe, M., Yoshinaga, K., Ogawa, M., Hayashi, S., Kunisada, T., Era, T., Sakakura, T. and Nishikawa, S. I. (1991). In utero manipulation of coat color formation by a monoclonal anti-c-kit antibody: two distinct waves of c-kit-dependency during melanocyte development. *EMBO J.* **10**, 2111-2118.

Nose, A. and Takeichi, M. (1986). A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos. *J. Cell Biol.* **103**, 2649-2658.

Ogawa, M., Matsuzaki, Y., Nishikawa, S., Hayashi, S. I., Kunisada, T., Sudo, T., Kina, T., Nakauchi, H. and Nishikawa, S. I. (1991). Expression and function of *c-kit* in hemopoietic progenitor cells. *J. Exp. Med.* **174**, 63-71.

Ogawa, M., Nishikawa, S., Yoshinaga, K., Hayashi, S., Kunisada, T., Nakao, J., Kina, T., Sudo, T., Kodama, H. and Nishikawa, S. I. (1993). Expression and function of c-Kit in fetal hemopoietic progenitor cells: transition from the early c-Kit-independent to the late c-Kit-dependent wave of hemopoiesis in the murine embryo. *Development* **117**, 1089-1098.

Orr-Urtreger, A., Bedford, M. T., Do, M. S., Eisenbach, L. and Lonai, P. (1992). Developmental expression of the α receptor for platelet-derived growth factor, which is deleted in the embryonic lethal *Patch* mutation. *Development* **115**, 289-303.

Palis, J., McGrath, K. E. and Kingsley, P. D. (1995). Initiation of hematopoiesis and vasculogenesis in murine yolk sac explants. *Blood* **86**, 156-163.

Risau, W. and Flamme, I. (1995). Vasculogenesis. *Annu. Rev. Cell Dev. Biol.* **11**, 73-91.

Robertson, E., Bradley, A., Kuehn, M. and Evans, M. (1986). Germ line transmission of genes introduced into cultured pluripotential cells by retroviral vector. *Nature* **323**, 445-448.

Schatteman, G. C., Morrison-Graham, K., Koppen, A. V., Weston, J. A. and Bowen-Pope, D. F. (1992). Regulation and role of PDGF receptor α subunit expression during embryogenesis. *Development* **115**, 123-131.

Schmitt, R. M., Bruyns, E. and Snodgrass, H. R. (1991). Hematopoietic development of embryonic stem cells in vitro: cytokine and receptor gene expression. *Genes Dev.* **5**, 728-740.

Shirayoshi, Y., Nose, A., Iwasaki, K. and Takeichi, M. (1986). N-linked oligosaccharides are not involved in the function of a cell-cell binding glycoprotein E-cadherin. *Cell Struct. Funct.* **11**, 245-252.

Smith, R. A. and Glomski, C. A. (1982). 'Hemogenic endothelium' of the embryonic aorta: Does it exist? *Dev. Comp. Immunol.* **6**, 359-368.

Sudo, T., Nishikawa, S., Ohno, N., Akiyama, N., Tamakoshi, M., Yoshida, H. and Nishikawa, S. I. (1993). Expression and function of the interleukin 7 receptor in murine lymphocytes. *Proc. Nat. Acad. Sci. USA* **90**, 9125-9129.

Takakura, N., Yoshida, H., Kunisada, T., Nishikawa, S. and Nishikawa, S. I. (1996). Involvement of platelet-derived growth factor receptor α in hair canal formation. *J. Inv. Dermatol.* **107**, 770-777.

Takakura, N., Yoshida, H., Ogura, Y., Kataoka, H., Nishikawa, S. and Nishikawa, S. I. (1997). PDGFRα expression during mouse embryogenesis: immunolocalization analyzed by whole-mount immunohistostaining using the monoclonal anti-mouse PDGFRα antibody APA5. *J. Histochem. Cytochem.* **45**, 883-893.

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.

Strum, K. and Tam, P. P. L. (1993). Isolation and culture of whole postimplantation embryos and germ layer derivatives. In *Guide to Techniques in Mouse Development* (ed. P. M. Wassarman and M. L. DePamphilis), pp. 164-190. Academic Press Inc, New York.

Vittet, D., Prandini, M. H., Berthier, R., Schweitzer, A., Martin-Sisteron, H., Uzan, G. and Dejana, E. (1996). Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. *Blood* **88**, 3424-3431.

Wiles, M. V. and Keller, G. (1991). Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* **111**, 259-267.

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.

Zorn, A. M. (1997). Cell-cell signaling: frog frizbees. *Curr. Biol.* **7**, 501-504.