

# Discordant developmental waves of angioblasts and hemangioblasts in the early gastrulating mouse embryo

Chie Furuta<sup>1</sup>, Hideo Ema<sup>1,\*</sup>, Shin-ichiro Takayanagi<sup>1</sup>, Takunori Ogaeri<sup>1</sup>, Daiji Okamura<sup>2</sup>, Yasuhisa Matsui<sup>2</sup> and Hiromitsu Nakauchi<sup>1,\*</sup>

Vasculogenesis and hematopoiesis are thought to arise in hemangioblasts, the common progenitors of cells in vessels and in blood. This scheme was challenged by kinetic analysis of vascular endothelial and hematopoietic progenitors in early gastrulating mouse embryos. The OP-9 co-culture system with a combination of cytokines permitted the detection of endothelial progenitors, as well as stroma-dependent hematopoietic progenitors. Endothelial progenitors were detected as early as embryonic day (E) 5.50, after which time their numbers increased. Stroma-dependent hematopoietic progenitors were detected at E6.75, the time point when hemangioblasts reportedly emerge. Colony-forming units in culture (CFU-c), most likely generated from stroma-dependent hematopoietic progenitors via contact with the microenvironment, were detected at E7.50, concomitant with the onset of primitive hematopoiesis in the yolk sac. The presence of nucleated erythrocytes and the expression of an embryonic-type globin in erythroid colonies derived from stroma-dependent hematopoietic progenitors and from CFU-c support the notion that these progenitors coordinately establish primitive hematopoiesis. Using Oct3/4 promoter-driven GFP transgenic mice, early endothelial progenitors, stroma-dependent hematopoietic progenitors, and CFU-c were all shown to express the Oct3/4 transcription factor. Among Oct3/4-positive cells, both endothelial and hematopoietic progenitors were present in the CD31-positive fraction, leaving a subset of endothelial progenitors in the CD31-negative fraction. These data imply that Oct3/4-positive mesoderm gives rise to CD31-negative angioblasts, CD31-positive angioblasts and CD31-positive hemangioblasts. We propose a distinct developmental pathway in which the angioblast lineage directly diverges from mesoderm prior to and independent of hemangioblast development.

**KEY WORDS:** Mouse, Embryo, Hematopoiesis, Vasculogenesis, Angioblast, Hemangioblast, Flk1, Tie2, CD31, Oct3/4

## INTRODUCTION

Hematopoiesis in the mouse begins in the blood islands of the yolk sac (YS) at E7.5 (Haar and Ackerman, 1971; Moore and Metcalf, 1970). Nucleated red blood cells in blood islands produce embryonic globins (Barker, 1968; Brotherton et al., 1979; Palis et al., 1999; Steiner and Vogel, 1973) that predominate in the circulation through E12.5, followed by eventual replacement with enucleated red blood cells that produce adult type globins. Progenitors of these definitive erythrocytes are detected initially in the YS, and later on in the paraaortic splanchnopleure (P-Sp)/aorta-gonad-mesonephros (AGM) region, the placenta and the fetal liver (Gekas et al., 2005; Houssaint, 1981; Palis et al., 1999). In a process presumed to be independent of these two types of erythropoiesis, hematopoietic stem cells (HSCs) arise in the P-Sp/AGM region (Cumano et al., 1996; Cumano et al., 2001; Medvinsky and Dzierzak, 1996; Muller et al., 1994) and the YS (Matsuoka et al., 2001; Palis et al., 2001; Palis et al., 1999; Yoder et al., 1997).

The formation of blood cells is closely correlated with that of vascular endothelial cells in the YS. This led to the hypothesis that both cell types arise from a common precursor, the hemangioblast (Eichmann et al., 1997; Murray, 1932; Sabin, 1920; Wagner, 1980). Endothelial and hematopoietic lineages share a number of expressed

genes (Asahara et al., 1997; Fina et al., 1990; Kabrun et al., 1997; Kallianpur et al., 1994; Millauer et al., 1993; Young et al., 1995). Much work in the past has focused on the isolation and identification of hemangioblasts among cells that have in vitro differentiated from embryonic stem (ES) cells (Choi et al., 1998; Kennedy et al., 1997; Nishikawa et al., 1998; Yamashita et al., 2000). Blast colony-forming cells (BL-CFCs) were found as candidate hemangioblasts in this model system (Choi et al., 1998). Recently, BL-CFCs were isolated also from the developing mouse embryo (Huber et al., 2004). All of these studies support the common origin of vascular and hematopoietic cells.

Little attention has been paid to endothelial progenitors, namely angioblasts. In an avian system, two distinct endothelial lineages have been described for aorta formation (Pardanaud et al., 1996). One of these, derived from splanchnopleural mesoderm, is associated with hematopoiesis. The other, without hematopoietic potential, is derived from paraxial mesoderm (Pardanaud et al., 1996). A similar mechanism has been speculated to operate in mammals (Hatzopoulos et al., 1998), but there is no compelling evidence particularly for early vasculogenesis and hematopoiesis. It is difficult to perform fate-mapping studies for a specific lineage such as vascular endothelium in the developing mouse embryo. We therefore decided to use a sensitive functional assay for endothelial and hematopoietic progenitors to determine their developmental kinetics in early gastrulating mouse embryos prior to the onset of YS hematopoiesis.

We found that a significant number of angioblasts can be detected one day before hematopoietic activity is detected. In an attempt to isolate angioblasts and hemangioblasts, we found that both cells expressed Flk1 and Tie2 receptor tyrosine kinases in the mouse embryo. We also found that both cells express Oct3/4, a member of

<sup>1</sup>Laboratory of Stem Cell Therapy, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan. <sup>2</sup>Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University, 4-1 Seiryomachi, Sendai, 980-8575, Japan.

\* Authors for correspondence (e-mail: hema@ims.u-tokyo.ac.jp; nakauchi@ims.u-tokyo.ac.jp)

the POU transcription factor family, which is likely to play a crucial role in the maintenance of the potentials in ES and embryonic carcinoma cells, and in primordial germ cells (PGCs) (Niwa et al., 2000; Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990). Furthermore, we obtained data suggesting that CD31 [platelet endothelial cell adhesion molecule 1, a member of the immunoglobulin super family (Newman, 1999)] expression can divide Oct3/4-positive cells into an angioblast or hemangioblast lineage.

## MATERIALS AND METHODS

### Mice

Female C57BL/6 (B6) mice were mated with male B6 mice or with Oct3/4-GFP heterozygous male mice that had been crossbred with B6 mice. In these transgenic mice, expression of GFP is under the control of the Oct3/4 promoter (Yoshimizu et al., 1999). Mice were allowed to mate naturally at night; offspring of females in which vaginal plugs were found the next morning were designated as of age embryonic day 0.5 (E0.5) as of midnight the night of mating. At specified time points, embryos were dissected out from uteri.

### Staging of mouse embryos

E5.50, E5.75 and E6.00 embryos were staged as described previously (Okamura et al., 2005). Pre-streak (PS), early streak (ES), mid-streak (MS), late streak (LS), no allantoic bud (OB), early allantoic bud (EB), late allantoic bud (LB), and early head-fold (EHF) stages were assigned developmental ages of E6.25, E6.50, E6.75, E7.00, E7.25, E7.50, E7.75 and E8.00, respectively (Downs and Davies, 1993).

### Cells

Embryos were treated with 0.1% dispase and 0.1% collagenase (Invitrogen, Carlsbad, USA) or 0.05% trypsin-EDTA in PBS (Sigma, St Louis, USA) at 37°C for 10 minutes. Single-cell suspensions were made by gentle pipetting. Cells were washed with 10% fetal calf serum (FCS) in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) (Gibco, Carlsbad, USA). One embryo equivalent was used as a unit for cell doses because the number of cells per embryo varies remarkably, depending on embryonic stage.

### Co-culture with OP-9 cells

We used a monolayer of OP-9 stromal cells to detect endothelial and hematopoietic progenitors in the mouse embryos (Kanatsu and Nishikawa, 1996; Nakano et al., 1994; Takakura et al., 1998). To perform the co-culture experiments, OP-9 cells were seeded at  $6 \times 10^3$  cells/cm<sup>2</sup> in 24-well plates that had been coated with 0.1% gelatin in PBS for 15 minutes at room temperature (RT). The following day, dissected embryonic cell suspensions were transferred onto a confluent layer of OP-9 cells. Cells were cultured in  $\alpha$ -MEM containing 10% FCS supplemented with 10 ng/ml of mouse interleukin (IL) 3, 10 ng/ml of human thrombopoietin (TPO), 10 ng/ml of mouse stem cell factor (SCF), 2 IU/ml of human erythropoietin (EPO) and 10 ng/ml of human vascular endothelial growth factor (VEGF), and maintained at 37°C in humidified air with 5% CO<sub>2</sub>. On day 7 of culture, colonies of 10<sup>3</sup> or more, small, round cells were recorded. The cells composing the colonies were collected and centrifuged onto glass slides (Thermo Electron Corporation, Waltham, USA). After staining with Hemacolor (Merck, Darmstadt, Germany), cells were morphologically examined and specified as neutrophils, macrophages, erythrocytes or megakaryocytes. These colonies were assigned to the category of stroma-dependent hematopoietic colonies, distinguished from hematopoietic colonies directly formed in methylcellulose as described below. After hematopoietic cells were removed, cells adhering to OP-9 cell layers were immunostained with rat anti-mouse CD31 (PECAM1) antibody (BD Biosciences, Franklin Lakes, USA) to identify endothelial cell colonies. Plates were fixed with 4% paraformaldehyde for 15 minutes at room temperature. Cells were treated with 5% donkey serum (Sigma) for 10 minutes at room temperature. After anti-CD31 antibody diluted 1:200 was added, cells were incubated overnight at 4°C. Cy3-conjugated donkey anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, USA) was used

at a 1:500 dilution to detect CD31 expression. Finally, 4',6-diamidino-2-phenylindole (DAPI) (Sigma) was added at 1:1000 and the plates were viewed under an inverted fluorescence microscope (Leica, Wetzlar, Germany).

For limiting-dilution experiments, 96-well plates were used. Diluted concentrations between 0.01 and 0.2 embryo equivalent cells per well, or between 0.01 and 0.06 embryo equivalent cells per well were used for detecting hematopoietic colonies or endothelial colonies. Twenty to 60 wells were prepared for each concentration. The frequencies of hematopoietic and endothelial progenitors were estimated by using L-Calc (StemCell Technologies, Vancouver, Canada).

### Methylcellulose colony assays

One embryo equivalent of cells was plated in 1.2% methylcellulose containing 30% FCS, 1% BSA,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 10 ng/ml IL3, 10 ng/ml TPO, 10 ng/ml SCF, 2 IU/ml EPO and 10 ng/ml VEGF. Cultures were maintained at 37°C in humidified air with 5% CO<sub>2</sub>. Hematopoietic colonies were counted on day 7 of culture, and cells composing colonies were morphologically examined.

In the re-plating experiments, embryonic cell suspensions were first cultured with OP-9 cells in the presence of the cytokines listed above. On day 0 to day 4 of culture, cells were treated with 0.05% trypsin-EDTA in PBS (Sigma) for 3 minutes. After incubation with 10% FCS in  $\alpha$ -MEM, cells were plated into methylcellulose for colony assays, as described above.

### Flow cytometry

Cells from embryos around E7.5 were incubated with phycoerythrin-labeled anti-Flk1, anti-Tie2, or anti-CD31 antibody (e-Bioscience, San Diego, USA) on ice for 30 minutes, followed by washes with 10% FCS in  $\alpha$ -MEM. Propidium iodide was used to exclude dead cells. Analysis and sorting were performed on a MoFlo high-speed cell sorter (Cytomation, Fort Collins, USA). Oct3/4-GFP<sup>+/+</sup> embryos were distinguished from Oct3/4-GFP<sup>-/-</sup> embryos under a fluorescent microscope (Leica), and were used for sorting experiments.

### RT-PCR

Oct3/4-GFP<sup>+</sup> cells and Oct3/4-GFP<sup>-</sup> cells were directly sorted into Eppendorf tubes containing 1.0 ml of Trisol-LS (Invitrogen). Total RNA was extracted in the presence of 2  $\mu$ g of collagen as a carrier, and reverse transcribed into cDNA using ThermoScript RT-PCR System (Invitrogen) and oligo-dT primer, according to the manufacturer's instruction. An aliquot of cDNA was used to measure the relative amount of *Gapdh* signal by real-time (RT) PCR using the ABI-7900 system (Applied Biosystems, Foster City, USA). The volume of cDNA solution containing 10,000 copies of *Gapdh* was determined using TaqMan Rodent *Gapdh* control (Applied Biosystems). Normalized cDNAs were amplified using the following primers:

*Oct3/4*, 5'-ACAACAATGAGAAGTTCAGG-3' and 5'-GTGTCCTGTAGCCTCATACTC-3';

*Sox2*, 5'-ACGCAAAAACCGTGATGCCGAC-3' and 5'-CGTTTGCC-TTAAACAAGACCACG-3';

*Nanog*, 5'-AGGGTCTGCTACTGAGATGCTCTG-3' and 5'-CAAC-CACTGGTTTTTCTGCCACCG-3';

brachyury, 5'-TGCAGTCCATGATAACTGG-3' and 5'-TACTGGCT-GTCAGAAATGTC-3';

*Flk1*, 5'-ATCTCCAGAACAGTAAGCGAAA-3' and 5'-TCCTGAG-TCAGCGTGAA-3'; and

*Gapdh*, 5'-ATTGTCAGCAATGCATCCTGC-3' and 5'-TCATACTTG-CAGGTTTTCTCC-3'.

After RNA extraction from individual hematopoietic colonies, the expression of  $\beta$ -globin genes was similarly examined, without the normalization process, using the following primers:

Beta major globin, 5'-CTGACAGATGCTCTCTTGGG-3' and 5'-CACAACCCAGAAACAGACA-3'; and

Beta H1 globin, 5'-AGTCCCCATGGAGTCAAAGA-3' and 5'-CTC-AAGGAGACCTTTGCTCA-3'.

PCR was carried out using 38 cycles of 95°C for 15 seconds, 56°C for 15 seconds and 72°C for 20 seconds.

## RESULTS

### Frequency of endothelial progenitors or stroma-dependent hematopoietic progenitors in the E7.5 mouse embryo

We first examined cells from embryos around E7.5 (E7.5 cells) for differentiation potential along endothelial and hematopoietic lineages, using the OP-9 co-culture system in the presence of IL3, TPO, EPO, SCF and VEGF. We detected round cells emerging as early as day 2 of culture. These cells tended to proliferate continuously on the stromal cell layer and gave rise to colonies. On day 7 of culture, we counted the number of colonies as stroma-dependent hematopoietic colonies. Such colonies were not seen to emerge after day 7 of culture. Colonies were individually collected from the co-cultures and morphologically examined if they contained neutrophils, macrophages, erythrocytes or megakaryocytes. Most of these colonies turned out to be of the hematopoietic lineage. Representative colonies are shown in Fig. 1A, parts a,b.

Endothelial colonies were almost invisible under an inverted microscope. The identification of such colonies required immunostaining with an anti-CD31 antibody. After staining, cells expressing CD31 were visible as endothelium-like beds and networks on the monolayer of OP-9 cells. Fig. 1A, parts c and d, demonstrates a typical endothelial colony derived from E7.5 cells.

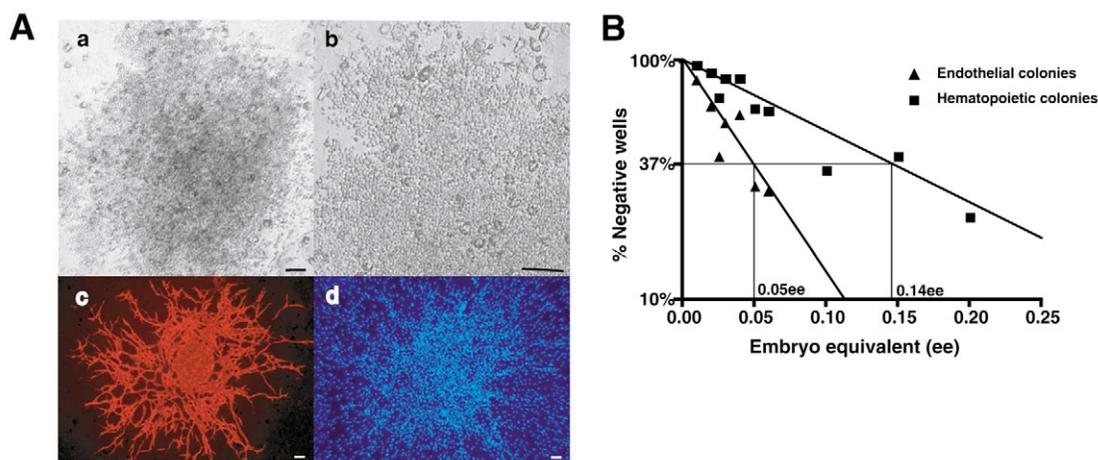
In order to calculate the numbers of endothelial and hematopoietic colonies per embryo around E7.5, we performed a limiting dilution assay. Each dose was assayed in 20 to 60 wells, or more, except for the doses of 0.2 embryo equivalents and 0.15 embryo equivalents, which were assayed in 10 wells. As shown in Fig. 1B, this analysis successfully predicted the frequencies to be on the order of one per 0.05 embryo equivalent for endothelial progenitors and one per 0.14 embryo equivalent for stroma-dependent hematopoietic progenitors. In other words, one embryo around E7.5 has approximately 20 endothelial progenitors and seven stroma-dependent hematopoietic progenitors that can be detected in the OP-9 co-culture system, suggesting that there are about three times more endothelial progenitors than stroma-dependent hematopoietic progenitors at this stage of embryonic development.

### Temporal kinetics of endothelial progenitors, stroma-dependent hematopoietic progenitors and CFU-c in embryos at early gastrulation

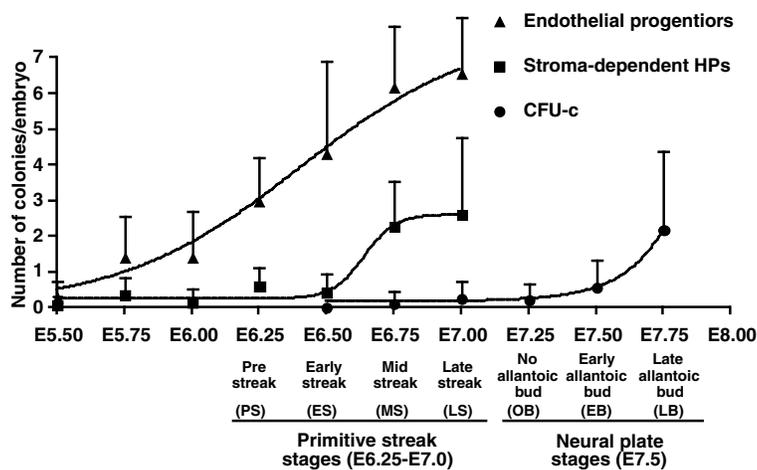
To investigate the kinetics of endothelial and hematopoietic progenitors in embryos from early-stage gastrulation, we performed semi-quantitative assays based on the OP-9 coculture and methylcellulose colony assays. The key factor in these experiments was careful dissection to obtain intact embryos, precisely staged at E5.50 to E7.75 (see Materials and methods). We thus could examine progenitor numbers every 0.25 embryonic days.

Fig. 2 shows the numbers of endothelial progenitors, stroma-dependent hematopoietic progenitors and CFU-c expressed in mean  $\pm$  s.d. per embryo at each stage of the embryos. Endothelial progenitors were detected in E5.50 embryos. At this stage of development, only four endothelial colonies were detectable among a total of 14 embryos examined. The number of endothelial colonies gradually increased thereafter. As a result, from E5.50 to E7.00 the number of endothelial progenitors expanded on average approximately 30-fold. A simple calculation implies that this expansion requires at least four divisions of progenitor cells in 1.5 days. If this is the case, cell cycle progression takes place on average every 9 hours or less.

Stroma-dependent hematopoietic progenitors were also detectable as early as E5.50. However, only one macrophage/erythrocyte colony was formed at this stage ( $n=14$  embryos). Unlike that of endothelial progenitors, the number of stroma-dependent hematopoietic progenitors remained below one per embryo equivalent until E6.50. The number of stroma-dependent hematopoietic progenitors then abruptly increased, with an approximately 6-fold expansion in a half day. Of note is that the ratio of the number of endothelial progenitors to that of hematopoietic progenitors in mid streak (MS)- and late streak (LS)-stage embryos appeared to be around 3.0. The consistency of these results with data obtained by limiting dilution analysis supports the overall accuracy of this semiquantitative assay.



**Fig. 1. Frequency of endothelial or hematopoietic progenitors in E7.5 embryos.** (A) Stroma-dependent hematopoietic colonies (a,b) and vascular endothelial colonies (c,d) are representatively shown. These colonies were formed by cells obtained from E7.5 embryos in co-culture with OP-9 stromal cells. Endothelial colonies were immunostained with anti-CD31 antibody. Red, CD31; blue, DAPI. Scale bar: 100  $\mu$ m. (B) Limiting-dilution analysis of endothelial progenitors and stroma-dependent hematopoietic progenitors in E7.5 embryos. Cells were plated in 96-well plates and were co-cultured with OP-9 stromal cells for 7 days in the presence of IL3, TPO, SCF, EPO and VEGF. The frequency of endothelial progenitors was estimated to be one in 0.05 embryo equivalents. The 95% confidence interval (CI) was 1/38-1/56. The frequency of stroma-dependent hematopoietic progenitors was estimated to be one in 0.14 embryo equivalent, and the 95% CI was 1/115-1/181.



**Fig. 2. Temporal kinetics of endothelial and hematopoietic progenitors in early gastrulating embryos.** The numbers of endothelial and stroma-dependent hematopoietic progenitors, and CFU-c per embryo were measured in precisely staged embryos from E5.5 to E7.75. Five to 14 embryos were examined for endothelial and hematopoietic progenitors at each time point. Seven to 11 embryos were examined for CFU-c at each time point. Data are expressed in mean $\pm$ s.d. per embryo. Sigmoid dose-response curves were used for approximation.

The earliest CFU-c was detected at E6.75, but its frequency was very low, one in eight embryos. As with stroma-dependent hematopoietic progenitors, the number of CFU-c did not significantly increase until E7.25, but showed a marked increase immediately thereafter (Fig. 2). All CFU-c gave rise to macrophage, macrophage/erythrocyte or erythrocyte/megakaryocyte colonies. Representative colonies are shown in Fig. S1A in the supplementary material. RT-PCR analysis of colonies showed that all of these colonies expressed  $\beta$ H1 globin (see Fig. S1B in the supplementary material).

This study clarified the time points for the emergence of endothelial progenitors and stroma-dependent hematopoietic progenitors. Both of these went on to form distinct developmental waves afterwards, followed by the CFU-c developmental wave. Endothelial progenitors appeared in embryos earlier and more frequently than did stroma-dependent hematopoietic progenitors.

### Locations of endothelial and hematopoietic progenitors in the embryo

To examine whether endothelial progenitors and stroma-dependent hematopoietic progenitors are located in a particular anatomical region of the developing mouse embryo, we carefully dissected E7.75 late bud stage embryos into the YS, allantois and embryo proper. As shown in Table 1, endothelial colonies were formed by all three of these parts of the embryo. The allantois contained significantly fewer endothelial progenitors than did the other parts. Stroma-dependent hematopoietic colonies were detected in the YS and embryonic proper, but not in the allantois. These data indicate that vasculogenic and hematopoietic activities are not restricted to the YS, although the allantois has little such activities, in the E7.75 embryo.

### Re-plating to methylcellulose of OP-9 co-cultured embryonic cells

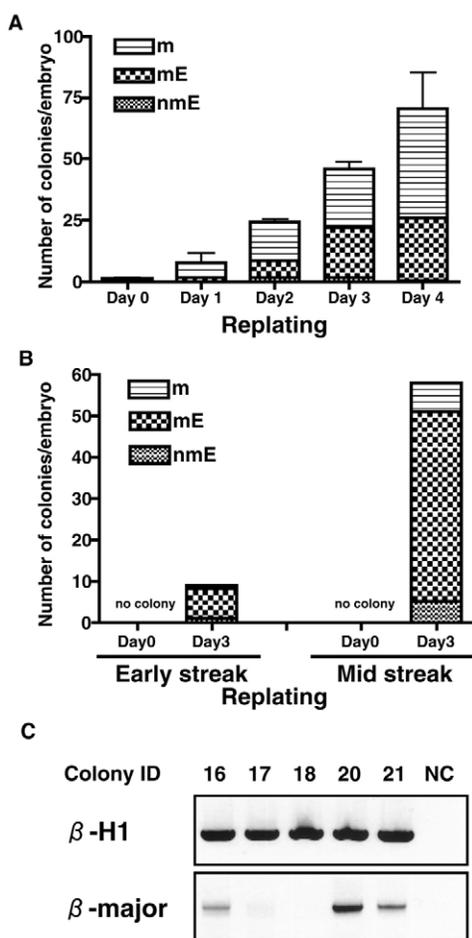
In order to study the relationship between stroma-dependent hematopoietic progenitors detected by the OP-9 coculture system and CFU-c detected by methylcellulose colony assay, we postulated that like long-term culture-initiating cells (Dexter et al., 1977; Lemieux et al., 1995), stroma-dependent hematopoietic progenitors from embryos around or before E7.5 need contact with OP-9 cells to become CFU-c. To verify this idea, one embryo-equivalent of E7.5 cells were seeded on OP-9 cells per well. On day 0, without culture, all cells in a well were transferred into the methylcellulose colony assay. On day 1, 2, 3 or 4 of co-culture, cells recovered from OP-9 co-cultures were plated into the methylcellulose colony assay. As shown in Fig. 3A, the number of CFU-c steadily increased along with the time in co-culture with OP-9 cells. OP-9 cells together with a combination of cytokines probably promoted the differentiation and/or maturation of hematopoietic progenitors into the CFU-c stage. The number of CFU-c showed about a 75-fold expansion by 4 day of culture. Most of these colonies were macrophage or macrophage/erythrocyte colonies.

It was possible that a small number of CFU-c level cells present in embryos formed secondary CFU-c in co-cultures with OP-9 cells and contributed to an expansion of CFU-c. To address this issue, we specifically selected ES- and MS-stage embryos for examination by re-plating assay, assuming that embryonic cells at these stages would not directly give rise to in vitro colonies. As expected, no colony was detected without the co-culture (Fig. 3B). After 3 days of co-culture, cells from both ES- and MS-stage embryos formed macrophage, macrophage/erythrocyte, and neutrophil/macrophage/erythrocyte colonies. Interestingly, more CFU-c were generated from MS-stage embryonic cells than from ES-stage embryonic cells. We then

**Table 1. Anatomical locations of endothelial and hematopoietic progenitors in E7.75 embryos**

Experiment (number of embryos)	Yolk sac		Allantois		Embryo proper	
	Endothelial colonies	Hematopoietic colonies	Endothelial colonies	Hematopoietic colonies	Endothelial colonies	Hematopoietic colonies
1 (5)	11 $\pm$ 2.2	6.0 $\pm$ 1.0	0.40 $\pm$ 0.55	0 $\pm$ 0	7.6 $\pm$ 2.5	1.80 $\pm$ 1.10
2 (5)	19 $\pm$ 3.1	8.8 $\pm$ 2.8	1.80 $\pm$ 1.50	0 $\pm$ 0	23.0 $\pm$ 5.6	1.20 $\pm$ 1.10
3 (3)	17 $\pm$ 5.6	6.3 $\pm$ 1.5	0.67 $\pm$ 1.20	0 $\pm$ 0	17.0 $\pm$ 1.0	0.34 $\pm$ 0.58

LB-stage (E7.75) embryos were dissected into the yolk sac, allantois or embryo proper by using tungsten needles. Single cells from each part were co-cultured with OP-9 cells in the presence of a combination of cytokine factors for 7 days. The numbers of endothelial and hematopoietic colonies formed are expressed as the mean $\pm$ s.d. per embryo.



**Fig. 3. Replating of embryonic cells into methylcellulose.** (A) E7.5 cells per embryo were co-cultured with OP-9 cells in the presence of IL3, TPO, SCF, EPO and VEGF. On day 0, 1, 2 or 4, after treatment with trypsin, single cells were replated into methylcellulose with IL3, TPO, SCF, EPO and VEGF. Following 7 days of further culture, colonies formed were picked up. Cytospin preparations were made and were stained with Hemacolor. This experiment used a total of 20 E7.5 embryos (three MS-, four LS-, seven OB-, three EB-, three LB-stage embryos). (B) Data from a total of four embryos are shown. Two embryos were of ES stage and two embryos were of MS stage. nmE, neutrophil/macrophage/erythrocyte; mE, macrophage/erythrocyte; m, macrophage. (C) RT-PCR analysis for  $\beta$ H1 and  $\beta$ -major globin expression in individual hematopoietic colonies that were formed after contact with OP-9 cells for 3 days.

examined globin expression in erythroid colonies. As is representatively shown in Fig. 3C, most colonies expressed  $\beta$ H1 globin.

We suggest that like the *in vitro* generation of CFU-c from hematopoietic progenitors via contact with OP-9 cells, stroma-dependent hematopoietic progenitors or hemangioblasts *in vivo* establish primitive hematopoiesis after interaction with their microenvironment (Figs 2, 3).

### Endothelial and hematopoietic differentiation potential in Flk1<sup>+</sup>, Tie2<sup>+</sup>, or CD31<sup>+</sup> E7.5 cells

To characterize endothelial and hematopoietic progenitors, these cells need to be highly purified from embryos. We were first interested in two tyrosine receptor kinases, Flk1 and Tie2, as positive

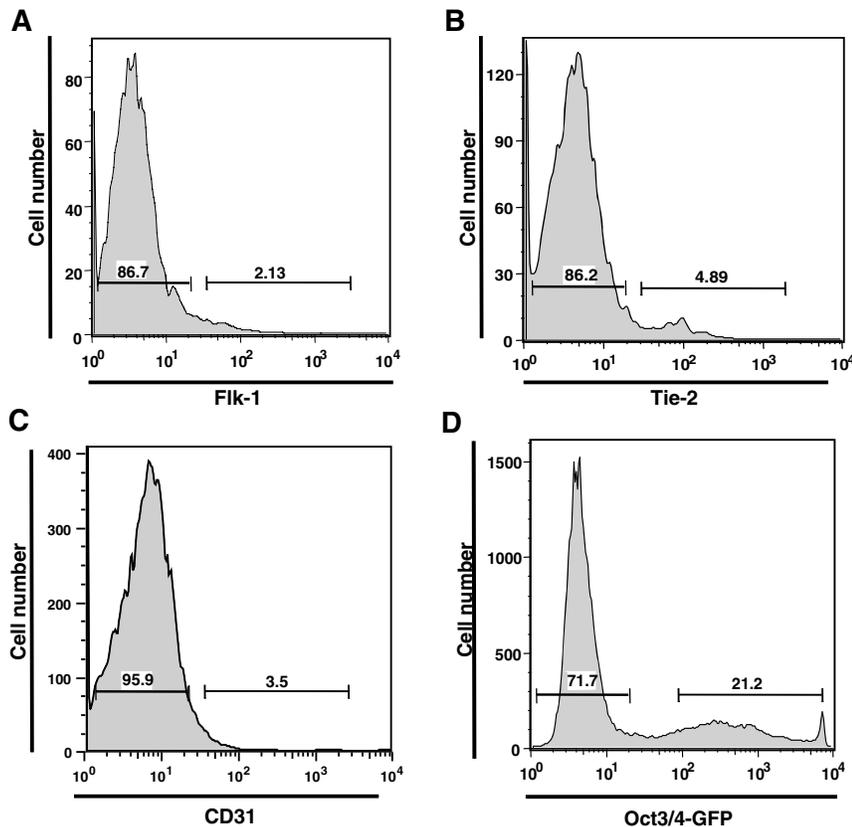
markers, because these molecules were reportedly expressed on both endothelial and hematopoietic progenitors or on hemangioblasts (Faloon et al., 2000; Huber et al., 2004; Kabrun et al., 1997; Nishikawa et al., 1998; Takakura et al., 1998; Yamashita et al., 2000). When E7.5 cells were stained with anti-Flk1 or anti-Tie2 antibody, and analyzed by flow cytometry, approximately 6% or 3% of the cells showed expression of Flk1 or Tie2, respectively (Fig. 4A,B). Flk1<sup>-</sup> and Flk1<sup>+</sup> cells were simultaneously sorted by flow cytometry, and were cultured with OP-9 cells in the presence of a combination of cytokines to compare their potentials for differentiation along endothelial and hematopoietic lineages. As shown in Table S1 in the supplementary material, in two experiments both endothelial and stroma-dependent hematopoietic progenitors were detected in Flk1<sup>+</sup> cells, but not in Flk1<sup>-</sup> cells. However, in the following three experiments a few endothelial or stroma-dependent hematopoietic progenitors were also detectable among Flk1<sup>-</sup> cells, whereas Flk1<sup>+</sup> cells consistently exhibited both differentiation potentials. Overall, the frequency of endothelial progenitors among Flk1<sup>+</sup> E7.5 cells was calculated as being in the range of one in 27-74 cells. The frequency of stroma-dependent hematopoietic progenitors among Flk1<sup>+</sup> E7.5 cells was in the range of one in 114-398 cells.

Tie2<sup>-</sup> and Tie2<sup>+</sup> E7.5 cells were also separated by flow cytometry, and their differentiation potentials were compared (see Table S2 in the supplementary material). While endothelial and stroma-dependent hematopoietic progenitors were detected in both the Tie2<sup>-</sup> and the Tie2<sup>+</sup> fractions, Tie2<sup>+</sup> cells were significantly enriched in such progenitors. The frequency of endothelial progenitors among Tie2<sup>+</sup> cells was estimated as being in the range of one in 18-89 cells; that of stroma-dependent hematopoietic progenitors was in the range of one in 38-46 cells. These results show that Flk1 and Tie2 can be used as positive markers for selecting endothelial and stroma-dependent hematopoietic progenitors, but selection for neither Flk1 nor Tie2 alone can completely isolate these progenitors.

After staining with anti-CD31 antibody, E7.5 cells were analyzed by flow cytometry. As shown in Fig. 4C, approximately 4% of the cells showed expression of CD31. CD31<sup>-</sup> and CD31<sup>+</sup> E7.5 cells were separately examined (Table 2). Interestingly, CD31<sup>-</sup> cells gave rise to only the endothelial lineage, whereas CD31<sup>+</sup> cells gave rise to endothelial and hematopoietic lineages. The frequency of endothelial progenitors among CD31<sup>-</sup> cells was estimated as being in the range of one in 90-865 cells. The frequency of endothelial progenitors among CD31<sup>+</sup> cells was estimated as being in the range of one in 15-83 cells; that of stroma-dependent hematopoietic progenitors was in the range of one in 29-237 cells.

### Endothelial and hematopoietic differentiation potential in Oct3/4<sup>+</sup> E7.5 cells

We thought that Oct3/4 might be useful as another marker to isolate these two types of progenitors because hematopoietic progenitors and PGCs have much in common (Ginsburg et al., 1990; McLaren, 1992; Rich, 1995). We used transgenic mice engineered to express green fluorescent protein (GFP) under the control of an *Oct3/4* promoter (Yoshimizu et al., 1999) to examine this possibility. As shown in Fig. S2 (see the supplementary material), Oct3/4-GFP<sup>+/+</sup> E7.75 embryos expressed GFP in the epiblast, the primitive streak, the allantois and restricted regions of the yolk sac. Flow cytometric analysis of Oct3/4-GFP<sup>+/+</sup> embryos revealed that approximately 40% of E7.5 cells expressed GFP (Fig. 4D). GFP<sup>-</sup> and GFP<sup>+</sup> fractions of the cells were sorted and cultured. As shown in Table 3, all endothelial and hematopoietic colonies were formed by GFP<sup>+</sup> cells; none was formed by GFP<sup>-</sup> cells. The frequency of endothelial



**Fig. 4. Expression of Flk1, Tie2, CD31 or Oct3/4-GFP in E7.5 embryos.** Representative flow cytometric analyses of pooled embryonic cells around E7.5 are shown. (A) Cells stained with an anti-Flk1 antibody.  $6.0 \pm 11.1\%$  (mean  $\pm$  s.d.,  $n=9$ ) of the cells showed expression of Flk1. (B) Cells stained with an anti-Tie2 antibody.  $3.0 \pm 2.7\%$  (mean  $\pm$  s.d.,  $n=4$ ) of the cells showed expression of Tie2. (C) Cells stained with an anti-CD31 antibody.  $4.3 \pm 3.8\%$  (mean  $\pm$  s.d.,  $n=5$ ) of the cells showed expression of CD31. (D)  $39.0 \pm 25.3\%$  (mean  $\pm$  s.d.,  $n=6$ ) of the cells from Oct3/4-GFP<sup>+/+</sup> embryos expressed GFP.

progenitors in GFP<sup>+</sup> cells was calculated as being in the range of one in 104-344 cells, and that of hematopoietic progenitors was in the range of one in 257-1303 cells.

We next sought to determine whether CFU-c express Oct3/4 by using methylcellulose colony assays. As shown in Table S3 in the supplementary material, CFU-c were detected in GFP<sup>+</sup> cells, but not in GFP<sup>-</sup> cells. The frequency of CFU-c in GFP<sup>+</sup> cells was calculated as being in the range of one in 75-122 cells.

To verify endogenous expression of the *Oct3/4* gene in sorted GFP<sup>+</sup> cells, RT-PCR analysis was performed on GFP<sup>+</sup> or GFP<sup>-</sup> E7.5 cells. As shown in Fig. S3 in the supplementary material, expression of the *Oct3/4* gene was detected in GFP<sup>+</sup> cells, but not in GFP<sup>-</sup> cells. Interestingly, expression of *Nanog* and brachyury was similarly detected in GFP<sup>+</sup> cells. Because expression of *Sox2* was also detected, *Nanog* may be transcriptionally regulated by Oct3/4 and *Sox2* in these cells, as has been shown for ES cells (Rodda et al., 2005). Expression of brachyury and *Flk1* in GFP<sup>+</sup> cells suggests that BL-CFCs are included in these cells (Huber et al., 2004).

Flow cytometry analysis revealed that  $3.0 \pm 2.4\%$  (mean  $\pm$  s.d.,  $n=3$ ) of Oct3/4-GFP<sup>+</sup> E7.5 cells expressed CD31 (data not shown). Oct3/4-GFP<sup>+</sup>CD31<sup>-</sup> cells and Oct3/4-GFP<sup>+</sup>CD31<sup>+</sup> cells were sorted from E7.5 cells by flow cytometry and were examined for differentiation potentials by the OP-9 co-culture system in three independent experiments (Table 4). In one of the four cultures from one experiment, one stroma-dependent hematopoietic colony and 28 endothelial colonies were formed by Oct3/4-GFP<sup>+</sup>CD31<sup>-</sup> cells. In all remaining cultures, Oct3/4-GFP<sup>+</sup>CD31<sup>-</sup> cells, as expected, gave rise to only endothelial colonies and not to stroma-dependent hematopoietic colonies. By contrast, Oct3/4-GFP<sup>+</sup>CD31<sup>+</sup> cells gave rise to both endothelial and hematopoietic colonies. The frequency of endothelial progenitors among Oct3/4-GFP<sup>+</sup>CD31<sup>-</sup> cells was estimated as being in the range of one in 238-349 cells. The frequency of endothelial progenitors among Oct3/4-GFP<sup>+</sup>CD31<sup>+</sup> cells was estimated as being in the range of one in 16-23 cells; that of stroma-dependent hematopoietic progenitors was in the range of one in 55-165 cells.

**Table 2. Isolation of endothelial and hematopoietic progenitors based on the expression of CD31**

Experiment	CD31 negative		CD31 positive	
	Endothelial colonies	Hematopoietic colonies	Endothelial colonies	Hematopoietic colonies
1	25	0/2252	3	2/249
2	22	0/19,037	32	2/474
3	26	0/8442	20	5/302
4	28	0/12,843	14	9/258
5	45	0/7135	7	1/180

Cells from embryos around E7.5 were separated into either CD31<sup>-</sup> or CD31<sup>+</sup> cells by flow cytometry. They were cultured with OP-9 cells in the presence of a combination of cytokine factors for 7 days. The numbers of endothelial or hematopoietic colonies per the number of cells plated are shown. A total of 58 embryos was used: 2 OB-, 6 EB- and 1 OB-stage embryo for experiment 1; 3 LS-, 3 OB-, 5 EB- and 3 LB-stage embryos for experiment 2; 1 LS-, 3 OB-, 1 EB- and 1 LB-stage embryo for experiment 3; 4 LS-, 4 OB-, 6 EB-, 4 LB- and 1 EHF-stage embryo for experiment 4; 4 LS-, 6 OB-, 2 EB- and 1 LB-stage embryo for experiment 5.

**Table 3. Isolation of endothelial and hematopoietic progenitors based on the expression of Oct3/4**

Experiment	Oct3/4-GFP negative		Oct3/4-GFP positive	
	Endothelial colonies	Hematopoietic colonies	Endothelial colonies	Hematopoietic colonies
1	0	0/6359	18	4/1877
2	0	0/4762	15	3/3908
3	0	0/9303	13	3/4466
4	0	0/24,987	39	13/10,009

Cells from Oct3/4-GFP<sup>+/+</sup> embryos around E7.5 were separated into either GFP<sup>-</sup> or GFP<sup>+</sup> cells by flow cytometry. They were cultured with OP-9 cells in the presence of a combination of cytokine factors for 7 days. The numbers of endothelial or hematopoietic colonies per the number of cells plated are shown. A total of 20 embryos was used: 1 LS-, 1 OB- and 2 LB-stage embryos for experiment 1; 1 LS- and 2 LB-stage embryos for experiment 2; 2 MS-, 1 LS-, 2 OB- and 2 LB-stage embryos for experiment 3; and 1 LS-, 1 OB- and 4 LB-stage embryos for experiment 4.

## DISCUSSION

Specific staging and delicate dissecting of mouse embryos, with a detection system using OP-9 cells, permitted quantitative evaluation of vasculogenesis and hematopoiesis every 0.25 embryonic days from E5.50 to E7.75. Three distinct waves, consisting of endothelial progenitors, stroma-dependent hematopoietic progenitors and CFU-c, were revealed for early embryonic stages (Fig. 2). The endothelial progenitors, supposedly equivalent to angioblasts that do not accompany hematopoiesis, appeared first at E5.50. The stroma-dependent hematopoietic developmental wave followed after approximately one day. Huber et al. have recently reported that hemangioblasts become detectable as BL-CFC at the midstream stage (E6.75) (Huber et al., 2004). Interestingly, the present study detected, at exactly the same time, a significant increase in the number of stroma-dependent hematopoietic progenitors. Hematopoietic activity of the stroma-dependent hematopoietic progenitors is likely to belong to the hemangioblasts at this stage. The third developmental wave was formed by CFU-c whose developmental potential was mostly restricted to macrophage, erythrocyte and megakaryocyte lineages. The number of CFU-c began to increase at E7.50. This is the time when YS hematopoiesis occurs in the neural plate stages. Stroma-dependent hematopoietic progenitors were able to give rise to CFU-c via contact with OP-9 cells after an interval as short as one day (Fig. 3). Based on the morphological analysis of erythrocytes and globin gene expression analysis, stroma-dependent hematopoietic progenitors together with CFU-c appear to represent primitive hematopoiesis in this time window of development.

The identification and characterization of angioblasts and hemangioblasts is one of the most crucial issues yet to be solved in the field of developmental hematopoiesis. To address this issue, these two types of progenitors need to be individually isolated and analyzed at the clonal level. High degrees of cell purification are essential for this purpose. Flk1<sup>+</sup> and Tie2<sup>+</sup> cells were significantly enriched in both endothelial and hematopoietic progenitors among E7.5 embryos. However, sorting for neither Flk1 nor Tie2 alone

could completely isolate these progenitors. Conversely, Oct3/4 expression exclusively marked all early endothelial progenitors, stroma-dependent hematopoietic progenitors, and CFU-c in E7.5 embryos (Table 3, see also Table S3 in the supplementary material). By E11.5, Oct3/4-GFP<sup>+</sup> cells were only found in the AGM region, whereas other hematopoietic sites, such as the YS and the fetal liver, did not express Oct3/4 (C.F., unpublished). Because endothelial and hematopoietic progenitors were detected in Oct3/4-GFP<sup>-</sup> AGM cells, it is likely that only PGCs remain to express Oct3/4 by this stage of development. In this regard, Oct3/4 can be a marker for early vasculogenesis and primitive hematopoiesis, although the role of Oct3/4 in endothelial and hematopoietic progenitors remains to be determined. Nonetheless, it is interesting to know whether Oct3/4 expression distinguishes primitive hematopoiesis from definitive hematopoiesis or not because hemangioblasts have been reported to generate primitive and definitive erythropoiesis (Huber et al., 2004).

This study showed that CD31 is a candidate marker to separate hemangioblasts from angioblasts. CD31 expression was examined because it is expressed in vascular endothelial cells (Newman, 1994), endothelial progenitors (Redick and Bautch, 1999) and HSCs (Baumann et al., 2004). We observed that in E7.5 embryos the CD31<sup>+</sup> fraction was significantly enriched in both endothelial and hematopoietic progenitors (Table 2). Interestingly, a subset of endothelial progenitors remained in the CD31<sup>-</sup> fraction (Table 2). As expected, among Oct3/4-positive cells, both endothelial and hematopoietic progenitors were detected in the CD31<sup>+</sup> fraction, and only endothelial progenitors were detected in the CD31<sup>-</sup> fraction in most cases. The Oct3/4-GFP signal was stronger in the CD31<sup>-</sup> fraction than in the CD31<sup>+</sup> fraction (data not shown). Because the internal cell mass and primitive streak show strong signal for Oct3/4-GFP, these data imply that downregulation of Oct3/4 expression is associated with upregulation of CD31 expression. Our interpretation is that Oct3/4<sup>+</sup>CD31<sup>-</sup> mesoderm gives rise to Oct3/4<sup>+</sup>CD31<sup>-</sup> angioblasts or Oct3/4<sup>+</sup>CD31<sup>+</sup> hemangioblasts. However, angioblasts should also be present in Oct3/4<sup>+</sup>CD31<sup>+</sup> cells because the frequency of endothelial progenitors was greater than that of stroma-dependent

**Table 4. Isolation of endothelial and hematopoietic progenitors based on the expression of Oct3/4 and CD31**

Experiment	Oct3/4-GFP positive, CD31 negative		Oct3/4-GFP positive, CD31 positive	
	Endothelial colonies	Hematopoietic colonies	Endothelial colonies	Hematopoietic colonies
1	43	0/15,021	12	2/196
2	52	0/15,725	15	6/330
	48	0/15,725	19	2/330
3 (n=4)	24±3.1	0.25±0.50/5717	14±1.5	2.3±2.6/327

GFP<sup>+</sup> cells from Oct-3/4-GFP transgenic E7.5 embryos were separated into either CD-31<sup>-</sup> or CD-31<sup>+</sup> cells by flow cytometry. They were cultured with OP-9 cells in the presence of a combination of cytokine factors for 7 days. The numbers of endothelial or hematopoietic colonies per the number of cells plated are shown. A total of 34 embryos was used: 3 LS-, 2 OB-, 1 EB- and 1 LB-stage embryo for experiment 1; 4 LS-, 9 OB-, 4 EB- and 1 LB-stage embryo for experiment 2; 1 OB-, 7 LB- and 1 EHF-stage embryo for experiment 3.

hematopoietic progenitors among this population. It was difficult to locate Oct3/4<sup>+</sup>CD31<sup>-</sup> cells or Oct3/4<sup>+</sup>CD31<sup>+</sup> cells in embryos. However, given that CD31 is expressed in the allantois and the YS (Redick and Bautch, 1999), and that there is little hematopoietic activity in the allantois (Table 1), Oct3/4<sup>+</sup>CD31<sup>+</sup> cells may reside mainly in the YS. Of interest is to know whether Oct3/4<sup>+</sup>CD31<sup>-</sup> cells and Oct3/4<sup>+</sup>CD31<sup>+</sup> cells play distinctive roles in embryonic vasculogenesis.

BL-CFCs were isolated from the brachyury<sup>+</sup>Fli1<sup>+</sup> fraction in the E7.5 embryo (Huber et al., 2004). RT-PCR revealed that Oct3/4<sup>+</sup> cells expressed brachyury and *Fli1* (see Fig. S3 in the supplementary material), supporting the notion that hemangioblasts exist in this population. We have attempted to detect single angioblasts in the Oct3/4<sup>+</sup>CD31<sup>-</sup> population and single hemangioblasts in the Oct3/4<sup>+</sup>CD31<sup>+</sup> population from E7.5 embryos. Unfortunately, we have so far not succeeded in this sort of clonal analysis. The frequency of hemangioblasts in Oct3/4<sup>+</sup>CD31<sup>+</sup> cells seems too low to permit hemangioblast detection. In addition, the OP-9 co-culture system seems not to be sensitive enough to detect them at the clonal level.

It seems widely accepted that hemangioblasts are the first generation of endothelial- and hematopoietic-lineage cells. This study, on the contrary, suggests that angioblasts diverge from mesoderm prior to the appearance of hemangioblasts; thus before the onset of primitive hematopoiesis. This manner of vasculogenesis and hematopoiesis resembles that in avian aorta formation (Pardanaud et al., 1996), and somewhat resembles that in mouse cardiovascular development (Hatzopoulos et al., 1998). Studies of the developmental fate of embryonic cells have suggested that primitive hematopoiesis takes place before and independently of endothelial development in the mouse YS (Kinder et al., 1999). This study supports the independent emergence of endothelial- and hematopoietic-lineage cells, but indicates that endothelial lineage begins to develop earlier than hematopoietic lineage.

Hemangioblasts can be defined as one of the subsets of so-called hematogenic endothelial cells (Smith and Glomski, 1982). These are endothelial-like progenitors that developmentally acquire hematopoietic activity in proper microenvironments (Fraser et al., 2003). It remains uncertain to what extent hematogenic endothelial cells contribute to vasculogenesis, particularly after mid-gestational stages. It is possible that the hematopoietic activity of hematogenic endothelial cells may increase as vasculogenic potential declines. Once integrated into vascular structures, these cells may play only a small role in further vasculogenesis. Alternatively, after vasculogenic potential is lost, these hematogenic endothelial cells may not be necessarily co-localized with endothelium (Bertrand et al., 2005).

We postulated that hematogenic endothelial cells might have the potential to give rise to HSCs. We transplanted two embryo equivalents of E7.5 whole embryos into lethally irradiated adult mice along with 2 × 10<sup>5</sup> competitor total bone marrow cells by either intravenous (*n*=12) or intramedullary (*n*=7) routes. In both transplantation experiments, we could not detect long-term reconstitution. But transient and low level reconstitution (<0.5% chimerism) was observed 8 weeks post-transplantation in one mouse transplanted by vein and in two mice transplanted via the bone marrow (data not shown). Perhaps the environment in the bone marrow of irradiated adult mice is not appropriate for homing and proliferation of embryonic HSCs in E7.5 embryos, even if HSCs exist in such embryos. Fraser et al. attempted to reconstitute busulfan-treated newborn mice with E8.5-E10.5 embryonic cells (Fraser et al., 2002). Low-level long-term reconstitution was

observed after transplantation with E9.5 or E10.5 embryonic cells, but not with E8.5 cells. Hematogenic endothelium presumably needs to mature to a certain stage for developing HSCs to be transplantable. More suitable assays for embryonic HSCs, such as in utero transplantation, should be attempted (Yoder, 2004).

This study establishes a simple framework to investigate the mechanisms underlying the development of vascular and hematopoietic lineages in mammals. We finally propose that a distinctive developmental pathway exists in which the angioblast lineage directly diverges from mesoderm prior to and independently of hemangioblast development.

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/14/2771/DC1>

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**Table S1. Isolation of endothelial and hematopoietic progenitors based on the expression of Flk1**

Experiment	Flk1 negative		Flk1 positive	
	Endothelial colonies	Hematopoietic colonies	Endothelial colonies	Hematopoietic colonies
1	0	0/33,612	14	2/382
2	0	0/2300	11	4/813
3	1	0/21,408	13	2/795
4	0	1/21,408	15	7/795
5	2	1/27,335	15	5/624

Cells from embryos around E7.5 were separated into either Flk1<sup>-</sup> or Flk1<sup>+</sup> cells by flow cytometry. They were cultured with OP-9 cells in the presence of a combination of cytokine factors for 7 days. The numbers of endothelial or hematopoietic colonies per the number of cells plated are shown. A total of 99 embryos was used: 10 LS-, 4 OB- and 29 LB-stage embryos for experiment 1; 3 OB-, 1 EB-, 9 LB- and 4 EHF-stage embryos for experiment 2; 1 MS-, 4 LS-, 5 OB-, 1 EB- and 14 LB-stage embryos for experiments 3 and 4; and 1 ES-, 1 MS-, 1 LS-, 4 OB-, 3 EB- and 4 LB-stage embryos for experiment 5.

**Table S2. Isolation of endothelial and hematopoietic progenitors based on the expression of Tie2**

Experiment	Tie2 negative		Tie2 positive	
	Endothelial colonies	Hematopoietic colonies	Endothelial colonies	Hematopoietic colonies
1	1	0/13,850	4	0/357
2	4	1/12,949	5	2/91
3	23	2/5988	16	11/413

Cells from embryos around E7.5 were separated into either Tie2<sup>-</sup> or Tie2<sup>+</sup> cells by flow cytometry. They were cultured with OP-9 cells in the presence of a combination of cytokine factors for 7 days. The numbers of endothelial or hematopoietic colonies per the number of cells plated are shown. A total of 33 embryos was used: 3 ES-, 2 MS-, 1 LS-, 4 OB- and 5 LB-stage embryos for experiment 1; 2 ES-, 3 MS-, 3 LS- and 1 EB-stage embryo for experiment 2; 3 OB-, 2 EB- and 4LB-stage embryos for experiment 3.

**Table S3. Presence of CFU-c in Oct3/4-GFP<sup>+</sup> E7.5 cells**

Experiment	Number of CFU-c (mean±s.d.)/cells	
	Oct3/4-GFP negative	Oct3/4-GFP positive
1	26±5.1/3220	0±0/1071
2	27±2.9/2021	0±0/667
3	29±1.2/2953	0±0/962

Cells from Oct3/4-GFP transgenic E7.5 embryos were separated into either GFP<sup>-</sup> or GFP<sup>+</sup> cells by flow cytometry. They were cultured with methylcellulose in the presence of a combination of cytokine factors for 7 days. The numbers of CFU-c are shown in mean±s.d. (*n*=3). A total of 27 embryos was used: 2 LS-, 3 OB-, 1 EB and 1 LB-stage embryo for experiment 1; 2 LS-, 1 OB-, 3 EB and 1 LB-stage embryo for experiment 2; 2 LS-, 4 OB-, 2 EB and 5 LB-stage embryos for experiment 3.