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Lineage analysis of the hemangioblast as defined by FLK1 and SCL expression

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SUMMARY

Accumulating studies support the idea that a common progenitor, termed the hemangioblast, generates both hematopoietic and endothelial cell lineages. To better define the relationship between these cell lineages, we have generated knock-in embryonic stem (ES) cells carrying a non-functional human CD4 at the *Scl* locus. By using in vitro differentiated *Scl*$^{+/CD4}$ ES cells, we demonstrate that FLK1 and SCL are molecular determinants of the hemangioblast. Furthermore, our studies demonstrate that hematopoietic and endothelial cells develop via distinct, sequential generation of FLK1 and SCL-expressing cells. FLK1$^+$CD4$^-$ cells first arise in developing embryoid bodies. The *Scl* gene is turned on within FLK1$^+$CD4$^-$ cells to give rise to FLK1$^+$CD4$^+$ cells. Alternatively, a subpopulation of the initial FLK1$^+$CD4$^-$ cells remains as SCL negative. Within the FLK1$^+$CD4$^+$ cells, FLK1 is down regulated to generate FlLK1$^-$CD4$^+$ cells. Replating studies demonstrate that hematopoietic progenitors are enriched within FLK1$^+$CD4$^+$ and FLK1$^-$CD4$^+$ cells, while endothelial cells develop from FLK1$^+$CD4$^+$ and FLK1$^+$CD4$^-$ cell populations.

Key words: Hemangioblast, Hematopoiesis, Vasculogenesis, FLK1, SCL, Mouse

INTRODUCTION

Studies over the past 100 years have shown that blood cells develop in close proximity to the vascular system during embryogenesis. In mice, mesodermal cells, which have migrated from the primitive streak, form aggregates to establish blood islands in the yolk sac at around embryonic day 7 (E7). Over the next 12 hours, the central cells within the blood islands give rise to primitive blood cells, while the peripheral cells differentiate into endothelial cells. These blood islands subsequently fuse to form the first extra-embryonic vascular network. The close developmental association of the hematopoietic and endothelial cell lineages within the yolk sac of the developing embryo has led to the hypothesis that they arise from a common precursor, the hemangioblast (Sabin, 1920; Murray, 1932; Wagner, 1980).

Gene-targeting studies have demonstrated that FLK1 (Kdr – Mouse Genome Informatics), a receptor tyrosine kinase, and SCL, a basic helix-loop-helix transcription factor, are crucial for hematopoietic and endothelial cell development. In mouse embryos, *Flk1* expression can be detected in the presumptive mesodermal yolk sac blood island progenitors as early as E7 (Yamaguchi et al., 1993; Dumont et al., 1995). Mice deficient in FLK1 do not develop blood vessels or yolk sac blood islands, and die between E8.5 and E9.5 (Shalaby et al., 1995). In chimeric aggregation studies with wild-type embryos, *Flk1*$^{-/-}$ ES cells fail to participate in vessel formation or to contribute to primitive or definitive hematopoiesis, suggesting that *Flk1* inactivation results in cell autonomous endothelial and hematopoietic defects (Shalaby et al., 1997). Mice carrying homozygous mutations at the *Scl* locus die at around E10.5 because of defective embryonic hematopoiesis (Shivdasani et al., 1995; Robb et al., 1995). The requirement of SCL in adult hematopoietic system has been shown in chimeric mice generated by injecting *Scl*$^{-/-}$ ES cells into the wild-type blastocysts (Porcher et al., 1996). None of the hematopoietic cells in these chimeric mice developed from *Scl*$^{-/-}$ ES cells, suggesting a functional role for SCL in adult hematopoiesis. Subsequent studies have shown that vasculogenesis in the *Scl*$^{-/-}$ yolk sac occurs normally, but that remodeling of the primary vascular plexus is defective (Visvader et al., 1998; Elefanty et al., 1999).

The in vitro differentiation model of ES cells has proven to be valuable for studies of cell lineage development. Hematopoietic cells develop within embryoid bodies (EBs, in vitro differentiated ES cells) faithfully following in vivo developmental kinetics (Kennedy et al., 1997; Choi et al., 1998; Palis et al., 1999). As in the developing embryo, primitive erythroid cells develop prior to definitive hematopoietic populations (Keller et al., 1993; Palis et al., 1999). Endothelial

cells within EBs also follow similar kinetics, in that they develop from FLK1$^+$ mesodermal cells (Vittet et al., 1996; Nishikawa et al., 1998; Nishikawa, 2001). By using in vitro differentiated ES cells, we previously identified blast colony forming cells (BL-CFCs) as a long pursued common progenitor of hematopoietic and endothelial cells, the hemangioblast (Choi et al., 1998) (reviewed by Choi, 2002). More importantly, BL-CFCs are a transient cell population: they develop prior to primitive erythroid cells, are most abundant in day 2.75-3.25 EBs and disappear shortly thereafter.

We have previously demonstrated that BL-CFCs expressed FLK1 (Faloon et al., 2000) and that *Scl*$^{-/-}$ EBs failed to generate blast colonies, the progeny of BL-CFCs, in vitro (Faloon et al., 2000; Robertson et al., 2000). These studies suggest that SCL is crucial for hemangioblast development and that the hemangioblast can be identified as the FLK1$^+$SCL$^+$ cell population. To understand better the relationship between FLK1 and SCL expression in the differentiation of hematopoietic and endothelial cell lineages, we have introduced a non-functional human CD4 gene (*CD4*) encoding extracellular and transmembrane domains into one allele of *Scl*. This strategy allows us to detect SCL-expressing cells by using monoclonal antibodies against human CD4 and flow cytometry analyses. Kinetic analyses of FLK1 and human CD4 expression of in vitro differentiated *Scl*$^{+/CD4}$ ES cells and cell sorting experiments for hemangioblast, hematopoietic and endothelial cells demonstrated that hematopoietic and endothelial cells developed via the sequential generation of FLK1- and SCL-expressing cells.

In this paper, CD4 refers to the non-functional human CD4 gene/protein.

MATERIALS AND METHODS

Construction of the *Scl*-human CD4 knock-in targeting vector and isolation of the knock-in clones

The 5′ homology arm containing ~7.4 kb *Bam*HI to *Not*I DNA fragment was isolated from the *Scl* 2A genomic clone. The extracellular and transmembrane domain of the human CD4 (*CD4*) gene (kindly provided by Dr Kenneth Murphy at Washington University) was knocked-in in-frame into the *Not*I site of the exon IV. This *Not*I site is 10 nucleotides downstream of the initiation codon. The 3′ homology arm containing ~4 kb *Not*I to *Sal*I DNA fragment was isolated from the *Scl* 2A genomic clone. The 3′ arm was first blunt ligated into the *Xho*I site located downstream of the PGK-neomycin cassette of the pLNTK. The 5′ homology-CD4 fragment was blunt ligated into the *Sal*I site of the pLNTK/3′ homology arm. The targeting construct also contains a thymidine kinase gene. R1 ES cells were electroporated with a linearized SCL targeting vector construct and selected with 500 μg/ml G418 and 2×10^{-6} M gancyclovior (FIAU). G418 and FIAU resistant clones were picked after ~10 days of selection and expanded for further analyses.

ES differentiation, blast colony and hematopoietic replating

Mouse ES cells were maintained on STO feeder cells in the presence of leukemia inhibitory factor (LIF). EBs were generated as described (Kennedy et al., 1997; Choi et al., 1998). Blast colonies were generated by replating sorted CD4$^+$ cells from day 2.75-3 EBs in the presence of VEGF (5 ng/ml), kit ligand (KL, 1% conditioned medium or 100ng/ml purified) and D4T endothelial cell conditioned medium (CM) (Kennedy et al., 1997; Choi et al., 1998) at 25%. Erythroid and myeloid colony assays were carried out as described previously (Faloon et al., 2000). Briefly, cells sorted from day 6 and day 8 EBs were cultured in methyl cellulose containing 10% plasma-derived serum (PDS, Antech; Texas), 5% protein free hybridoma medium (PFHM2, Gibco/BRL), ascorbic acid (12.5 μg/ml), L-glutamine (2 mM), transferrin (300 μg/ml; Boehringer Mannhein) and MTG (4.5×10^{-4} M), together with the following cytokines: KL (1% conditioned medium), IL3 (1% conditioned medium), Epo (2 units/ml), IL1 (5 ng/ml), IL6 (5 ng/ml), IL11 (10 ng/ml), G-CSF (30 ng/ml), M-CSF (5 ng/ml) and GM-CSF (3 ng/ml). Hematopoietic colonies were counted 7-10 days later. IL1, IL6, IL11, G-CSF and M-CSF were purchased from R&D Systems. KL was obtained from a medium conditioned by CHO cells transfected with a KL expression vector (kindly provided by Genetics Institute). Epo was purchased from Amgen (Thousand Oaks, CA), and IL3 was obtained from a medium conditioned by X63 Ag8-653 myeloma cells transfected with a vector expressing IL3 (Karasuyama and Melchers, 1988).

FACS analysis

For FACS analysis of FLK1 and CD4 expression, EBs were treated with 7.5 mM EDTA/PBS (pH 7.4) for 2 minutes. Cells were centrifuged, resuspended in staining medium (4% FCS in PBS), passed through a 20-gauge needle to generate single cell suspension, and counted. After centrifugation, cells were resuspended to a density of 5×10^6 cells/ml in 2.4G2 supernatant to block antibodies from binding to Fc receptors II and III (CD16 and CD32) (Unkeless, 1979). Cells were placed into each well of a V-shaped 96-well plate at 5×10^5 cells/well followed by incubation on ice for 30 minutes. Subsequently, biotinylated mouse anti-human CD4 monoclonal antibody (CALTAG), freshly diluted in wash buffer (4% FCS in PBS), was added into each well and incubated on ice for 15 minutes. After three washes, freshly diluted streptavidin-allophycocyanin (Sav-APC; Pharmingen) and phycoerythrin (PE)-conjugated anti-FLK1 monoclonal antibody (Pharmingen) were added and incubated on ice in the dark for 15 minutes. Cells were washed three times, re-suspended in wash buffer, and transferred to 5 ml polypropylene tubes for analysis. A three-color FACS analysis of FLK1, human CD4 and endothelial or hematopoietic markers was carried out by staining cells first with endothelial/hematopoietic markers. FITC-conjugated anti-mouse CD31, FITC-conjugated anti-mouse CD34 or FITC-conjugated anti-mouse CD45 was added directly. When cells were stained with non-labeled anti-mouse VE-cadherin and anti-mouse Ter-119 antibodies, FITC-conjugated goat anti-rat IgG and FITC-conjugated goat anti-rat IgG_{2b}, respectively, were used to amplify the signals. All the antibodies were purchased from Pharmingen. Cells were subsequently stained with anti-human CD4 and anti-FLK1 antibodies as described above. Cells were analyzed on a FACS Caliber (Becton-Dickinson). FACS data were analyzed with CellQuest software (Becton-Dickinson).

Cell sorting and in vitro cultures of sorted cell populations

For FACS-cell sorting, single cell suspensions were prepared the same way as the FACS analyses, except that the EB cells were dissociated with trypsin (0.08%)/EDTA (0.36 mM)/PBS instead of 7.5 mM EDTA/PBS. Double-color staining and sorting for FLK1 and human CD4 cells were performed the same way as for FACS analysis. Prior to sorting, stained cells were filtered through 40-μm nylon mesh. Cells were sorted using FACS MoFlo (Becton-Dickinson), and the sorted cells were reanalyzed on a FACS Caliber. FLK1$^+$CD4$^-$ or FLK1$^+$CD4$^+$ sorted cells were cultured for an additional 20-48 hours in a bacterial petri dish in IMDM media containing 15% pre-selected FCS, ascorbic acid (50 μg/ml), L-glutamine (2 mM) and MTG (4.5×10^{-4} M) at a cell density of 2×10^5/ml.

Endothelial cell cultures and immunohistochemical staining

Cells sorted from day 6 EBs were plated onto type IV collagen

(Sigma)-coated, 24-well plates in IMDM media containing 15% pre-selected FCS, ascorbic acid (50 μg/ml), L-glutamine (2 mM), MTG (4.5×10^{-4} M) and VEGF (50 ng/ml) at a cell density of 2×10^4/well. Cells were cultured in humidified 37°C incubator with 5% CO_2 for 3-4 days. For immunohistochemical staining, adherent cells were washed with PBS, fixed for 10 minutes in PBS containing 4% paraformaldehyde (PFA) at 4°C, and washed twice (10 minutes each) in PBS. Following the wash, the endogenous peroxidase was quenched in methanol/30% hydrogen peroxide/10% sodium azide (50:10:1) for 1 hour at 4°C. Cells were washed twice and blocked with PBS containing 1% goat serum, 0.2% bovine serum albumin and 2% skim milk for 1 hour. Cells were then incubated with biotinylated anti-mouse CD31 (Pharmingen) overnight at 4°C. After three washes, cells were incubated with streptavidin-horseradish peroxidase (Pharmingen) for 1 hour at room temperature. Cells were then washed three times and incubated with a DAB kit to develop the color (Vector).

In situ hybridization

Sorted $CD4^+$ and $CD4^-$ cells, from day 5 EBs, were fixed in freshly prepared 4% paraformaldehyde in PBS. Cells were dehydrated through graded ethanols, xylene and embedded in paraffin wax. Sections (4 μm) were adhered to Superfrost Plus (VWR) microscope slides. Sections of E8.5 mouse embryos were placed on the slides and served as positive controls for *Scl* expression (Silver and Palis, 1997). In situ hybridization was performed essentially as described (McGrath et al., 1999), except probes were synthesized at 4.2×10^9 dpm/μg, and hybridization occurred at 50°C. Replicate slides were probed with a sense control probe and no signal above background was detected (not shown). Cells were photographed in brightfield and darkfield with a SPOT RT slider (Diagnostic Instruments) digital camera. Images were processed, pseudocolored and merged using Photoshop (Adobe Systems) and Fovea Pro 2 (Reindeer Graphics).

Gene expression analysis

RNA was purified from sorted $FLK1^+CD4^+$, $FLK1^+CD4^-$, and $FLK1^-CD4^-$ cells (from day 2.75 EBs), reverse transcribed, and poly-A tailed using terminal transferase. Total cDNA was amplified using oligo dT as a primer (5′GTTAACTCGAGAATTCT$_{24}$3′) (Brady et al., 1990). After one round of re-amplification using 1μl of the primary PCR products as template, PCR products were separated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with ^{32}P randomly primed cDNA probe corresponding to the 3′ end of the *L32*, *Flk1*, *Scl*, *Gata1*, *Gata2* and *Lmo2* genes. After the hybridization, the blot was washed at high stringency and exposed to an X-ray film.

RESULTS

Construction of human CD4 knock-in ES cells at the *Scl* locus

To isolate SCL-expressing cells, we introduced a non-functional human CD4 gene encoding extracellular and transmembrane domains (Bedinger et al., 1988) into one allele of *Scl*. The strategy for constructing a targeting vector is illustrated in Fig. 1. As shown, a CD4-loxPneo cassette was targeted in-frame into the *Not*I site of the fourth exon of *Scl*. The restriction enzyme, *Not*I, site is located 10 nucleotides downstream of the ATG initiation codon. As a result, *Scl* expression is disrupted, and CD4 expression in the modified cells should faithfully mimic the endogenous *Scl* expression. R1 ES cells were electroporated, selected, and individual colonies picked and screened by Southern blot analyses (Fig. 1). Clones with the correct targeting event were expanded and karyotyped. Karyotypically normal clones (clones 1-4, 1-19 and 1-68) were further subjected to in vitro analyses.

Kinetics of the development of FLK1 and CD4-expressing cells during EB differentiation

To establish that CD4 expression truly correlates with that of *Scl*, we first sorted $CD4^+$ and $CD4^-$ cells and subjected them to in situ hybridization for *Scl* expression. As shown in Fig. 2, the sorted $CD4^+$ cells showed strong hybridization to *Scl* antisense probe (Fig. 2A-D), while $CD4^-$ cells were negative for *Scl* expression (Fig. 2E-H). The levels of *Scl* expression in the sorted $CD4^+$ cells are similar to the endogenous levels in blood island cells of the E8.5 mouse embryo (Fig. 2I). These studies indicate that CD4 expression can be used as a surrogate marker for *Scl* expression.

After $CD4^+$ cells were shown to represent SCL expressing cells accurately, kinetic analyses of FLK1 and CD4 expression were performed using in vitro differentiated $Scl^{+/CD4}$ ES cells. Undifferentiated ES cells do not express FLK1 or SCL (Choi et al., 1998; Faloon et al., 2000). Upon differentiation, cells expressing FLK1 developed first in EBs and CD4-expressing cells were detected from day 2.75 in developing EBs (Fig. 2J), although the levels of CD4 expression at this stage were low. The low CD4 expression in early EBs could reflect the weak *Scl* promoter activity in early development and is consistent with studies by Elefanty, who characterized knock-in mice carrying a *lacZ* gene at the *Scl* locus (Elefanty et al., 1998; Elefanty et al., 1999). Cells expressing CD4 increased significantly over the next 24-48 hours (Fig. 2J). About 40% of the total EB cells expressed CD4 by day 5, and ~75% of the total EB cells expressed CD4 by day 6. The percentage of $CD4^+$ cells decreased thereafter and reached to ~20% by day 8. At earlier time points (days 2.75-3), all the $CD4^+$ cells also expressed FLK1. EBs from days 4-6 contained three distinct cell populations as follows: cells expressing only FLK1 ($FLK1^+CD4^-$), cells expressing only CD4 ($FLK1^-CD4^+$), and cells expressing both FLK1 and CD4 ($FLK1^+CD4^+$). In later EBs (day 8), the number cells expressing both FLK1 and CD4 was significantly lower, and there were predominantly $FLK1^+CD4^-$ or $FLK1^-CD4^+$ cells.

Developmental relationship between $FLK1^+CD4^-$, $FLK1^+CD4^+$ and $FLK1^-CD4^+$ cell populations

FACS analysis for FLK1 and CD4 expression suggested that $FLK1^+$ cells developed first followed by $CD4^+$ cells. To understand better the developmental relationship between $FLK1^+CD4^-$, $FLK1^+CD4^+$ and $FLK1^-CD4^+$ cells, we first FACS-sorted $FLK1^+$ and $FLK1^-$ cells from day 2.5 EBs and then cultured them separately for an additional 20 hours. $FLK1^+$ cells progressed to give rise to $FLK1^+CD4^+$ cells after 10 hours of in vitro culture, and $FLK1^-CD4^+$ cells were readily detected after 20 hours of culture. $FLK1^-$ cells progressed first to $FLK1^+$ and then to $FLK1^+CD4^+$ cells with time (Fig. 3A). We subsequently FACS-sorted $FLK1^+CD4^+$ and $FLK1^+CD4^-$ cell populations from day 4 EBs, cultured them for an additional 20 hours, and analyzed them for FLK1 and CD4 expression. As shown in Fig. 3B, $FLK1^+CD4^+$ cells readily gave rise to $FLK1^-CD4^+$ cells, indicating that FLK1 is downregulated within the $FLK1^+CD4^+$ cells to generate $FLK1^-CD4^+$ cell populations. Consistently, $FLK1^+CD4^-$ cells first gave rise to $FLK1^+CD4^+$ and then to $FLK1^-CD4^+$ cells

upon an additional 20-hour culture. FLK1^{-}CD4^{+} cells further increased after 48 hours of culture (not shown). These studies clearly argue that there is a distinct, developmental succession between FLK1^{+}CD4^{-}, FLK1^{+}CD4^{+} and FLK1^{-}CD4^{+} cells. The FLK1^{+}CD4^{-} cell population will initially develop. The *Scl* gene will be turned on within the FLK1^{+}CD4^{-} cells to give rise to the FLK1^{+}CD4^{+} cell population. Subsequently, the *Flk1* gene will be downregulated within the FLK1^{+}CD4^{+} cells to finally give rise to the FLK1^{-}CD4^{+} cells.

FLK1^{+}SCL^{+} cells from day 2.75 EBs are enriched for the hemangioblast

As discussed earlier, BL-CFCs express FLK1 (Faloon et al., 2000) and *Scl*$^{-/-}$ EBs fail to generate blast colonies that are the progeny of BL-CFCs (Faloon et al., 2000; Robertson et al., 2000). To determine if the BL-CFC cell population could be identified as FLK1^{+}SCL^{+}, day 2.75 EB cells were subjected to FACS analysis and cell sorting. If FLK1 and SCL are true determinants of the hemangioblast, it was expected that FLK1^{+}CD4^{+} cells were enriched for BL-CFCs. Therefore, FLK1^{+}CD4^{+}, FLK1^{+}CD4^{-} and FLK1^{-}CD4^{-} cells from day 2.75 EBs were sorted and subjected to blast colony assays. As shown in Fig. 4A, blast colonies developed predominantly from sorted FLK1^{+}CD4^{+} cells, not FLK1^{+}CD4^{-} or FLK1^{-}CD4^{-} cells. The small number of blast colonies that developed from FLK1^{+}CD4^{-} cells is most likely to be due to the CD4^{low} cells that could have been sorted as a negative cell population. Furthermore, secondary EBs mainly developed from FLK1^{-}CD4^{-} cells, strongly supporting the idea that FLK1^{-}CD4^{-} cells still contained undifferentiated ES cells.

To characterize further the FLK1^{+}CD4^{+}, FLK1^{+}CD4^{-} and FLK1^{-}CD4^{-} cells present within day 2.75 EBs, they were also subjected to gene expression analyses. RNA from FLK1^{+}CD4^{+}, FLK1^{+}CD4^{-} and FLK1^{-}CD4^{-} cells was subjected to global amplification of mRNA transcripts (Brady et al., 1990). The amplified PCR products were analyzed for

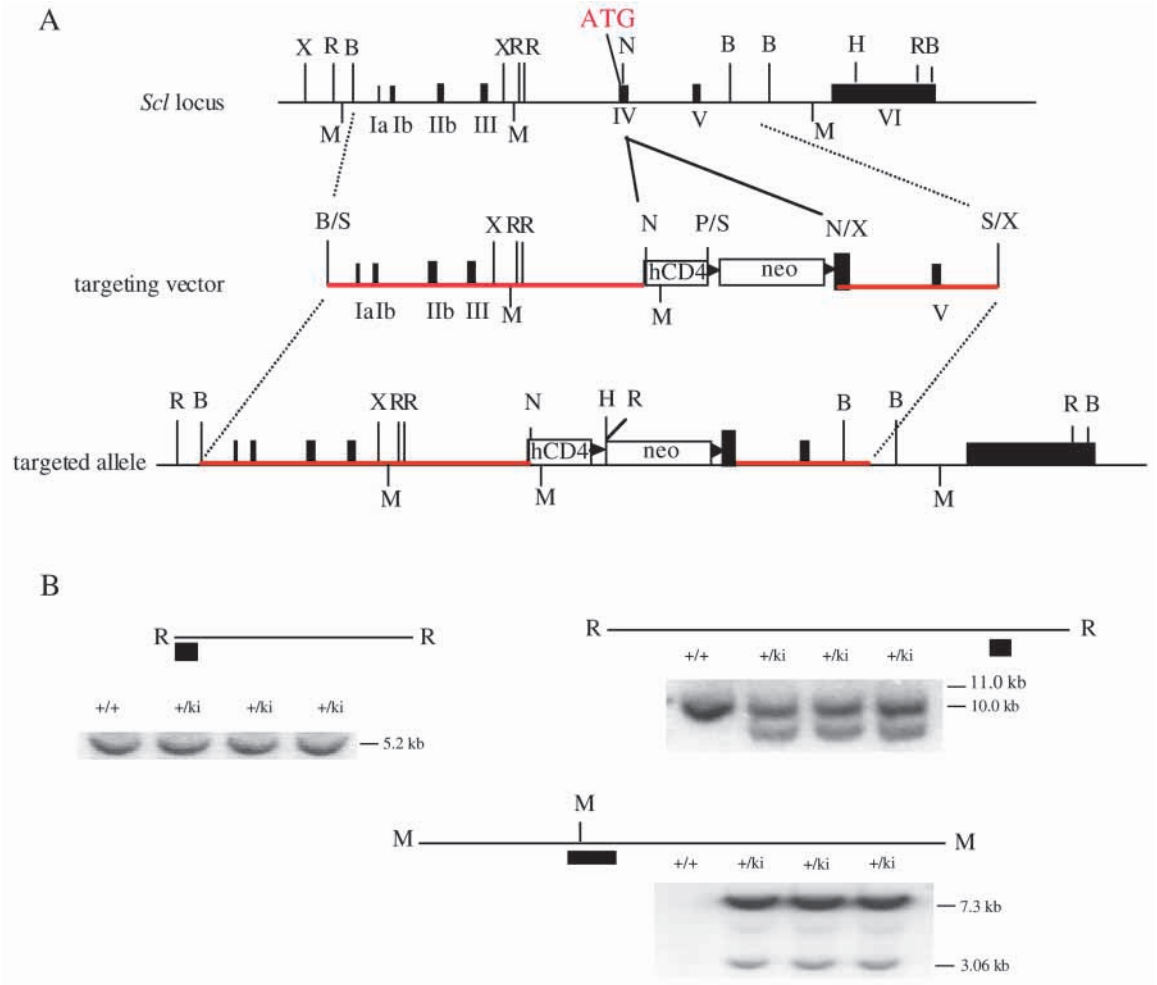

Fig. 1. Generation of human CD4 (CD4) knock-in ES cells. (A) Targeting strategy used to insert CD4 gene into the *Scl* locus is shown. The mouse *Scl* locus, targeting construct, and the targeted allele are shown. The black boxes and numbers below indicate the exons. The ATG codon starts within exon IV. (B) Southern blot analysis of the targeted allele. DNA was digested with enzymes indicated and run on an agarose gel. Top left, genomic DNA was digested with *Eco*RI and probed with genomic DNA as indicated below. Both targeted and wild-type alleles generated a 5.2 kb DNA band. Top right: genomic DNA was digested with *Eco*RI and probed with exon 6 probe as indicated below. The wild-type allele (upper band) and the targeted allele (lower band) are shown. Bottom: genomic DNA was digested with *Mun*I and probed with human CD4 gene. Only the targeted allele gave 7.3 and 3 kb DNA bands. The gel was run for 48-72 hours for good separation of DNA. The enzymes used are as follows: X, *Xho*I; R, *Eco*RI; N, *Not*I; B, *Bam*HI; H, *Hin*dIII; M, *Mun*I.

the expression of *Flk1*, *Scl*, *Gata1*, *Gata2* and *Lmo2* (Fig. 4B). As expected, *Flk1* was expressed within FLK1$^+$CD4$^-$ and FLK1$^+$CD4$^+$ cells. *Scl*, *Gata1*, *Gata2*, and *Lmo2* expression was greatly raised within FLK1$^+$CD4$^+$ cells compared with FLK1$^+$CD4$^-$ cells. None of these genes was expressed in FLK1$^-$CD4$^-$ cells. Taken together, our results strongly support the notion that hemangioblasts can be identified as FLK1$^+$SCL$^+$ cells.

FLK1 and SCL expression in hematopoietic and endothelial cells

To determine if FLK1 and SCL expression can define hematopoietic and endothelial cell populations, the nature of FLK1$^+$CD4$^-$, FLK1$^-$CD4$^+$, and FLK1$^+$CD4$^+$ cell populations, present in later stages of EB development (days 5-6), was determined by three-color FACS analyses for FLK1, CD4 and hematopoietic or endothelial cell markers. Cells gated on VE-cadherin$^+$, CD31$^+$ (PECAM-1$^+$), CD34$^+$, Ter-119$^+$, or CD45$^+$ were analyzed for FLK1 and CD4 expression. As shown in Fig. 5, cells expressing VE-cadherin, an endothelial cell marker, expressed both FLK1 and CD4, while cells expressing Ter119, an erythroid marker, expressed CD4 but not FLK1. CD31 and CD34, markers of both hematopoietic and endothelial cells, were expressed in both FLK1$^+$CD4$^+$ and FLK1$^-$ CD4$^+$ cells. CD45, a marker normally used for hematopoietic cells, was expressed in both FLK1$^+$CD4$^+$ and FLK1$^-$CD4$^+$ cells at day 6.

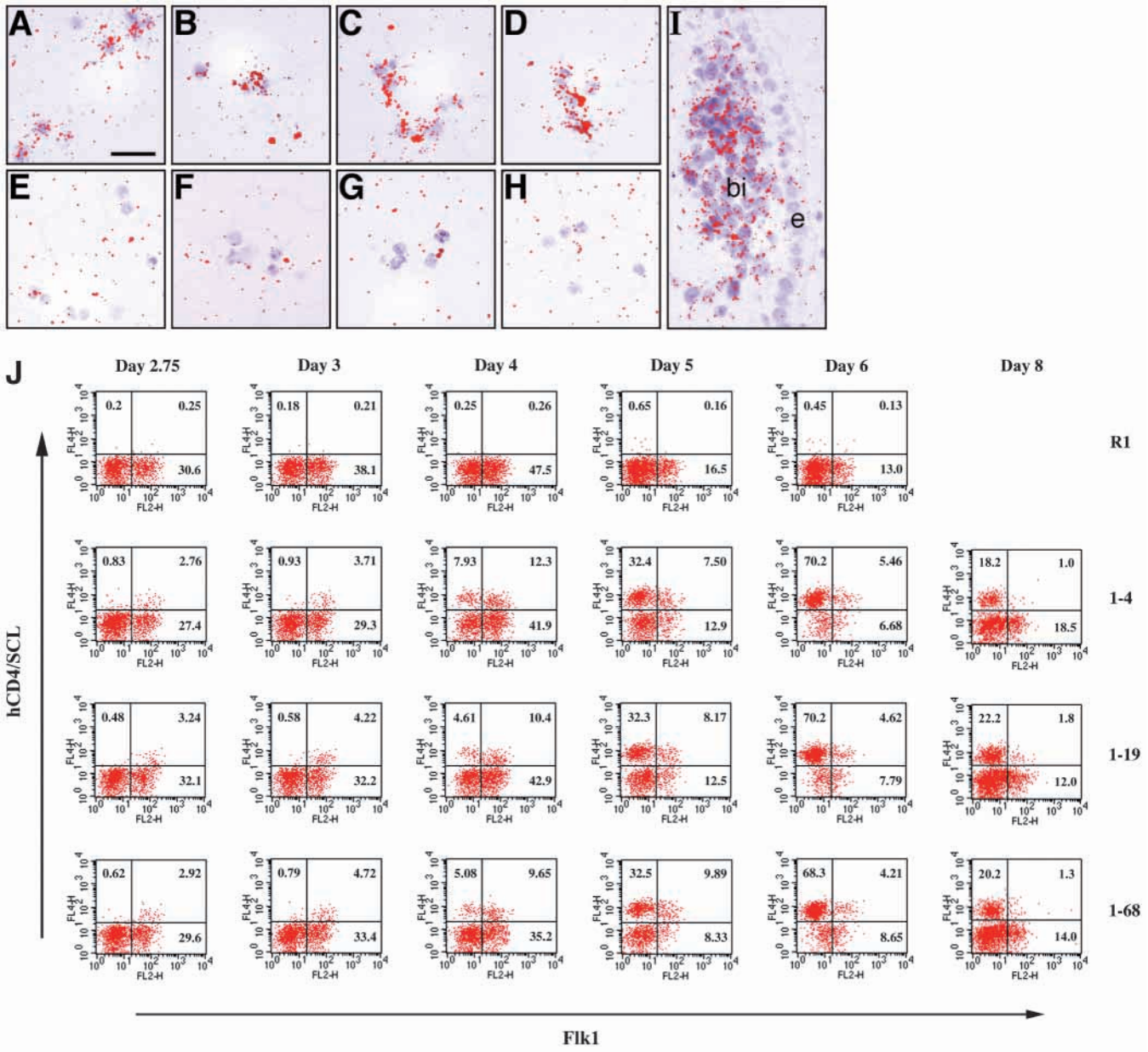

Fig. 2. (A-I) In situ hybridization of Scl. CD4$^+$ and CD4$^-$ cells were sorted from day 5 EBs and subjected to in situ hybridization. (A-D) CD4$^+$ cells; (E-H) CD4$^-$ cells; (I) E8.5 yolk sac. bi, blood islands; e, endoderm. (J) Kinetic analyses of FLK1 and SCL expression during EB development. In vitro differentiated ES cells (from day 2.75 to day 8) from wild-type R1 and three independent knock-in clones (1-4, 1-19, and 1-68) were subjected to FACS analyses for FLK1 and CD4 expression. Numbers in a given box indicate the percentage of cells that are FLK1$^+$CD4$^-$, FLK1$^+$CD4$^+$ or FLK1$^-$CD4$^+$.

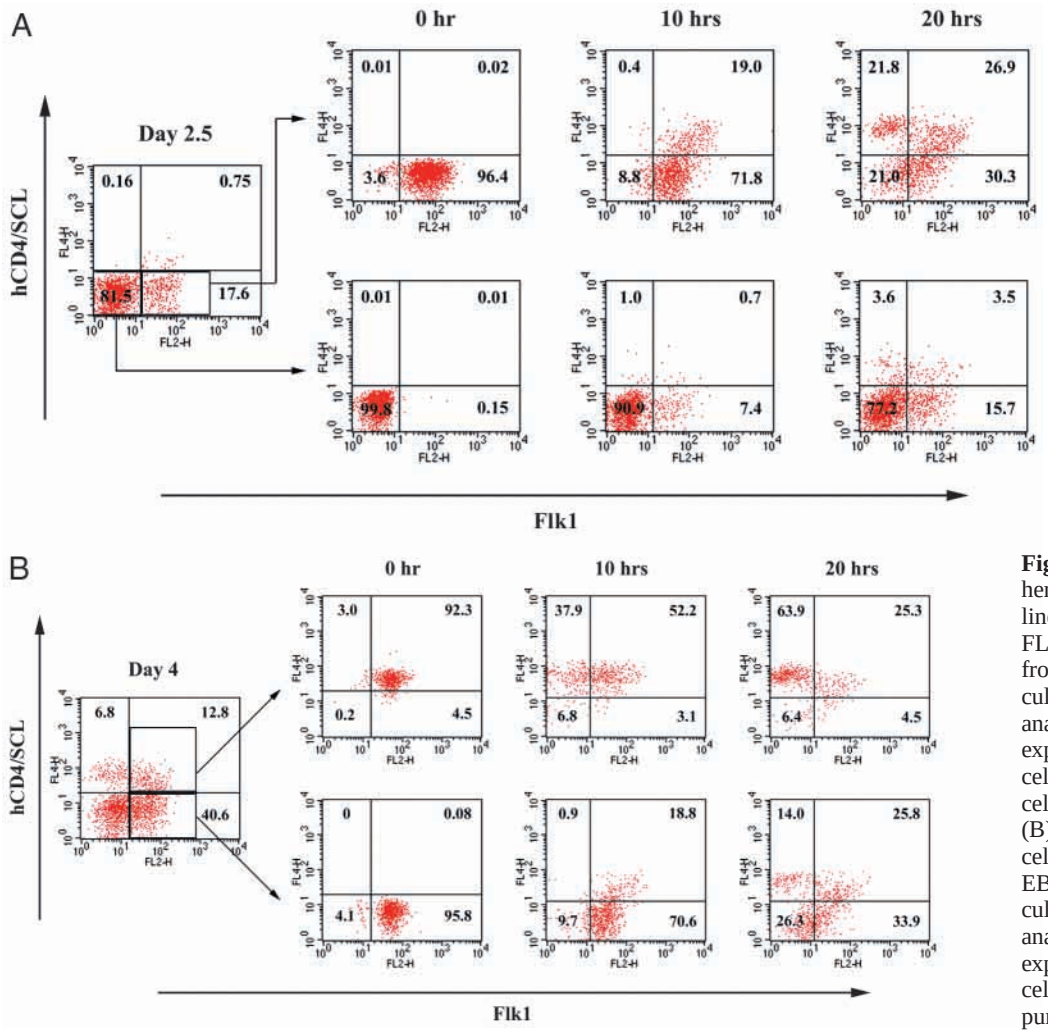

Fig. 3. In vitro progression of hematopoietic and endothelial cell lineages. (A) FLK1$^+$CD4$^-$ and FLK1$^-$CD4$^-$ cells were FACS-sorted from day 2.5 EBs and further cultured in vitro. The cells were then analyzed for FLK1 and CD4 expression for up to 20 hours. The cells at 0 hour indicate the starting cell population after sorting. (B) FLK1$^+$CD4$^+$ and FLK1$^+$CD4$^-$ cells were FACS-sorted from day 4 EBs and further subjected to in vitro culture as in A. The cells were analyzed for FLK1 and CD4 expression for up to 20 hours. The cells at 0 hours show the sorting purity.

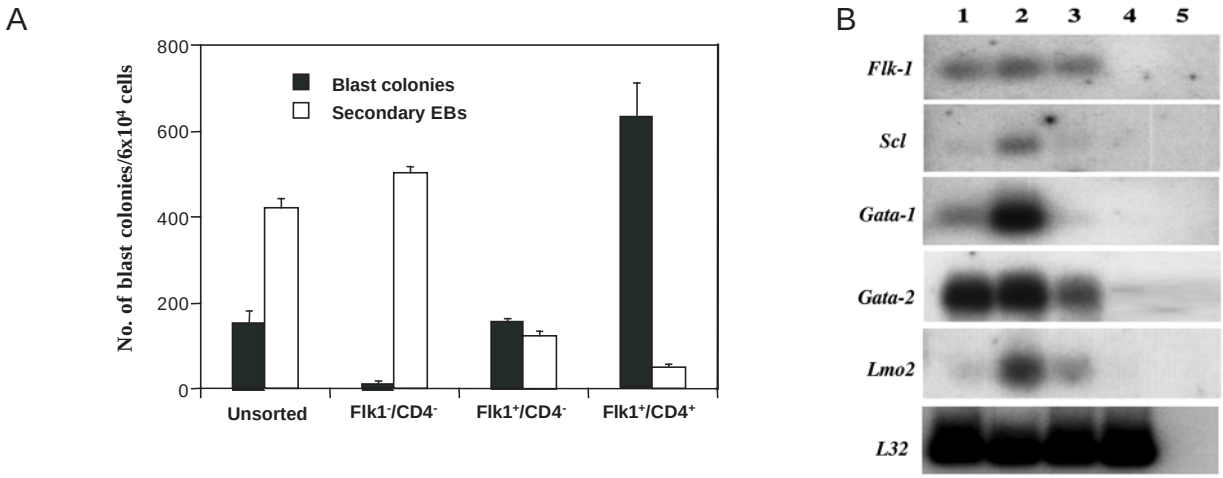

Fig. 4. FLK1$^+$CD4$^+$cells from D2.75 EBs are enriched for the hemangioblasts. (A) FLK1$^+$CD4$^+$, FLK1$^+$CD4$^-$ and FLK1$^-$CD4$^-$ cells were FACS-sorted and subjected to blast colony replating (6×10^4 cells/ml). The resulting blast colonies were counted 4 days later. Secondary EBs were also counted and shown. Error bars indicate standard deviations from triplicate plates. (B) Gene expression analysis. RNA from FLK1$^+$CD4$^+$, FLK1$^+$CD4$^-$ and FLK1$^-$CD4$^-$ cells were amplified and probed with *Flk1*, *Scl*, *Gata1*, *Gata2*, *Lmo2* and *L32*. 1, unsorted 2.75 EBs; 2, FLK1$^+$CD4$^+$; 3, FLK1$^+$CD4$^-$; 4, FLK1$^-$CD4$^-$; 5, water – a negative control for RT-PCR.

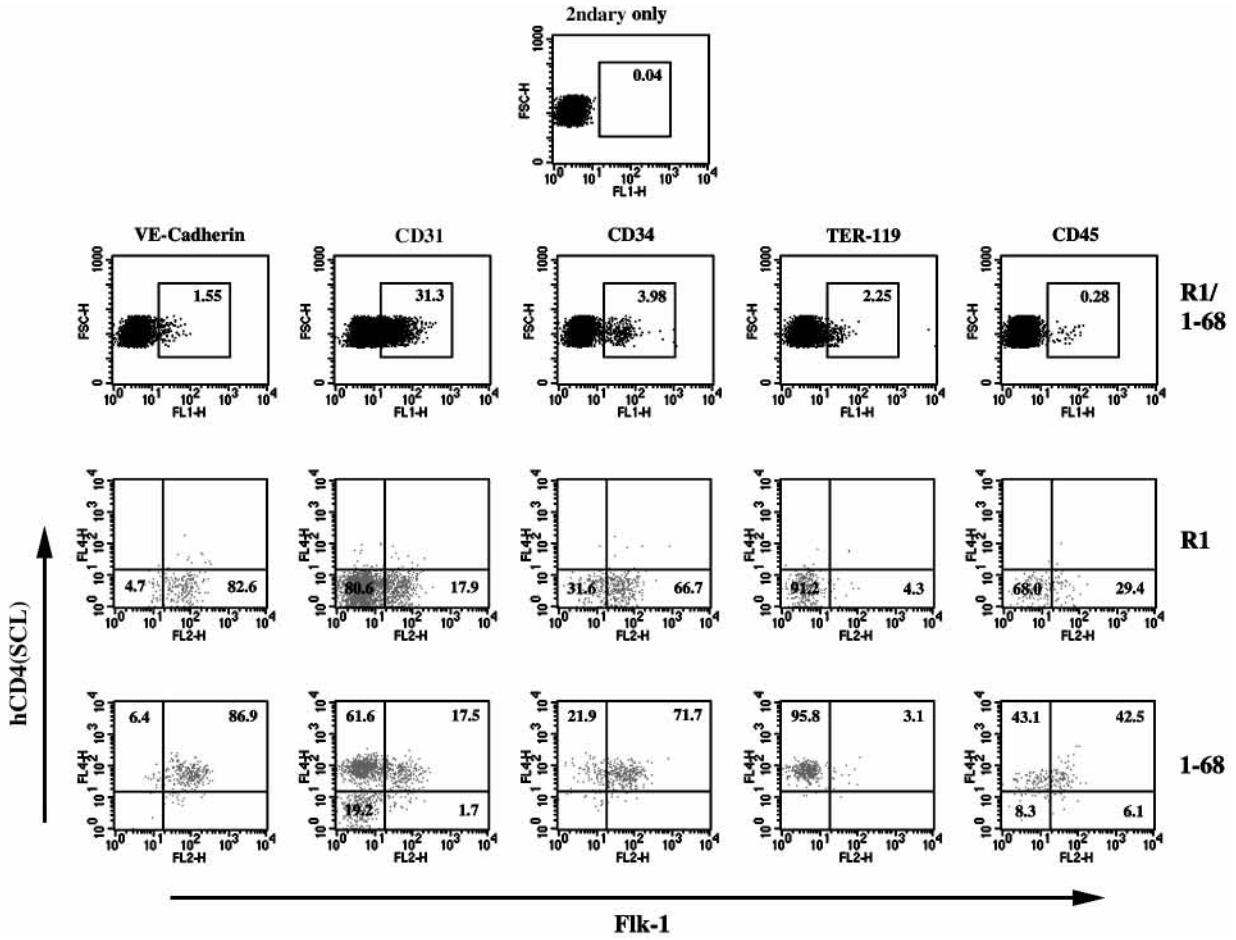

Fig. 5. Hematopoietic and endothelial cell analysis. Day 6 EB cells were subjected to three-color analyses for FLK1, CD4 and hematopoietic or endothelial cell markers. Cells gated on VE-cadherin$^+$, CD31$^+$, CD34$^+$, Ter-119$^+$ or CD45$^+$ were analyzed for FLK1 and CD4 expression.

CD45 was predominantly expressed in FLK1$^-$CD4$^+$ cells from later stages of EBs (day 8, not shown).

To ascertain if hematopoietic and endothelial progenitors can be isolated based on SCL and FLK1 expression, we FACS-sorted FLK1$^+$CD4$^-$, FLK1$^+$CD4$^+$ and FLK1$^-$CD4$^+$ cells from day 6 EBs, and subjected them to hematopoietic and endothelial cell assays. For the hematopoietic progenitor studies, sorted cells were replated in methylcellulose cultures with hematopoietic factors. As shown in Fig. 6A, hematopoietic colonies developed from both FLK1$^+$CD4$^+$ and FLK1$^-$CD4$^+$ cells. FLK1$^-$CD4$^+$ cells predominantly gave rise to erythroid colonies, while FLK1$^+$CD4$^+$ cells gave rise to macrophage and bi-potential erythroid/macrophage colonies. The endothelial progenitors were assayed by replating sorted cells onto type IV collagen-coated plates with VEGF and cultured for 4 days. Afterwards, the adherent cells were stained for CD31. As shown in Fig. 6B, endothelial cells developed from FLK1$^+$CD4$^-$ and FLK1$^+$CD4$^+$, but not from FLK1$^-$CD4$^-$ cells. No adherent cells developed from FLK1$^-$CD4$^+$ cells.

FLK1$^+$CD4$^+$ cells were rarely present in day 8 EBs, and two distinct cell populations, FLK1$^+$CD4$^-$ and FLK1$^-$CD4$^+$, were readily observed (Fig. 2). Again, these two cell populations were sorted and examined for their potential to generate hematopoietic or endothelial cells in cultures. As shown in Fig. 6C, all the hematopoietic colonies developed from FLK1$^-$CD4$^+$ cells, while the endothelial cells still developed from FLK1$^+$CD4$^-$ cells (not shown). Taken together, we conclude that hematopoietic progenitors initially develop from FLK1$^+$CD4$^+$ and FLK1$^-$CD4$^+$ cells and then from FLK1$^-$CD4$^+$ cells in later stages of EBs.

DISCUSSION

We have examined the expression of FLK1 and SCL by using the in vitro differentiation model of *Scl*$^{+/CD4}$ ES cells to further understand the development of hemangioblast, endothelial and hematopoietic cell progenitors. Our studies demonstrate that almost all the CD4$^+$(SCL$^+$) cells from day 2.75 EBs co-expressed FLK1 (Fig. 2J). Furthermore, sorted FLK1$^+$CD4$^+$ cells, but not FLK1$^+$CD4$^-$, readily generated blast colonies. Finally, gene expression analyses demonstrated that the expression of *Scl*, *Gata1*, *Gata2* and *Lmo2*, genes to be expressed in primitive hematopoietic compartment, was greatly enriched in FLK1$^+$CD4$^+$ cells compared with FLK1$^+$CD4$^-$ cells. Taken together, these studies indicate that FLK1$^+$SCL$^+$ cells present within day 2.75 EBs represent the hemangioblasts. In zebrafish, SCL is expressed in the lateral

mesoderm, which gives rise to hematopoietic, endothelial and pronephric lineages (Gering et al., 1998). Cells expressing both FLK1 and SCL appear to give rise to FLK1^{-}SCL^{+} hematopoietic and FLK1^{+}SCL^{-} endothelial cells (Gering et al., 1998). These observations predict that cells expressing both SCL and FLK1 represent hemangioblasts. Indeed, cells expressing both SCL and FLK1 increase dramatically when SCL is overexpressed in zebrafish embryos (Gering et al., 1998). As a result, both hematopoietic and endothelial cells also increase. The suggestive role for SCL in the specification of the hemangioblast formation comes from the observation that the expansion of FLK1^{+}SCL^{+} cells occurs at the expense of somitic and pronephric duct tissues (Gering et al., 1998). Furthermore, SCL expression is greatly reduced in zebrafish *cloche* (*clo*) mutants (Liao et al., 1998), which show defective hematopoietic and endothelial differentiation (Stainier et al., 1995). More importantly, ectopically expressed SCL can rescue, although incompletely, the hematopoietic and endothelial cell defects in these mutants. Additionally, Drake and Fleming (Drake and Fleming, 2000) have examined early mouse embryos for FLK1 and SCL expression. At E6.5, FLK1^{+}SCL^{+} cells were already present, albeit dispersed, in the extra-embryonic yolk sac. Primary vascular networks became evident in the regions where FLK1^{+}SCL^{+} cells were detected, suggesting that the initial FLK1^{+}SCL^{+} cells represent hemangioblasts.

The analyses of sorted cell populations have demonstrated that FLK1^{+} cells isolated from day 2.5 EBs progressed to give rise initially to FLK1^{+}CD4^{+} cells and subsequently to FLK1^{-}CD4^{+} cells. Consistently, sorted FLK1^{+}CD4^{-} cells from day 4 EBs progressed to give rise to FLK1^{+}CD4^{+} and then to FLK1^{-}CD4^{+} cell populations when cultured for an additional 24-48 hours in vitro, while FLK1^{+}CD4^{+} cells proceeded to give rise to FLK1^{-}CD4^{+} cells. As the hematopoietic progenitors were present within FLK1^{-}CD4^{+} and FLK1^{+}CD4^{+} cells in early EBs (day 4-6) and in FLK1^{-}CD4^{+} cells in later EBs (day 8, Fig. 2), we conclude that FLK1 expression within hematopoietic progenitors is downregulated (Fig. 7).

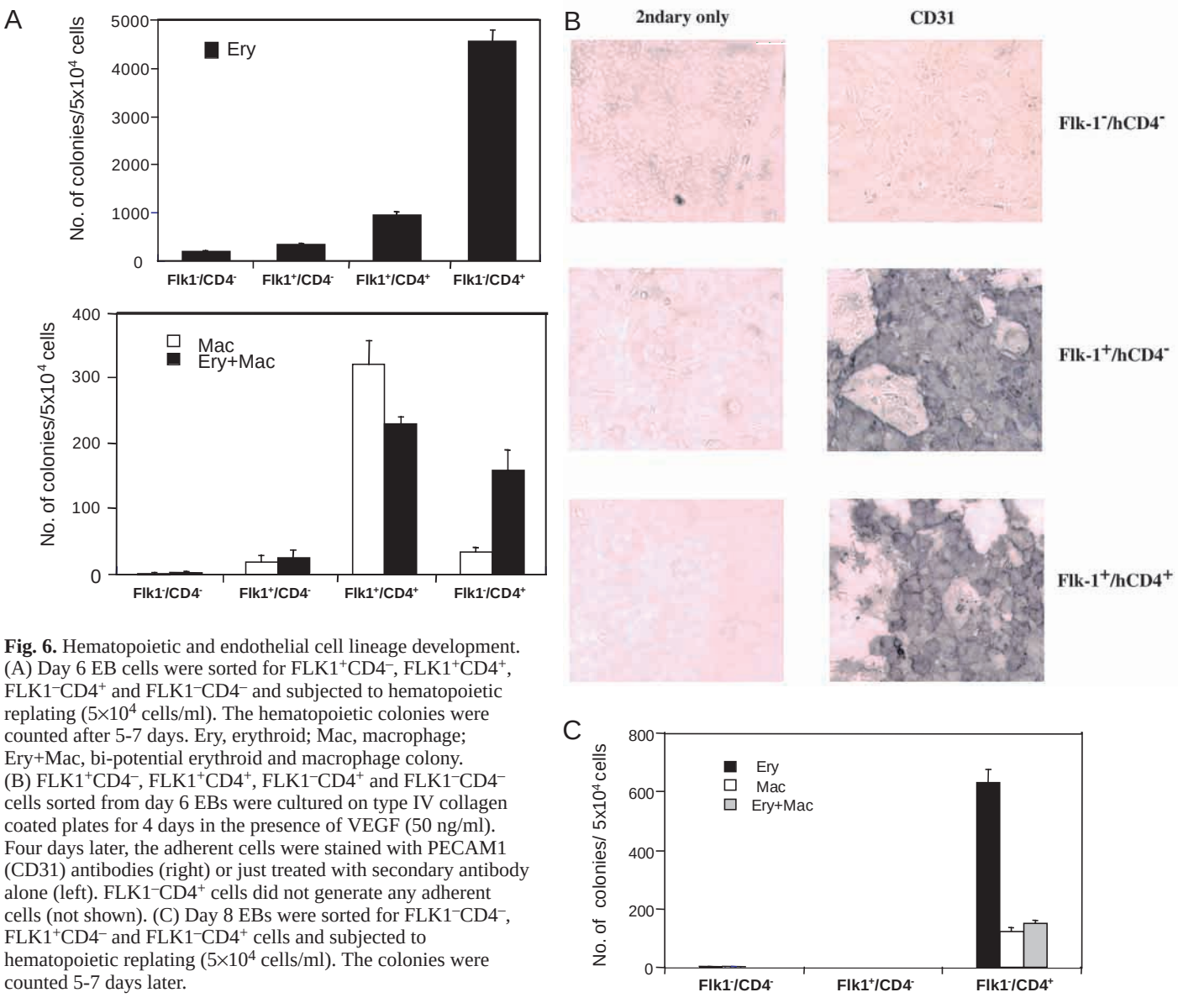

Fig. 6. Hematopoietic and endothelial cell lineage development. (A) Day 6 EB cells were sorted for FLK1^{+}CD4^{-}, FLK1^{+}CD4^{+}, FLK1^{-}CD4^{+} and FLK1^{-}CD4^{-} and subjected to hematopoietic replating (5×10^4 cells/ml). The hematopoietic colonies were counted after 5-7 days. Ery, erythroid; Mac, macrophage; Ery+Mac, bi-potential erythroid and macrophage colony. (B) FLK1^{+}CD4^{-}, FLK1^{+}CD4^{+}, FLK1^{-}CD4^{+} and FLK1^{-}CD4^{-} cells sorted from day 6 EBs were cultured on type IV collagen coated plates for 4 days in the presence of VEGF (50 ng/ml). Four days later, the adherent cells were stained with PECAM1 (CD31) antibodies (right) or just treated with secondary antibody alone (left). FLK1^{-}CD4^{+} cells did not generate any adherent cells (not shown). (C) Day 8 EBs were sorted for FLK1^{-}CD4^{-}, FLK1^{+}CD4^{-} and FLK1^{-}CD4^{+} cells and subjected to hematopoietic replating (5×10^4 cells/ml). The colonies were counted 5-7 days later.

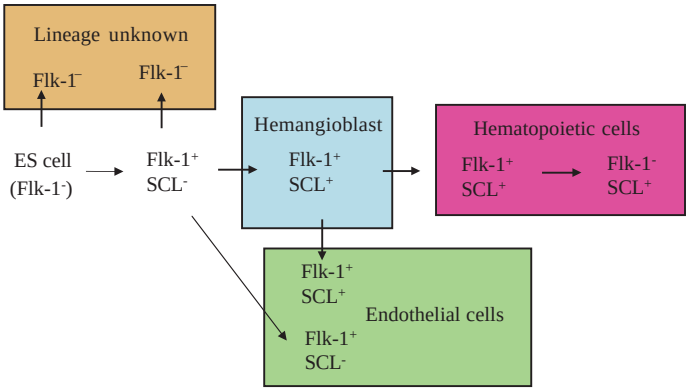

Fig. 7. Schematic diagram of the hematopoietic and endothelial cell lineage development within EBs.

Consistent with this interpretation, there were only FLK1^{-}CD4^{+} or FLK1^{+}CD4^{-} cell populations present in later stages of EBs (day 8). Previous studies have also demonstrated that hematopoietic progenitors were enriched within the FLK1^{+} cell populations derived from early EBs, but not later stages of EBs (Kabrun et al., 1997). Similarly, the FLK1^{+} cell population from E8.5 yolk sacs and whole embryos contained hematopoietic progenitors, while few FLK1^{+} cells present in day 12 fetal livers contained hematopoietic potential (Kabrun et al., 1997). Elefanty and colleagues knocked-in a bacterial *lacZ* gene to the *Scl* locus to follow SCL-expressing cells (Elefanty et al., 1998; Elefanty et al., 1999). Histochemical staining of *Scl*$^{+/lacZ}$ embryos for β-galactosidase activity showed that *lacZ* was expressed in hematopoietic and endothelial cells, as well as in the developing brain. Cell sorting and replating studies of β-galactosidase^{+} cells from fetal livers showed that erythroid and myeloid progenitors were present within β-galactosidase^{+} cells. Furthermore, β-galactosidase^{+} cell fractions from the bone marrow were enriched for erythroid, myeloid, lymphoid and CFU-S12 progenitors. These studies support the notion that SCL is expressed in hematopoietic progenitors, which could also include hematopoietic stem cells.

Our studies have also demonstrated that the development of endothelial cells can be followed by FLK1 and SCL expression (Fig. 7). Replating studies have demonstrated that endothelial cells developed from two distinct cell populations, FLK1^{+}CD4^{-} and FLK1^{+}CD4^{+} cells. The nature of the endothelial cells developing from the FLK1^{+}CD4^{-} and FLK1^{+}CD4^{+} cells is currently not known. Given the findings that vascular development occurs normally in *Scl*$^{-/-}$ embryos, but that subsequent vascular remodeling is defective in these embryos (Visvader et al., 1998), it is possible that FLK1^{+}CD4^{+} derived endothelial cells represent terminally differentiated mature endothelial cells. In avian systems, it has been well demonstrated that different mesodermal regions produce endothelial progenitors (Pardanaud et al., 1996). The somatopleural mesoderm, adjacent to the ectoderm, will give rise only to endothelial cell populations. The splanchnopleural mesoderm, adjacent to the endoderm, will give rise to both hematopoietic and endothelial cells. Given these observations, it is possible that endothelial cells that develop closely together with hematopoietic cells will express *Scl*, while endothelial cells that do not associate with hematopoietic cells will not express *Scl*. Further studies are required to address these issues.

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