

In vitro hematopoietic and endothelial potential of *flk-1*^{-/-} embryonic stem cells and embryos

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Communicated by Philippa Marrack, National Jewish Medical and Research Center, Denver, CO, December 21, 1998 (received for review November 5, 1998)

ABSTRACT Mice deficient in the Flk-1 receptor tyrosine kinase are known to die *in utero* because of defective vascular and hematopoietic development. Here, we show that *flk-1*^{-/-} embryonic stem cells are nevertheless able to differentiate into hematopoietic and endothelial cells *in vitro*, although they give rise to a greatly reduced number of blast colonies, a measure of hemangioblast potential. Furthermore, normal numbers of hematopoietic progenitors are found in 7.5-day postcoitum *flk-1*^{-/-} embryos, even though 8.5-day postcoitum *flk-1*^{-/-} embryos are known to be deficient in such cells. Our results suggest that hematopoietic/endothelial progenitors arise independently of Flk-1, but that their subsequent migration and expansion require a Flk-1-mediated signal.

The vascular endothelial growth factor (VEGF) receptor Flk-1 is known to play a key role in the regulation of embryonic vascular and hematopoietic development. In the mouse embryo, *flk-1* expression can be detected in presumptive mesodermal yolk-sac blood-island progenitors as early as 7.0 days postcoitum (dpc) (1, 2). As has been described recently (3), mice deficient in Flk-1 do not develop blood vessels or yolk sac blood islands and die between 8.5 and 9.5 dpc. In chimeric aggregation studies with wild-type embryos, *flk-1*^{-/-} embryonic stem (ES) cells fail to participate in vessel formation or to contribute to primitive or definitive hematopoiesis *in vivo*, suggesting that Flk-1 inactivation results in a cell autonomous endothelial and hematopoietic defect (4). Taken together, these studies suggest that Flk-1 may mark the common endothelial/hematopoietic precursor, the hemangioblast. The recently identified blast colony-forming cell (BL-CFC), an *in vitro* ES cell derivative, has confirmed the existence of such a cell. In response to VEGF, BL-CFCs form blast colonies containing Flk-1-expressing blast cells that give rise to primitive, definitive hematopoietic and endothelial cells *in vitro* (5, 6). Taken together, these findings strongly suggest that the VEGF/Flk-1 interaction is important for hemangioblast development.

Although all of these studies suggest that the Flk-1 receptor is important in embryonic endothelial and hematopoietic development, the precise nature of its role remains undefined. In particular, the contribution of Flk-1 to hemangioblast formation is unclear, as *flk-1*^{-/-} and *VEGF*^{+/-} mice show residual hematopoietic and endothelial activity (3, 7, 8) and Flk-1-deficient ES cells appear to retain a degree of hematopoietic potential *in vitro* (4). To define the developmental role of Flk-1 more systematically, we have characterized the hematopoietic and endothelial potential of *flk-1*^{-/-} ES cells and embryos in greater detail. And in particular, as the BL-CFC assay should allow the developmental consequences of Flk-1 deficiency during the very early VEGF-dependent stage of hematopoiesis to be analyzed quantitatively (5), we have assessed the ability of *flk-1*^{-/-} ES cells to give rise to blast

colonies. We report that Flk-1-deficient BL-CFCs appear to develop normally within *flk-1*^{-/-} embryoid bodies (EBs), but fail to respond to VEGF in forming blast colonies. Consequently, the number of blast colonies is greatly reduced. Nevertheless, Flk-1-deficient blast cells retain the potential to differentiate into hematopoietic and endothelial cells in culture. Consistent with this, *flk-1*^{-/-} vs. *flk-1*^{+/+} or *+/-* EBs show similar levels of hematopoietic and endothelial cell differentiation potential. We also demonstrate that 7.5-dpc *flk-1*^{-/-} embryos contain normal numbers of hematopoietic progenitors, even though 8.5-dpc *flk-1*^{-/-} embryos are known to be profoundly deficient in such cells. Taken together, our results suggest that Flk-1 is required not for the formation of hematopoietic/endothelial progenitors but rather for the subsequent migration and expansion of such cells.

MATERIALS AND METHODS

Flk-1 Protein Analysis. Day 7 EBs were pelleted and lysed in 1 ml of lysis buffer (1% Nonidet P-40/50 mM Tris-HCl, pH 7.5/150 mM NaCl/2 mM MgCl₂/1 μg/ml aprotinin/1 μg/ml leupeptin/100 μg/ml phenylmethylsulfonyl fluoride) on ice for 30 min with occasional mixing. The lysate was then spun at 14,000 rpm for 30 min, the resultant supernatant was incubated with normal rabbit serum (25 μl/ml lysate) for 1 hr on ice, 200 μl protein A Sepharose CL-4B (Pharmacia) beads (100 μg/ml) were added, and the mixture was incubated at 4°C for another hour with rocking. The sample was then spun at 13,000 rpm for 2 min, the supernatant was removed and mixed with Flk-1 antiserum (1 μg/ml final concentration) and incubated on ice for 1 hr, followed by the addition of 100 μl protein A Sepharose CL-4B beads (100 μg/ml) and incubation on ice with rocking for 1 hr. Immune complex-containing beads were then collected by spinning at 4°C for 15 sec and were washed three times in cold lysis buffer. Beads were mixed with gel-loading buffer (2% SDS/100 mM DTT/60 mM Tris-HCl, pH 6.8), boiled for 5 min, size separated by SDS/PAGE, and transferred to nitrocellulose. Finally, Flk-1-specific bands were detected with Flk-1 antiserum followed by chemiluminescent detection.

Cell Culture. ES cells were maintained on STO feeder cells in the presence of leukemia inhibitory factor (LIF). EBs, blasts, primitive erythroid (EryP), and myeloid colonies were generated as described previously (5). Briefly, blast colonies were generated by replating day 2.75 EBs in the presence of either VEGF (5 ng/ml), kit ligand (KL, 1% conditioned medium or 100 ng/ml purified) and D4T endothelial cell conditioned medium (25%) or

Abbreviations: VEGF, vascular endothelial growth factor; dpc, days postcoitum; ES, embryonic stem; BL-CFC, blast colony-forming cell; EB, embryoid body; LIF, leukemia inhibitory factor; EryP, primitive erythroid; RT-PCR, reverse transcription-PCR; FCS, fetal calf serum; FDG, fluorescein di-β-D-galactopyranoside; CDM, chemically defined medium; BMP-4, bone morphogenetic protein-4; KL, kit ligand; IL, interleukin; CSF, colony-stimulating factor; MTG, monothio glycerol.

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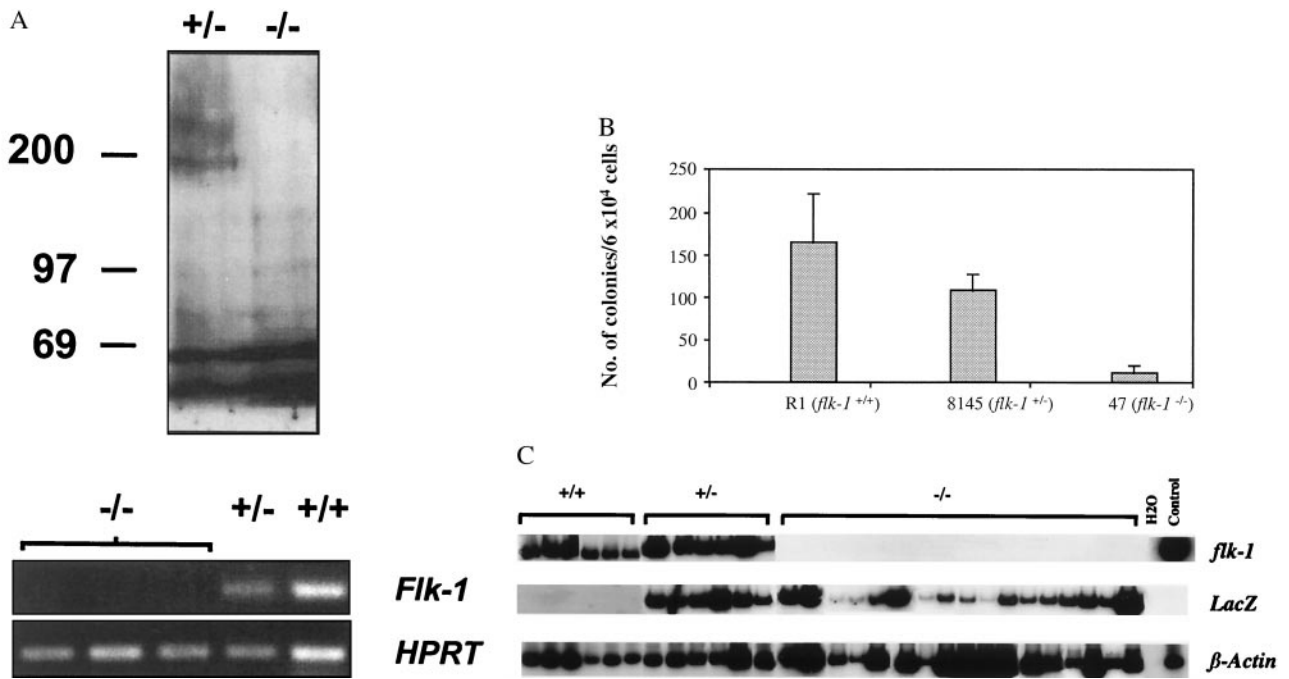


FIG. 1. (A) *flk-1*^{-/-} EBs do not express Flk-1 protein or *flk-1* RNA. Lysates of EBs derived from *flk-1*^{-/-} and +/- ES cells were analyzed by immunoprecipitation/immunoblotting by using polyclonal Flk-1 antiserum as described (19). The ≈200-kDa Flk-1-specific band is not seen in lysates from *flk-1*^{-/-} EBs (Upper). A Flk-1-specific band was also not detected in lysates of [³⁵S] methionine-labeled *flk-1*^{-/-} EBs, while it was readily detected in *flk-1*^{+/-}-derived samples (not shown). Total RNA was extracted from *flk-1*^{-/-}, +/-, and +/+ EBs, cDNA was prepared, and PCR was performed with *flk-1*-specific probes as described (10, 11, and see *Materials and Methods*). Whereas *flk-1*-specific bands were detected in *flk-1*^{+/-} and +/+ samples, they were not found in EBs derived from three independently derived *flk-1*^{-/-} ES clones (Lower). Control PCRs performed with RNA in the absence of reverse transcription were negative in all cases. *HPRT*-specific RT-PCR serves as an RNA/cDNA loading control. -/-, *flk-1*^{-/-}; +/-, *flk-1*^{+/-}; +/+, *flk-1*^{+/+}; numbers on left indicate position of molecular weight standards. (B) *flk-1*^{-/-} ES cells give rise to greatly reduced numbers of blast colonies. The number of blast colonies from day 2.75 *flk-1*^{-/-}, *flk-1*^{+/-}, and +/+ EBs is shown. Similar results were obtained from three independent replatings. An average number from one such replating, obtained from triplicate plates, is shown. Error bars indicate standard deviations. Whereas the number of blast colonies from *flk-1*^{+/-} EBs was somewhat lower than that from the *flk-1*^{+/+} EBs, the significance of the difference is not clear. R1, wild-type R1 ES cells; 8145, a *flk-1*^{+/-} ES cell line; 47, a *flk-1*^{-/-} ES cell line. (C) *flk-1* and *lacZ* expression in *flk-1*^{+/+}, +/-, and -/- blast colonies. Expression of *flk-1*, *lacZ*, and *β-actin* was determined by RT-PCR with ³²P dCTP added to the PCR reaction. Bands were then visualized by autoradiography. -/-, *flk-1*^{-/-}; +/-, *flk-1*^{+/-}; H₂O, PCR negative control. Day 6 EB cDNA, positive control.

VEGF (5 ng/ml) and D4T conditioned medium (25%). For the generation of endothelial cells, individual blast colonies were transferred to matrigel-coated (Collaborative Research) microtiter wells containing Iscove's modified Dulbecco's medium with 10% fetal calf serum (FCS) (HyClone), 10% horse serum (Biocell Laboratories), VEGF (5 ng/ml), insulin-like growth factor-1 (10 ng/ml), Epo (2 units/ml), basic fibroblast growth factor (10 ng/ml), interleukin-11 (50 ng/ml), KL (1% of the sup), endothelial cell growth supplement (100 ng/ml; Collaborative Research), L-glutamine (2 mM), and 4.5×10^{-4} M monothio glycerol (MTG) (Sigma). After 3 to 4 days of culture, nonadherent hematopoietic cells were removed by gentle pipetting, and the adherent cells were cultured for an additional 1–2 weeks in Iscove's modified Dulbecco's medium with 10% FCS (HyClone), 10% horse serum, VEGF (5 ng/ml), insulin-like growth factor-1 (10 ng/ml), basic fibroblast growth factor (10 ng/ml), endothelial cell growth supplement, 100 ng/ml; Collaborative Research), L-glutamine (2 mM), and 4.5×10^{-4} M MTG (Sigma) and then harvested by trypsinization for gene expression studies. EryP colonies were analyzed by replating day 4 EBs with plasma-derived serum (Antech; 10%), ascorbic acid (12.5 ng/ml), L-glutamine (2 mM), transferrin (300 ng/ml; Boehringer Mannheim), protein free hybridoma media II (GIBCO/BRL; 5%), and MTG (4.5×10^{-4} M) with erythropoietin (Amgen Biologicals, 2 units/ml). One ml of methylcellulose cultures containing 4×10^4 cells were put in 30 mm bacterial grade Petri dishes. EryP colonies were counted 4–6 days after replating. Other myeloid colonies were analyzed by replating day 9–10 EBs with plasma-derived serum (10%, Antech, Tyler, TX), ascorbic acid (12.5 ng/ml), L-glutamine (2 mM), transferrin (300 ng/ml; Boehringer Mann-

heim), protein-free hybridoma media II (GIBCO/BRL; 5%), and MTG (4.5×10^{-4} M) containing erythropoietin (2 units/ml), KL (1%), interleukin (IL)-3 (1%), IL-11 (25 ng/ml), granulocyte—macrophage colony-stimulating factor (CSF) (3 ng/ml), macrophage CSF (5 ng/ml), granulocyte CSF (30 ng/ml), IL-6 (5 ng/ml), LIF (1 ng/ml), and VEGF (5 ng/ml). Hematopoietic colonies were counted 7–10 days after the replating. IL-11, granulocyte—macrophage CSF, macrophage CSF, IL-6, LIF, and VEGF were purchased from R & D Systems. KL was obtained from medium conditioned by Chinese hamster ovary cells transfected with a KL expression vector (kindly provided by Genetics Institute) or purchased from R & D Systems. IL-3 was obtained from medium conditioned by X63 Ag8–653 myeloma cells transfected with a vector expressing IL-3 (9).

Gene Expression Analysis. RNA isolation and reverse transcription—PCR (RT-PCR) were performed as described (10, 11). All RNA samples were treated with DNase I (amplification grade from GIBCO/BRL) before cDNA synthesis to eliminate any contaminating genomic DNA. Specific primers used are as follows (5). Primers for *hprt*, β major, and β H1 span the intron, such that ≈1,100 bp (*hprt*), 1,296 bp (β major), and 1,053 bp (β H1) genomic DNA fragment will be amplified. None of the samples showed genomic DNA amplification (not shown). The size of PCR products is indicated in parentheses.

hprt, sense, 5'-GCTGGTGAAAAGGACCTCT-3'; antisense, 5'-CACAGGACTAGAACACCTGC-3' (249 bp). β -actin, sense, 5'-ATGAAGATCCTGACCGAGCG-3'; antisense 5'-TACTTGCCTCAGGACGGA GC-3' (443 bp). *flk-1*, sense, 5'-GCCCTGAGTCCTCAGGAC-3'; antisense, 5'-GGTCTCCACGCAGAAC-3' (320 bp). *lacZ* (*flk-1*), sense, 5'-

GCCCTGAGTCCTCAGGAC-3'; antisense, 5'-AAAGCGC-CATTCTGCCATTCA-3' (320bp). α -globin, sense, 5'-CTCTCTGGGGAAGACAAAAGCAAC-3'; antisense, 5'-GGTGCTAGCCAAGTCCACCAGCA-3' (331 bp). *flt-1*, sense, 5'-TGTGGAGAACTTGGTGACCT-3'; antisense, 5'-TG-GAGAACAGCAGGACTGATGAT-3' (504 bp). β H1, sense, 5'-AGTCCCCATGGAGTCAAAGA-3'; antisense, 5'-CTC-AAGGAGACCTTTGCTCA-3' (265 bp). β major, sense, 5'-CTGACAGACTCTCTTGGG-3'; antisense, 5'-CACAA-CCCCAGAAACAGACA-3' (578 bp). *tie-1*, sense, 5'-GTC-ACTGCCCTCCTGACTGG-3'; antisense, 5'-CGATGTACT-TGGATATAGGC-3' (208 bp). PECAM-1, sense, 5'-GTCA-TGGCCGTCGAGTA-3'; antisense, 5'-CTCCTCGGCATCT-TGCTGAA-3' (260 bp). *tie-2*, sense, 5'-TTGAAGT-GACGAATGAGAT-3'; antisense, 5'-ATTTAGAGCTGTC-TGGCTGT-3' (197 bp). VWF, sense, 5'-GTGGTGGC-ATGATGGAGAGTTA-3'; antisense, 5'-GCAAGGTCAC-AGAGGTAGCTGACT-3' (484 bp).

Fluorescein di- β -D-galactopyranoside (FDG)/Fluorescence-Activated Cell Sorter Analysis. EBs were trypsinized to produce a single-cell suspension. One $\times 10^6$ cells were resuspended in PBS/10% FCS (HyClone)/10 mM Hepes, pH 7.2/300 μ M chloroquine and incubated at 37°C for 30 min. Prewarmed 2 mM FDG (Molecular Probes) was added in equal volume to the cells and incubated for exactly 1 min. The FDG loaded into the cells (hypotonic state) and an isotonic shock was introduced with the addition of 1.8 ml of ice-cold staining medium [PBS/10% HyClone FBS/300 μ M chloroquine/1 mM phenylethyl β -D-thiogalactopyranoside (PETG) and 1 μ g/ml propidium iodide]. Cells were maintained at 4°C and in the dark until analysis. Endogenous β -galactosidase present in the lysosome was inhibited by pretreatment with chloroquine. In addition, a competitive inhibitor, PETG, was added to stop the reaction by outcompeting FDG from being cleaved by β -galactosidase.

Embryo Manipulation. Embryos were removed at 7.5 dpc (noon on the day of the vaginal plug is 0.5 days) and were processed immediately for methylcellulose hematopoietic colony assay with factors described above. At the time of dissection, ectoplacental cone and trophoblast cells corresponding to each embryo were cultured in ES cell medium without LIF for 10–14 days to ensure loss of maternal cells. Embryos were then genotyped by Southern blot analysis of cultured cell DNA (not shown).

RESULTS

***flk-1*^{-/-} EBs Give Rise to Reduced Numbers of Blast Colonies.** As previously described (4), *flk-1*^{-/-} ES cells were derived by G418 selection from *flk-1*^{+/-} cells produced by a gene-targeting event in which the *lacZ* gene was placed under the transcriptional control of *flk-1* regulatory elements by insertion into the first exon of *flk-1*. β -galactosidase activity therefore acts as a histochemical marker of Flk-1 expression. To ensure that the formation of hematopoietic or endothelial cells *in vitro* could not be attributed to contamination of

Table 1. Kinetic analysis of β -galactosidase-expressing cells in *flk-1*^{+/+}, *flk-1*^{+/-}, and *flk-1*^{-/-} EBs

Exp		D2.75	D4	D6	D9
1	<i>flk-1</i> ^{+/-}	3.3		31.97	18.83
	<i>flk-1</i> ^{-/-}	4.6		47.26	18.75
2	<i>flk-1</i> ^{+/-}	3.9	18.9	23.9	
	<i>flk-1</i> ^{-/-}	2.8	19.0	24.3	

β -galactosidase-expressing cells were stained with FDG and quantified by FACS. The % of β -galactosidase-expressing cells present in day 2.75–9 EBs in two separate representative experiments is shown. The percentage of β -galactosidase-expressing cells in *flk-1*^{+/-} EBs was similar to that of Flk-1-expressing cells as judged by Flk-1 monoclonal antibody staining (ref. 20, not shown).

flk-1^{-/-} cells with +/- or +/+ cells, we first verified that *in vitro*-differentiated *flk-1*^{-/-} EBs did not produce Flk-1 and did not contain *flk-1* transcripts (Fig. 1A).

Because the combined hematopoietic/endothelial defect observed in *flk-1*^{-/-} embryos was consistent with impaired hemangioblast formation, we anticipated that the BL-CFC assay might allow the developmental consequences of Flk-1 deficiency during the very early VEGF-dependent stage of hematopoiesis and vasculogenesis to be analyzed quantitatively. We therefore assessed the ability of *flk-1*^{-/-}, +/-, and +/+ ES cells to give rise to BL-CFCs, an *in vitro* measure of hemangioblast potential. When blast colony numbers were compared, far fewer blast colonies were obtained from *flk-1*^{-/-} EB cells compared with *flk-1*^{+/-} and +/+ cells (Fig. 1B). Moreover, the blast colonies produced from *flk-1*^{-/-} ES cells were much smaller than those from +/- and +/+ cells (data not shown), suggesting that the proliferative capacity of blast cells is limited in the absence of Flk-1.

Because blast colonies are believed to arise from Flk-1-expressing progenitors in a VEGF-dependent manner, it was unclear how they could have developed in *flk-1*^{-/-} EBs. Because it was possible that the blast colonies from *flk-1*^{-/-} EBs could have originated from Flk-1-negative progenitors in response to some other factor(s) present in the blast colony replating medium, the cell origin of blast cells from *flk-1*^{-/-} EBs was determined. We therefore analyzed individual *flk-1*^{+/+}, +/-, and -/- blast colonies (28, 23, and 23, respectively) for *flk-1* and β -galactosidase expression. By virtue of the *lacZ* gene-targeting event that underlies *flk-1* inactivation, Flk-1-deficient cells that express β -galactosidase would normally express *flk-1*. As illustrated in Fig. 1C, all *flk-1*^{+/+} and *flk-1*^{+/-} blast colonies expressed *flk-1*. In addition, all *flk-1*^{+/-} blast colonies analyzed expressed *lacZ*. Whereas *flk-1*^{-/-} blast colonies did not, as expected, express *flk-1*, they did however express *lacZ*. Thus, it is most likely that blast colonies from *flk-1*^{-/-} EBs have arisen from the same Flk-1 (or β -galactosidase)-expressing precursors as have their +/- and +/+ counterparts.

Flk-1-Deficient Cells Proliferate Normally Within *flk-1*^{-/-} EBs. Whereas the reduced number of blast colonies found in *flk-1*^{-/-} EBs was consistent with impaired BL-CFC formation in

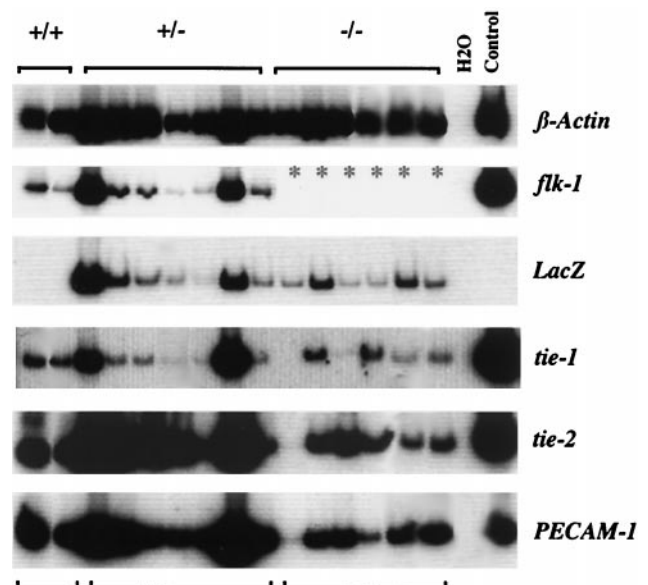


FIG. 2. Expression of endothelial markers in blast colony-derived adherent cells. RNA obtained from adherent cells generated from *flk-1*^{+/+}, +/-, and -/- blast colonies was analyzed by RT-PCR for the expression of *flk-1*, *lacZ*, and other endothelial transcripts. *c-fms* expression level was high in the first sample in the -/- series (lane 10 from the left, not shown). *, not done. H₂O, PCR negative control. Day 6 EB RNA, positive control.

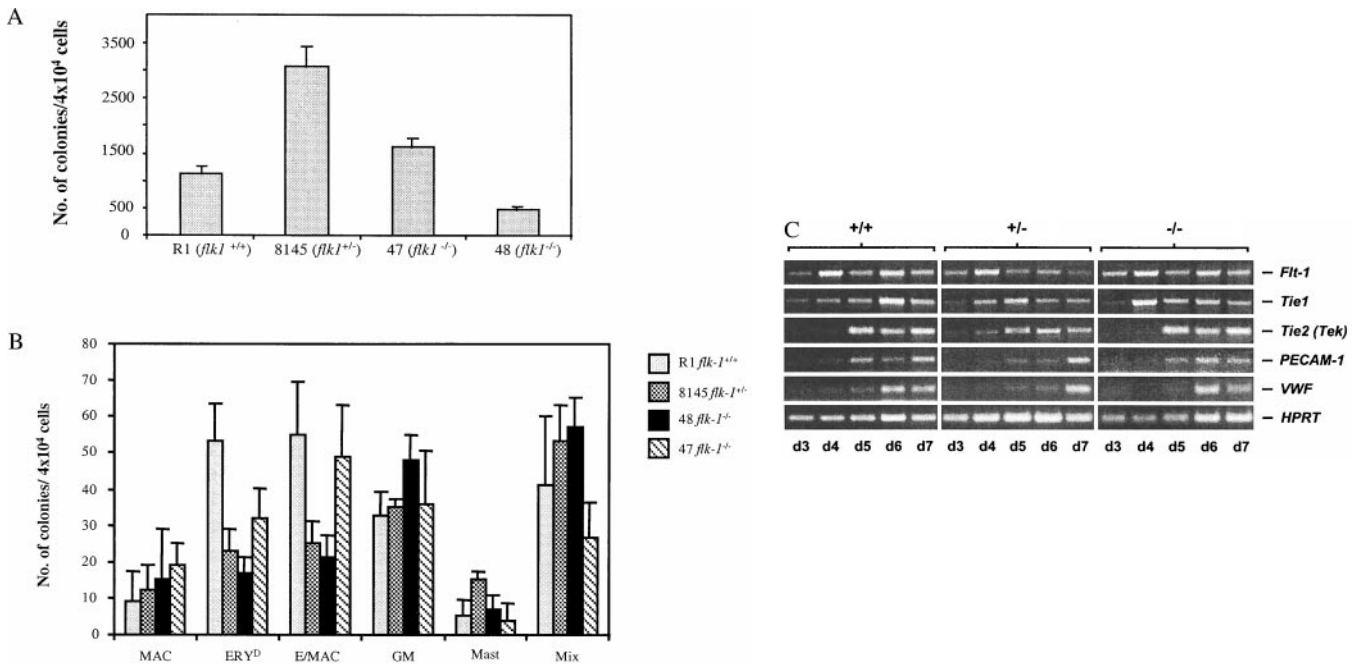


FIG. 3. (A) *flk-1*^{-/-} ES cells give rise to EryP colonies *in vitro*. EryP progenitors were quantified from day 4 *flk-1*^{-/-}, +/− and +/+ EBs using methylcellulose colony assay. R1, wild-type R1 ES cells; 8145, a *flk-1*^{+/-} line; 47/48, two independent *flk-1*^{-/-} ES cell lines. Results obtained from three independent replatings were similar. An average number from one such replating, obtained from triplicate plates, is shown. Error bars indicate standard deviations. The difference in the number of EryP colonies between 47 and 48 could reflect clonal variations. (B) *flk-1*^{-/-} ES cells give rise to definitive erythroid and myeloid progenitors. Types of day 9 colonies produced: MAC, macrophage; ERY^D, definitive erythroid; Ery/MAC, mixed erythroid/macrophage; GM, granulocyte/macrophage; Mast, mast cell; Mix, mixed myeloid. Results obtained from three independent replatings were similar. An average number from one such replating, obtained from triplicate plates, is shown. Error bars indicate standard deviations. In all cases, while clonal variation is observed among cell lines used, overall, the hematopoietic potential of *flk-1*^{-/-} cells was indistinguishable from that of their +/− and +/+ counterparts. (C) Appearance of endothelial marker genes during differentiation of *flk-1*^{-/-}, +/−, +/+ ES cells. RT-PCR was performed with total RNA prepared from timed EBs, but with primers specific to a series of endothelial marker genes. The appearance of endothelial markers in *flk-1*^{-/-} day 3–day 7 EBs is indistinguishable from that of their +/− and +/+ counterparts. Marker gene expression was not detectable on day 0 (not shown). Transcripts detected are indicated on the right; d3–d7, days of differentiation; −/−, *flk-1*^{-/-}; +/−, *flk-1*^{+/-}; +/+, *flk-1*^{+/+}.

the absence of Flk-1, it was also possible that *flk-1*^{-/-} BL-CFCs have developed normally, but then have failed to respond to VEGF to form blast colonies. The observation that *flk-1*^{-/-}, +/−, and +/+ EBs were indistinguishable in size (data not shown) suggested that Flk-1-deficient cells were capable of extensive proliferation within *flk-1*^{-/-} EBs in non-VEGF-dependent manners. To determine whether Flk-1-deficient cells developed normally within *flk-1*^{-/-} EBs, we used β -galactosidase expression to identify Flk-1-deficient (but β -galactosidase⁺) cells within *flk-1*^{-/-} EBs. β -galactosidase-expressing cells were quantified in days 2.75–9 *flk-1*^{-/-} and +/− EBs by fluorescence-activated cell sorter analysis by using the fluorescein-conjugated β -galactosidase substrate FDG (12, 13). As shown in Table 1, *flk-1*^{-/-} and +/− β -galactosidase⁺ cell numbers were similar in day 2.75 EBs and increased in parallel with ongoing EB differentiation. Thus, Flk-1-deficient precursors were able to proliferate normally within *flk-1*^{-/-} EBs. We concluded therefore that the reduction of blast colony number and size was most likely because of the failure of Flk-1-deficient BL-CFCs to proliferate in response to VEGF when they are replated in methylcellulose cultures.

The Developmental Potential of Flk-1-Deficient (*lacZ*-Expressing) Blast Cells *in Vitro* Is Not Impaired. Because blast cells have been shown to give rise to both hematopoietic and endothelial cells (5), we next determined whether Flk-1-deficient blast cells retained this ability. Individual blast colonies were therefore transferred from methylcellulose to liquid culture containing both hematopoietic and endothelial cell growth factors (5). Sixty-nine of 156 (44%) and 29 of 72 (40%) of blast colonies from day 2.75 *flk-1*^{+/+} and *flk-1*^{+/-} EBs, respectively, gave rise to both hematopoietic and endothelial

cells. More importantly, 92 of 280 (33%) of *flk-1*^{-/-} blast colonies, from day 2.75, also gave rise to both lineages, as assessed by the formation of hematopoietic cells with signs of hemoglobinization and adherent cells. The adherent cells that developed from *flk-1*^{-/-} blast colonies were indistinguishable in morphology from those from +/+ or +/− blast cells and all expressed *PECAM-1*, *tie-1*, and *tie-2*, characteristic of endothelial cells (Fig. 2). Furthermore, while adherent cells derived from *flk-1*^{+/+} and +/− blast colonies expressed *flk-1*, all *flk-1*^{-/-}-derived cells expressed *lacZ*. BL-CFCs found in *flk-1*^{-/-} EBs therefore retain the potential for both endothelial and hematopoietic differentiation. Thus, Flk-1 expression is not an absolute requirement for the formation, or differentiation, of BL-CFCs/hemangioblasts.

***flk-1*^{-/-} ES Cells Are Capable of Hematopoietic and Endothelial Differentiation *in Vitro*.** Based on the observation that *flk-1*^{-/-} ES cells were able to give rise to BL-CFCs that retained dual-lineage hemangioblast potential, we proceeded to determine the extent to which hematopoietic/endothelial differentiation potential was preserved. We therefore quantified hematopoietic progenitors found in day 4 and day 9 *flk-1*^{-/-}, +/−, and +/+ EBs (14). As shown in Fig. 3A, *flk-1*^{-/-} EBs contained a similar number of EryP progenitors compared with those of *flk-1*^{+/+} and *flk-1*^{+/-} EBs. Furthermore, in contrast to the profound hematopoietic deficiency observed in 8.5 dpc *flk-1*^{-/-} embryos (3), the ability of *flk-1*^{-/-} ES cells to give rise to definitive erythroid and to macrophage/myeloid progenitors was indistinguishable from that of their heterozygous counterparts (Fig. 3B). Thus, the hematopoietic differentiation of *flk-1*^{-/-} ES cells *in vitro* did not recapitulate the block to hematopoiesis previously observed in Flk-1-deficient embryos (3) and did not

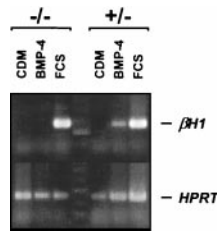


FIG. 4. Flk-1-deficient ES cells do not initiate β HI globin expression under BMP-4-induced serum free differentiation conditions. *flk-1*^{-/-} and +/- ES cells were differentiated in CDM in the presence or absence of added FCS or BMP-4, as described. Specifically, CDM contained Iscove's modified Dulbecco's medium plus Ham's F-12 medium (both from GIBCO/BRL, Life Technologies, Grand Island, NY) at a 1:1 ratio, and supplemented with Glutamax-I (GIBCO/BRL)/BSA, 5 mg/ml (Boehringer Mannheim)/1X lipids (100 × chemically defined lipid concentrate; GIBCO/BRL)/human transferrin, 15 μg/ml (Boehringer Mannheim)/monothioglycerol, 450 μM (Sigma)/insulin, 7 μg/ml (Boehringer Mannheim)/LIF, 1 units/ml (GIBCO/BRL). Expression of β HI embryonic globin transcripts was detected by RT-PCR 5 days after differentiation. Whereas differentiating *flk-1*^{-/-} and +/- ES cells both expressed β HI in the presence of added FCS, only *flk-1*^{+/-} cells did so after the addition of BMP-4. Identical results were obtained in six separate experiments performed with two independent *flk-1*^{-/-} ES cell lines (not shown). The absence of β HI expression in *flk-1*^{-/-} CDM+ and BMP-4+ lanes was confirmed by Southern blotting with an internal β HI-specific oligonucleotide (not shown). -/-, *flk-1*^{-/-}; +/-, *flk-1*^{+/-}.

appear to manifest the cell autonomous hematopoietic defect seen in chimeric aggregation studies (4).

In parallel with this study, we also examined the *in vitro* endothelial differentiation potential of *flk-1*^{-/-} ES cells. Embryonic vascular development is characterized both *in vivo* and *in vitro* by the sequential activation of a series of "endothelial-specific" genes in a manner that is believed to reflect successive endothelial maturation steps (15). We therefore examined the expression of a series of such endothelial marker genes by RT-PCR at sequential time points of *in vitro* differentiation. As shown in Fig. 3C, with the exception of *flk-1* transcripts, which were absent, the appearance of *flt-1*, *tie-1*, *tek* (*tie-2*), *PECAM-1*, and *VWF* transcripts during *flk-1*^{-/-} ES cell differentiation was indistinguishable from that observed in *flk-1*^{+/-} and +/+ EBs. Notably, *flk-1*^{-/-} EBs contained *tie-1* transcripts, which were absent in Flk-1-deficient embryos (3). Thus, as we had already observed in our hematopoietic assays, Flk-1-deficient endothelial differentiation proceeded further *in vitro* than *in vivo*, as well.

The *In Vitro* Hematopoietic Potential of *flk-1*^{-/-} ES Cells Is Assay Dependent. To determine whether the *in vitro* hematopoietic and endothelial potential of *flk-1*^{-/-} ES cells might somehow be related to the specific culture conditions used, we next assessed their hematopoietic potential by using serum-free conditions (16). In this system, ES cells are differentiated in chemically defined medium (CDM) in the absence of serum, thereby avoiding the potentially confounding influence of factors found in serum. Hematopoietic differentiation can be triggered by the addition of bone morphogenetic protein-4 (BMP-4), which induces the formation of postero-ventral mesoderm, and the onset of hematopoiesis is scored by the detection of embryonic globin (β HI) mRNA by RT-PCR. As shown in Fig. 4, the addition of serum to *flk-1*^{+/-} and -/- ES cells differentiating in CDM resulted in the induction of β HI expression. In addition, as expected, differentiation in CDM in the absence of BMP-4 did not result in β HI expression by either cell type. The addition of BMP-4, however, induced β HI expression in *flk-1*^{+/-} cells but not in their -/- counterparts. Thus, under these conditions, Flk-1-deficient ES cells did appear to manifest a hematopoietic defect that could be identified *in vitro*.

Hematopoietic Progenitors Are Present in 7.5-dpc *flk-1*^{-/-} Embryos. Previous studies indicated that 8.5-dpc *flk-1*^{-/-} embryos contained virtually no hematopoietic progenitors (3). Stud-

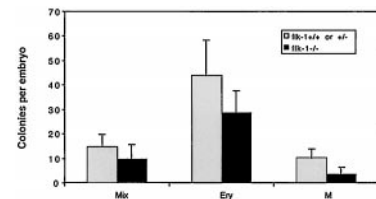


FIG. 5. 7.5-dpc *flk-1*^{-/-} embryos contain hematopoietic progenitors. 7.5-dpc embryos (resulting from *flk-1*^{+/-} × +/- matings) were treated with 0.25% collagenase/20% FCS, and cells were then plated in 1% methylcellulose in the presence of insulin (10 ng/ml), erythropoietin (2 units/ml), KL (100 ng/ml), IL-1 (650 units/ml), IL-3 (100 units/ml), granulocyte—macrophage CSF (50 units/ml) and macrophage CSF (100 units/ml) as described. Embryos were genotyped by Southern blot analysis of cultured ectoplacental cone and trophoblast cells (ref. 3; data not shown). Colonies were scored after 7 and 10 days of incubation. Mix, mixed colony; Ery, erythroid colony; M, myeloid/macrophage colony. Results from total eight embryos that are +/- or -/- and four embryos that are +/+ are shown.

ies of chimeric mice generated by the aggregation of *flk-1*^{-/-} and +/+ ES cells revealed that Flk-1-deficient cells manifest a cell autonomous defect in hematopoiesis. Analysis of these chimeric embryos also suggested that this defect might relate to the failure of Flk-1-deficient cells to migrate appropriately from the posterior primitive streak to the extra-embryonic site of blood island formation and possibly also to intraembryonic sites of early hematopoiesis (4). If *Flk-1*^{-/-} cells truly failed to migrate to establish hematopoiesis and angiogenesis in the yolk sac, one would predict that it might be possible to rescue cells with hematopoietic potential from earlier embryos. We therefore isolated and genotyped 7.5-dpc embryos by Southern blot analysis of cultured ectoplacental cone and trophoblast cells (data not shown) and quantified erythroid, mixed, and myeloid hematopoietic progenitors as had previously been done at 8.5 dpc (3). As shown in Fig. 5, 7.5-dpc embryos contained hematopoietic progenitors of all types, in numbers only slightly lower than those of their +/- and +/+ counterparts. Thus, Flk-1-deficient embryos have the potential to initiate hematopoietic differentiation pathways.

DISCUSSION

In this report, we show that although *flk-1*^{-/-} ES cells manifest a cell autonomous developmental defect *in vivo*, depending on the culture conditions used, they are able to give rise to primitive and definitive hematopoietic cells, endothelial cells, and to common hematopoietic/endothelial precursors *in vitro*. Furthermore, we demonstrate that *flk-1*^{-/-} embryos do have the potential to produce hematopoietic (and endothelial) cells, although this ability is not normally realized *in vivo*. Cells capable of contributing to hematopoiesis do arise in *flk-1*^{-/-} embryos, but they are detectable only during a brief developmental window before 8.5 dpc. Thereafter, such cells are no longer found. How can these disparate observations be reconciled? Our data are most consistent with the notion that common endothelial/hematopoietic precursors arise independently of a Flk-1 signal. In the absence of a functional Flk-1 receptor, however, such potential hematopoietic/endothelial cells are unable to complete this differentiation pathway: First, the observation that 7.5-dpc *flk-1*^{-/-} embryos contain essentially normal numbers of hematopoietic progenitors, while 8.5-dpc yolk sacs contain practically none, is consistent with the idea that Flk-1-deficient precursors have failed to migrate appropriately to sites of future hematopoiesis and therefore have failed to receive the appropriate inductive or survival signals required for blood formation. This view is consistent with the reported accumulation of Flk-1-deficient cells in non-extraembryonic mesoderm, such as amnion, of *flk-1*^{-/-/+} chimeric embryos (4) and is further supported by the recent demonstration that VEGF functions as a chemoattractant for angioblast migration in *Xenopus* (17). Second, the reduced num-

ber and size of Flk-1-deficient colonies produced in the VEGF-dependent BL-CFC assay, contrasted with the essentially normal proliferation of Flk-1-deficient precursors during the formation of *flk-1*^{-/-} EBs, suggests that the proliferation of Flk-1-deficient precursors may be impaired *in vivo* as well.

Because *flk-1*^{-/-} ES cells could also differentiate and give rise to hematopoietic cells on OP9 stromal cells (not shown; ref. 18), it appears, at least in part, that serum may contain factor(s) that can deliver a VEGF/Flk-1 equivalent or bypassing signal *in vitro*, thereby promoting differentiation or proliferation or preventing cell death. This model requires that migration- and differentiation-blocked Flk-1-deficient cells are nevertheless mature enough to respond to such signals *in vivo*. The ability of 7.5-dpc *flk-1*^{-/-} embryos to give rise to hematopoietic colonies with kinetics that are similar to those of +/+ or +/- embryos supports this idea. The nature of this serum effect remains to be defined. We believe, however, that it is likely not mediated by an alternative VEGF receptor, Flt1. Whereas *flk-1*^{-/-} EBs and blast cells do express *flt-1* transcripts (Fig. 3C and data not shown), as do *flk-1*^{-/-} embryos (3), not only has Flt1 not been shown to mediate a proliferative signal, but the addition of VEGF and/or placenta growth factor (PIGF), a ligand for Flt-1, does not rescue Flk-1-deficient hematopoiesis under serum-free conditions (not shown). Furthermore, the addition of PIGF to blast colony replating media does not enhance blast colony formation (not shown).

Whereas total Flk-1 inactivation is known to result in lethal impairment of vascular and hematopoietic development (3), the role of specific Flk-1 receptor/substrate interactions in mediating hematopoietic/endothelial progenitor migration, proliferation, and differentiation remains obscure. The ability to isolate Flk-1-expressing progenitors and to generate ES cells and animals bearing more subtle Flk-1 mutations that interrupt individual receptor/substrate interactions, while leaving others intact, may provide insight into these questions.

We thank Wendy Horsfall, Fouad Shalaby, and Xiang-Fu Wu for technical assistance. We also thank Daved Fremont, Christopher Nelson, and Doreen Salli for critical comments on the manuscript. This work was supported by grants from the Medical Research Council of Canada and The Bayer/Canadian Red Cross Society Research and Development Fund (to A.C.S.) and from the National Institutes of Health (to K.C.).

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