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Basic fibroblast growth factor positively regulates hematopoietic development

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SUMMARY

Recently identified BLast Colony Forming Cells (BL-CFCs) from in vitro differentiated embryonic stem (ES) cells represent the common progenitor of hematopoietic and endothelial cells, the hemangioblast. Access to this initial cell population committed to the hematopoietic lineage provides a unique opportunity to characterize hematopoietic commitment events. Here, we show that BL-CFC expresses the receptor tyrosine kinase, Flk1, and thus we took advantage of the BL-CFC assay, as well as fluorescent activated cell sorter (FACS) analysis for Flk1$^+$ cells to determine quantitatively if mesoderm-inducing factors promote hematopoietic lineage development. Moreover, we have analyzed ES lines carrying targeted mutations for fibroblast growth factor receptor-1 (*fgfr1*), a receptor for basic fibroblast growth factor (bFGF), as well as *scl*, a transcription factor, for their potential to generate BL-CFCs and Flk1$^+$ cells, to further define events leading to hemangioblast development. Our data suggest that bFGF-mediated signaling is critical for the proliferation of the hemangioblast and that cells expressing both Flk1 and SCL may represent the hemangioblast.

Key words: Hemangioblast, Hematopoiesis, Vasculogenesis, bFGF, BL-CFC, Flk1, scl.

INTRODUCTION

The establishment of blood islands in the extra-embryonic yolk sac marks the onset of hematopoiesis and vasculogenesis in the developing embryo. These blood islands develop from aggregates of mesodermal cells that colonize the presumptive yolk sac at approximately 7 days post-coitum (dpc). Despite great interest in understanding signals/factors governing the initiation of hematopoietic differentiation in the developing embryo, very little knowledge exists regarding mechanisms involved in hematopoietic specification. Most likely, factors involved in mesoderm induction/patterning events, such as bFGF, activin A, and bone morphogenetic proteins (BMPs), also regulate hematopoietic specification. However, methods previously available to address such an issue have been limited. For example, the use of mice lacking mesoderm-inducing factor genes to delineate the roles of specific function in hematopoietic commitment is complicated by early embryonic lethality prior to blood formation (Deng et al., 1994; Yamaguchi et al., 1994; Mishina et al., 1995; Winnier et al., 1995). Furthermore, tools to study hematopoietic commitment, i.e. the origin of the initial cell population committed to hematopoietic cell lineage, have also been limited.

In vitro differentiated ES cells (reviewed by Keller, 1995) are valuable for the study of early hematopoietic lineage differentiation, as demonstrated by the identification of the very primitive, hematopoietic committed cells which otherwise have been elusive in embryos (Choi et al., 1998). Blast-colony-forming cells (BL-CFCs), generated from in vitro differentiated progeny (embryoid bodies, EBs) of embryonic stem (ES) cells, represent the long sought common progenitor of hematopoietic and endothelial cells, the hemangioblast (Sabin, 1920; Murray, 1932; Wagner, 1980). They form blast colonies in response to vascular endothelial growth factor (VEGF) in methylcellulose cultures. Cells within the blast colonies, blast cells, express a set of genes common to both hematopoietic and endothelial lineages, including *scl*, *CD34* and the VEGF receptor, *Flk1*. Most importantly, primitive, definitive hematopoietic, and endothelial cell progenitors are present within blast cells as revealed by replating in medium containing both hematopoietic and endothelial cell growth factors (Choi et al., 1998; Kennedy et al., 1997).

Several studies suggest that mesoderm-inducing factors are able to affect EB development, as well as hematopoietic differentiation. For example, the addition of bFGF or activin A to differentiating EBs enhances *brachyury* gene expression, a marker for mesodermal tissue (Yamada et al., 1994). This observation suggests that cells within EBs can respond to

external signals, and therefore argues that one could utilize this system for examining factors involved in hematopoietic specification. Johansson and Wiles (1995) have demonstrated that BMP4 induced the expression of the embryonic globin gene, *βH1*, from day-5 EBs generated in serum free, chemically defined medium (CDM). Similarly, Huber et al. (1998) demonstrated that BMP4 in combination with activin A or bFGF induces the generation of erythroid cells in *Xenopus* animal cap cultures. These studies suggest that hematopoietic development could be governed by a combination of several, rather than individual, mesoderm inducing factors.

Since BL-CFCs represent the earliest committed hematopoietic precursors so far identified, they provide an ideal model for studying factors that regulate the onset of hematopoietic development. In this study, we investigated the effect of bFGF and Activin A on the generation of the BL-CFC and Flk1$^+$ cells and analyzed *fgfr1*$^{-/-}$ and *scl*$^{-/-}$ ES cells to further characterize factors/signals regulating hemangioblast development. Our results suggest that bFGF or Activin A, singularly or in concert, positively regulate hematopoietic development and that Activin A-mediated BL-CFC generation requires bFGF-mediated signals. Our studies also suggest that the hemangioblast expresses both Flk1 and SCL.

MATERIALS AND METHODS

ES differentiation, BL-CFC, and hematopoietic progenitor analysis

ES cells were maintained on STO feeder cells in the presence of LIF. EBs were generated as described by Choi et al. (1998). Basic FGF or activin A was added at the initiation of EB differentiation. The resulting EBs (day-2.75) were analyzed for the blast colonies, as well as, FACS analysis for Flk1$^+$ cells. Basic FGF was purchased from Upstate Biotechnology (Lake Placid, NY), and activin A was kindly provided by Dr A. Parlow at NIDDK's National Hormone & Pituitary Program.

Blast colonies, primitive erythroid, and myeloid colonies were generated as described previously (Keller et al., 1993; Choi et al., 1998). Briefly, blast colonies were generated from day-2.75-3 EBs in the presence of VEGF (5 ng/ml), KL (1% conditioned medium or 100 ng/ml purified) and D4T conditioned medium (CM) at 25%. Primitive erythroid progenitors from day-4 EBs were analyzed in the presence of plasma derived serum (PDS, Antech; 10%), ascorbic acid (12.5 ng/ml), L-glutamine (2 mM), transferrin (300 μg/ml; Boehringer Mannheim), protein free hybridoma media II (PFHMII, Gibco-BRL; 5%), and MTG (4.5×10^{-4} M) with erythropoietin (Amgen, 2 units/ml). EryP colonies were counted 4-6 days after replating. Other myeloid progenitors were analyzed by replating day-9 to -10 EBs in PDS (10%, Antech), ascorbic acid (12.5 ng/ml), L-glutamine (2 mM), transferrin (300 μg/ml; Boehringer Mannheim), protein free hybridoma medium II (PFHMII, Gibco-BRL; 5%), and MTG (4.5×10^{-4} M) containing Epo (2 units/ml), KL (1%), IL-3 (1%), IL-1 (10 ng/ml), IL-11 (25 ng/ml), GM-CSF (3 ng/ml), M-CSF (5 ng/ml), G-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-1, IL-11, GM-CSF, M-CSF, IL-6, LIF and VEGF were purchased from R&D Systems. KL was obtained from medium conditioned by CHO cells transfected with a KL expression vector (kindly provided by Genetics Institute) or purchased from R&D Systems. Epo was purchased from Amgen (Thousand Oaks, CA). IL-3 was obtained from medium conditioned by X63 Ag8-653 myeloma cells transfected with a vector expressing IL-3 (Karasuyama and Melchers, 1988).

FACS analysis

For Flk1 monoclonal antibody staining (Kabrun et al., 1997), EBs were treated with trypsin/EDTA for 3 minutes, cells were passed through a 20-gauge needle to produce a single cell suspension, counted, and centrifuged. Cells were resuspended to a concentration of 5×10^6 cells/ml in 2.4G2 supernatant/2% HI rat serum to block antibody from binding to Fc gamma 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 anti-Flk1 antibody freshly diluted in wash buffer (20%FCS/PBS/0.1% sodium azide) was added and incubated on ice for 15 minutes. Cells were washed 3 times with 2% FCS/PBS/0.1% sodium azide. Streptavidin-phycoerythrin (secondary reagent) (Pharmingen) freshly diluted in wash buffer was added and incubated on ice in the dark for 10 minutes. Cells were washed for a total of 3 times, resuspended in wash buffer and transferred to 5 ml polypropylene tubes for analysis. Cells were analyzed on a FACSCaliber (Becton-Dickinson). FACS data were analyzed with CellQuest software (Becton-Dickinson). For Flk1$^+$ cells sorting, cells were treated the same except that they were filtered through 40 μm nylon mesh prior to sorting. Flk1$^+$ cells were sorted using FACSVantage (Becton-Dickinson).

β-galactosidase activity within *Flk1*$^{+/-}$ EBs was measured as described by Schuh et al. (1999). Briefly, EBs were trypsinized to produce a single cell suspension. 1×10^6 cells were resuspended in PBS, 10% FCS (Hyclone), 10 mM Hepes, pH 7.2 and 300 μM chloroquine and incubated at 37°C for 20 minutes. Pre-warmed 2 mM FDG (Molecular Probes) was added in equal volume to the cells and incubated for exactly 1 minute. The FDG was 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 μM chloroquine, 1 mM phenylethyl β-D-thiogalactopyranoside (PETG) and 1 μg/ml propidium iodide (PI)). Cells were maintained at 4°C in the dark until analysis. Endogenous β-galactosidase present in the lysosome was inhibited by pre-treatment with chloroquine. In addition, the competitive inhibitor, PETG, was added to stop the reaction by outcompeting FDG from being cleaved by β-galactosidase.

For endothelial cell marker staining, EBs were grown in methylcellulose or liquid culture, collected and treated with collagenase (0.25%/20%FCS/PBS) for 1 hour and gently disassociated to single cells by passage through a 20 gauge needle. After the Fc receptor blocking step (see above) cells were stained with the appropriate primary antibody. The following antibodies were used in these experiments: biotinylated anti-Flk1 (89B3A5), biotinylated anti-PECAM-1 (MEC 13.3; Pharmingen), anti-P-selectin (RB40.34; Pharmingen), anti-VE-cadherin (11D4.1; Pharmingen) and anti-CD34-FITC (RAM34; Pharmingen). The secondary reagent for the unconjugated antibodies was goat anti-rat IgG-FITC (Caltag Laboratories).

Gene expression analysis

RNA isolation and RT-PCR were performed as described by Chomczynski and Sacchi (1987); Chelly et al. (1988). All RNA samples were treated with DNaseI (amplification grade from Gibco/BRL) before cDNA synthesis to eliminate any contaminating genomic DNA. Specific primers used are as follows (Keller et al., 1993; Johansson and Wiles, 1995).

HPRT, sense, 5′CACCTGGCACTCTCCACCTTC3′; antisense, 5′GCTGGTGAAAAGGACCTCT3′.

rex1, sense, 5′CGTGTAACATACACCATCCG3′, antisense, 5′GAAATCCTCTTCCAGAATGG3′.

fgf5, sense 5′GGCAGAAGTAGCGCGAGC3′; antisense 5′CCGTAAATTTGGCACTTG3′.

nodal, sense, 5′CCGTCCCCTCTGGCGTACATG3′; antisense 5′GACCTGAGAAGGAATGACGG3′.

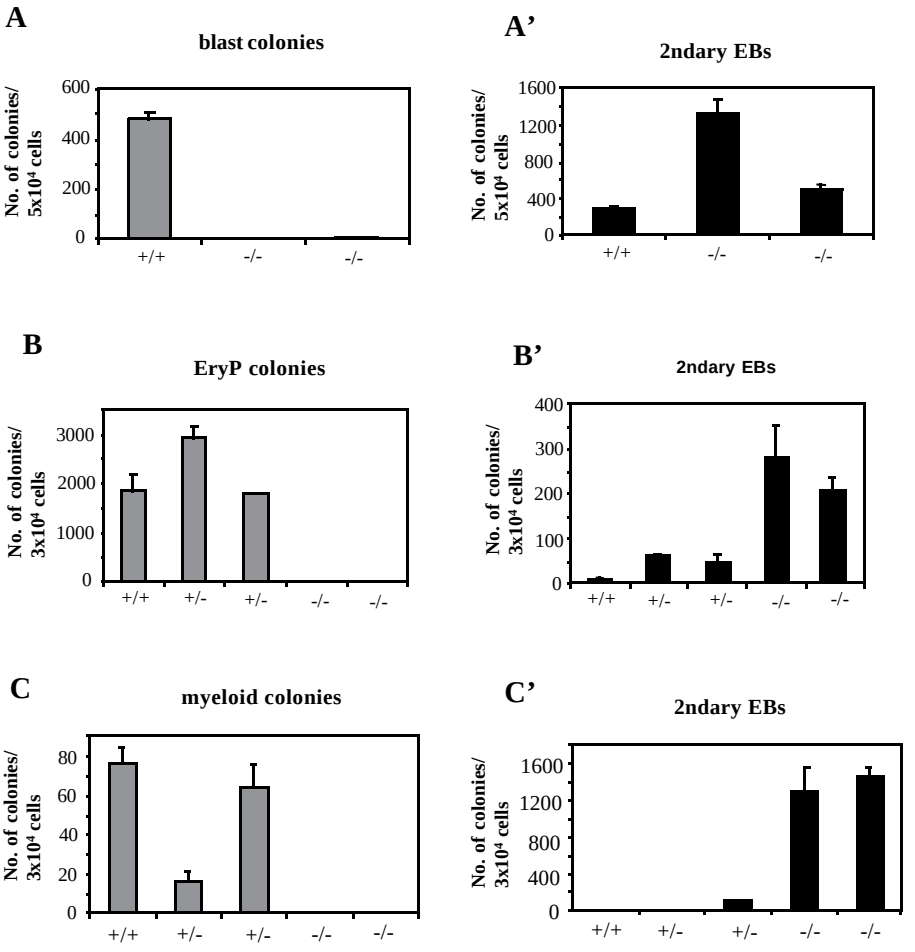


Fig. 1. Hematopoietic development is greatly impaired in *fgfr1*$^{-/-}$ EB cells. *fgfr1*$^{-/-}$ ES cells were differentiated in vitro and day-2.75, day-4, and day-9 EBs were analyzed for BL-CFC (A-A'), primitive erythroid (B-B'), and other myeloid progenitors (C-C'), respectively. One representative result out of 5 independent experiments is shown. Error bars indicate standard deviations from triplicate plates. +/+, one *fgfr1*$^{+/+}$, *fgfr1*$^{+/-}$, two independent *fgfr1*$^{+/-}$ lines, and *fgfr1*$^{-/-}$, two independent *fgfr1*$^{-/-}$ lines, are shown. Myeloid colonies include definitive erythroid, macrophage, granulocyte/macrophage and mixed colonies.

GATA-4, sense 5′CTAAGCTGTCCCCACAAGGC3′; antisense 5′CAGAGCTCCACCTGGAAAGG3′.

Myf5, sense 5′GAAGGCTCCTGTATCCCCTCAC3′; antisense 5′GTTCTCCACCTGTTCCCTCAGC3′.

α-cardiac MHC, sense 5′CTCGCAGAACACCAGCC3′; antisense 5′GTAGGTGAGCTCCTTGAT3′.

β-H1, sense 5′AGTCCCCATGGAGTCAAAGA3′; antisense 5′CTCAAGGAGACCTTTGCTCA3′.

β-major, sense 5′CTGACAGATGCTCTCTTGGG3′; antisense 5′CACAAACCCCAGAAACAGACA3′.

T gene, sense, 5′CATGTACTCTTTCTTGCTGG3′; antisense, 5′GGTCTCGGGAAAGCAGTGGC3′.

BMP2, sense, 5′GAATCAGAACACAAGTCAGT; antisense, 5′GTTTGTGTTTGGCTTGACGC3′.

BMP4, sense, 5′TGTGAGGAGTTTCCATCACG; antisense, 5′CAGCGAAGGACTGCAGGGCT3′.

TGFβ1, sense, 5′CCAAAGACATCTCACACAGT3′; antisense, 5′TAACGCCAGGAATTGTTGCT3′.

VEGF, sense, 5′TCAGAGAGCAACATCACCAT3′; antisense, 5′ACCGCCTCGGCTTGTCACAT3′.

scl, sense, 5′ATTGCACACACGGGATTCTG3′; antisense, 5′GAATTCAGGGTCTTCCTTAG3′.

Flk1, sense, 5′CACCTGGCACTCTCCACCTTC3′; antisense, 5′GATTTCATCCCACTACCGAAAG3′.

KL, sense, 5′GACTGTGTGCTCTCTTCAAC3′; antisense, 5′CTTGCAAAACCTCCAGAGTC3′.

c-kit, sense, 5′TGTCTCTCCAGTTTCCCTGC3′; antisense, 5′GCAAGGACAAGGGAACCTCTG3′.

Single cell RT-PCR was performed following Brady et al. (1990). Briefly, single Flk1^{+} cells were picked into 4 μl of lysis/1st strand buffer by micromanipulation, reverse transcribed, poly(A) tailed using terminal transferase. Total cDNA was amplified using oligo(dT) as a primer (5′GTTAACTCGAGAATTCT$_{24}$3′). After one round of reamplification using 1 μl of the primary PCR products as template, PCR products were separated on an agarose gel, transferred to a nylon membrane, hybridized with ^{32}P randomly primed cDNA probe corresponding to the 3′ end of the *L32*, *Flk1* or *fgfr1* gene. After the hybridization, the blot was washed at high stringency and exposed to an X-ray film.

RESULTS

fgfr1$^{-/-}$ ES cells show defective hematopoietic development

In an effort to determine if bFGF plays a role in hematopoietic development, we initially analyzed *fgfr1*$^{-/-}$ ES cells in vitro for their potential to give rise to BL-CFCs and hematopoietic cells. *fgfr1*$^{-/-}$ ES cells differentiated poorly in vitro (not shown), suggesting that the Fgfr1 mediated signal is important for EB cell development. Hematopoietic development was also defective in *fgfr1*$^{-/-}$ EBs, such that the number of blast colonies, primitive erythroid and myeloid progenitors were all greatly reduced in *fgfr1*$^{-/-}$ EBs compared to those in *fgfr1*$^{+/+}$ EBs (Fig. 1A-C). The number of secondary EBs, which arise from undifferentiated ES cells within EB cells, was greatly increased as shown in Fig. 1A'-C'. This suggested that the Fgfr1-mediated signal is necessary for ES cell differentiation and also for hematopoietic lineage development.

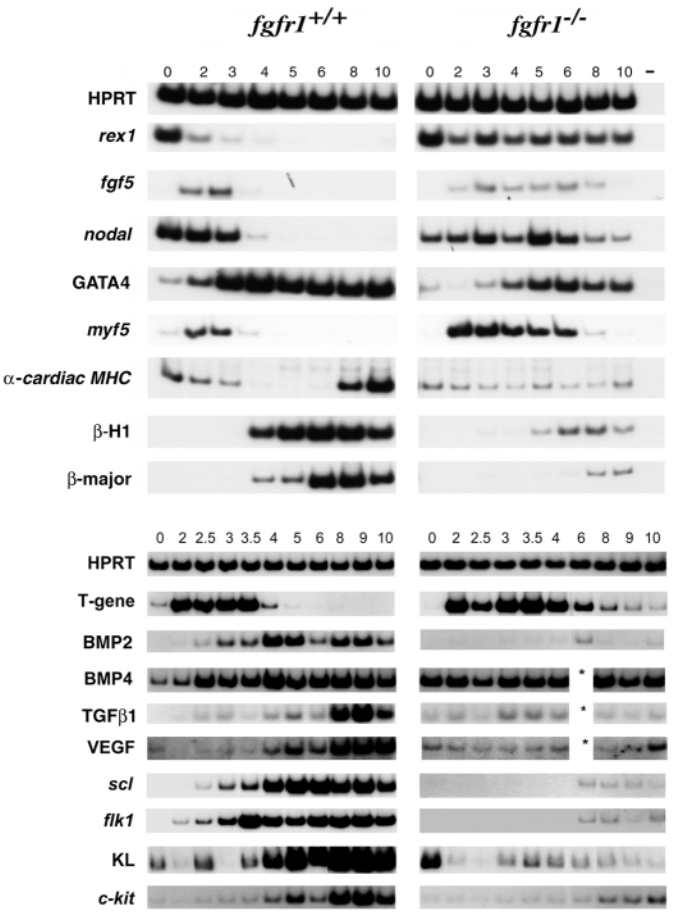


Fig. 2. Gene expression analysis. Numbers on top indicate number of days of EB differentiation. * indicates samples not examined. The size of PCR products are as follows: HPRT, 249 bp; *rex1*, 129 bp; *fgf5*, 331 bp; *nodal*, 321 bp; GATA-4, 327 bp; *myf-5*, 384 bp; alpha cardiac myosin heavy chain, 406 bp; βH1, 278 bp; β-major, 578 bp; T, 313 bp; BMP2, 272 bp; BMP4, 324 bp; TGFβ1, 172 bp; VEGF, 355 bp; *scl*, 321 bp; *Flk1*, 239 bp; KL, 592 bp; and *c-kit*, 210 bp. The PCR products of VEGF include additional 151 and 283 bp DNA and KL an additional 508 bp DNA which are not shown here.

To better understand the hematopoietic defect observed in *fgfr1*$^{-/-}$ EBs, wild-type, as well as, *fgfr1*$^{-/-}$ EBs obtained from different time points were subjected to gene expression analysis using semi-quantitative RT-PCR. As shown in Fig. 2, *rex1*, expressed in the inner cell mass and undifferentiated ES cells, was down regulated as EBs developed in wild type cells. However, *rex1* was still expressed in *fgfr1*$^{-/-}$ EBs even at day 10, confirming the notion that *fgfr1*$^{-/-}$ ES cells differentiated poorly in vitro and gave rise to secondary EBs when replated. The expression of *fgf5*, *nodal* and *T* gene in primitive ectoderm and the early primitive streak region and mesoderm, respectively, was still detectable in later *fgfr1*$^{-/-}$ EBs but not in *fgfr1*$^{+/+}$ EBs. The expression of BMP2 and TGFβ1 was greatly reduced in *fgfr1*$^{-/-}$ EBs compared to controls. However, BMP4 gene expression was not affected. This result suggested that the Fgfr1 signal is necessary for the BMP2 and TGFβ1 gene expression, but not for the expression of BMP4. The expression of VEGF and GATA4, in primitive endoderm, visceral endoderm and parietal endoderm (Miquerol et al., 1999) and visceral endoderm (Arceci et al., 1993), respectively, was down regulated in *fgfr1*$^{-/-}$ EBs without kinetic change. The expression of the cardiac α-myosin heavy chain gene was down regulated in late *fgfr1*$^{-/-}$ EBs (days 8 and 10), although the expression of *myf5*, the first myogenic marker, was up regulated in *fgfr1*$^{-/-}$ EBs. The expression of genes including *Flk1*, *scl*, *c-kit*, kit ligand (KL), *βH1*, and β-major globin, was greatly reduced in *fgfr1*$^{-/-}$ EBs compared to controls, confirming the finding that the hematopoietic differentiation is impaired in *fgfr1*$^{-/-}$ EBs.

The addition of bFGF during EB differentiation increases BL-CFC frequency

As the overall *in vitro* differentiation potential of *fgfr1*$^{-/-}$ ES cells was impaired, it was difficult to evaluate whether bFGF plays a role, if any, in hematopoietic differentiation. Therefore, we assessed the effect of bFGF on hematopoietic development by adding it during EB differentiation and determining if it could affect BL-CFC development. As shown in Fig. 3A, EBs differentiated for 2.75 days in the presence of bFGF contained higher cell numbers compared to control EBs. This suggested that bFGF enhanced EB proliferation and/or differentiation and is consistent with the finding that Fgfr1 is important for embryonic cell proliferation (Deng et al., 1994; Yamaguchi et al., 1994) and EB differentiation. If hematopoietic progenitors expanded preferentially within EBs in response to bFGF, we expected that the BL-CFC frequency would increase as well. In contrast, if the effect of bFGF were to enhance EB cell proliferation non-specifically, one would predict that the BL-

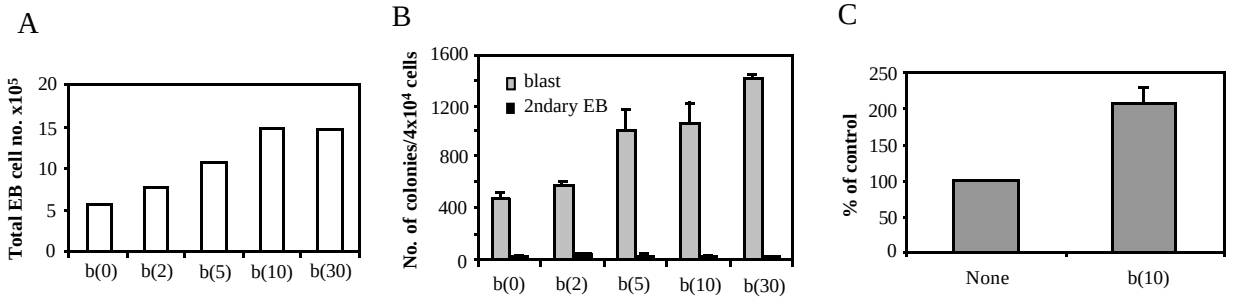


Fig. 3. The effect of bFGF on BL-CFC development. EBs from CCE ES cells were generated in the presence of bFGF at different concentrations and examined for BL-CFCs. A and B are from the same experiment. (A) Total EB cell number generated from 60,000 ES cells. (B) Blast colony number per 4×10^4 EB cells. (C) Combined results from 7 independent experiments are shown as a percentage of control. Error bars indicate standard error mean. Number in parenthesis indicate bFGF concentrations per ml (ng/ml).

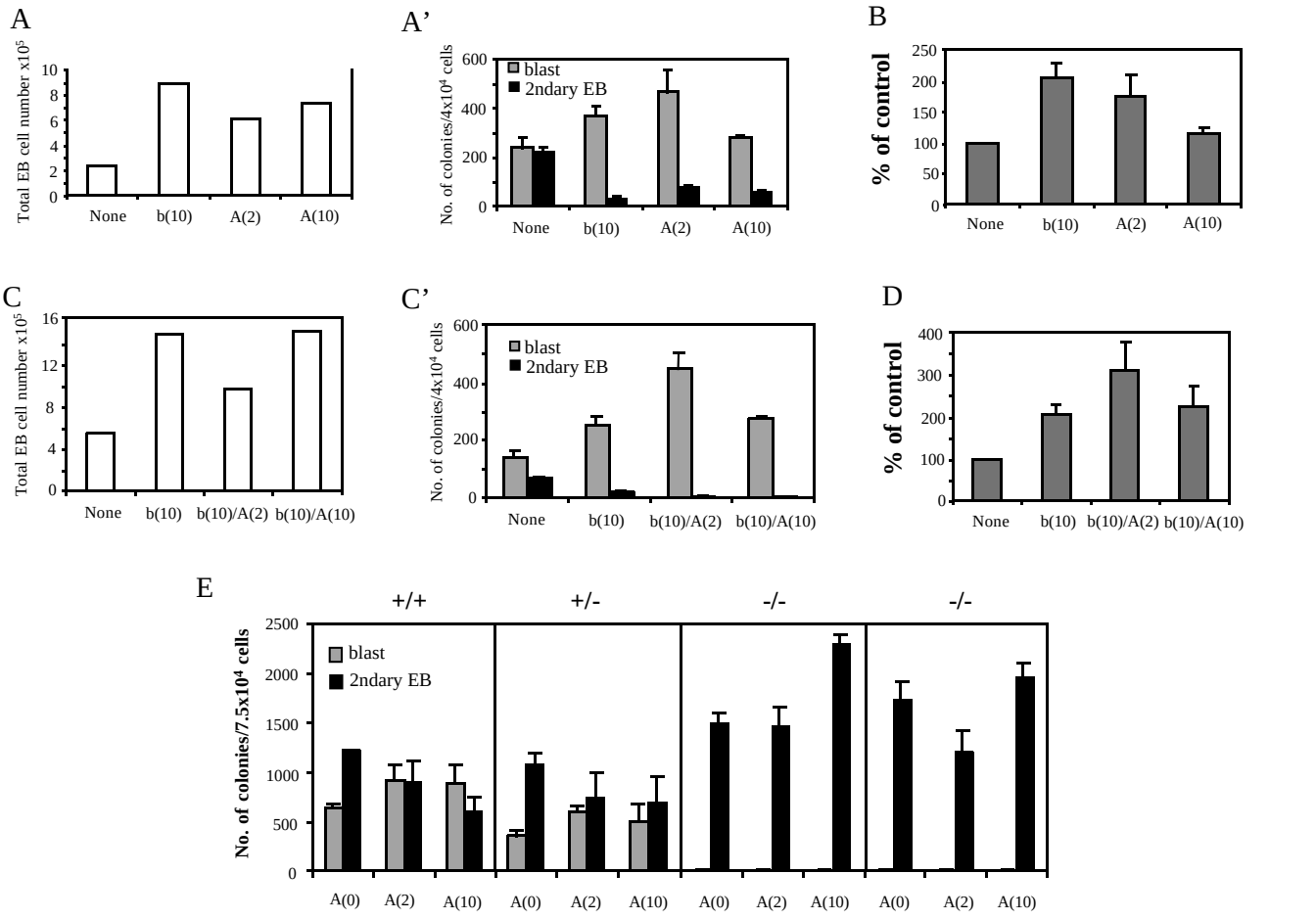


Fig. 4. BL-CFC frequency increases further in the presence of both bFGF and activin A. Basic FGF and activin A were added to CCE EB differentiation, singly or together, and the resulting blast colonies were scored. b, bFGF; A, activin A. Numbers in parenthesis indicate the factor concentrations used per ml (ng/ml). One representative result, A,A'and C,C', is shown. Error bars indicate standard deviations from triplicate plates. (B,D) Results obtained from 7 independent experiments are shown as a percentage of control. Error bars indicate standard error mean. E, Activin A-mediated BL-CFC development requires bFGF-mediated signals. *fgfr1*$^{+/+}$, *fgfr1*$^{+/-}$ and *fgfr1*$^{-/-}$ EBs were generated in the presence of activin A and the resulting blast colonies were examined. +/+, *fgfr1*$^{+/+}$; +/–, *fgfr1*$^{+/-}$; *fgfr1*$^{-/-}$, two independent *fgfr1*$^{-/-}$ lines. Numbers in parentheses indicate the activin A concentrations used (ng/ml).

CFC frequency would remain the same. As shown in Fig. 3B and C, EBs grown for 2.75 days in the presence of bFGF contained a higher BL-CFC frequency compared to those from control EBs. The increase in blast colony number in the presence of bFGF was dose-dependent, such that the number of colonies increased with higher concentrations of bFGF used. The developmental potential of blast cells generated from bFGF-stimulated EBs remained the same, since a similar percentage of blast colonies gave rise to both hematopoietic and endothelial cells (30/48 from control and 34/48 blast colonies from EBs differentiated with bFGF). Taken together, it is likely that bFGF not only enhanced EB proliferation and/or differentiation, but also preferentially promoted the development of hematopoietic committed progenitors.

Activin A and bFGF show additive or synergistic effect on BL-CFC generation

In *Xenopus*, it has been shown that basic FGF and activin A synergize in inducing mesoderm and that mesoderm induction by activin A requires FGF-mediated signals (Cornell and Kimelman 1994; LaBonne and Whitman 1994). To determine if bFGF and Activin A could also synergize in BL-CFC development, EBs were developed in the presence of bFGF, activin A, or bFGF and activin, replated, and the number of resulting blast colonies was compared. As shown in Fig. 4A,B, activin A also promoted generation of BL-CFC. The increase in BL-CFC frequency by activin A was more obvious at the lower concentrations of activin A used. Furthermore, the number of blast colonies from EBs obtained with activin A and bFGF together was much greater than that from EBs with activin or bFGF alone (Fig. 4C,D). Again, this increase was more apparent at lower concentrations of activin A. From these data, we concluded that bFGF or activin A singularly promoted BL-CFC development and that these two factors showed an additive or synergistic effect on BL-CFC generation.

To determine if activin A-mediated BL-CFC development also requires the Fgfr1 signal, *fgfr1*$^{+/+}$, *fgfr1*$^{+/-}$, and *fgfr1*$^{-/-}$ ES cells were differentiated in vitro, in the presence of activin A, and the resulting blast colonies were examined. As shown in Fig. 4E, both *fgfr1*$^{+/+}$ and *fgfr1*$^{+/-}$ EB cells gave rise to an increased number of blast colonies in the presence of activin A. However, *fgfr1*$^{-/-}$ EBs developed in the presence of activin A still failed to generate any

BL-CFCs, and only gave rise to secondary EBs when replated. This suggests that activin A cannot rescue the hematopoietic defect observed in *fgfr1*$^{-/-}$ ES cells and that activin A-mediated BL-CFC generation requires the Fgfr1 signal.

Blast colonies develop from Flk1^{+}, but not Flk1^{-}, cell population

We have previously demonstrated that vascular endothelial growth factor (VEGF) is critical for BL-CFCs to form blast colonies in methylcellulose cultures and that blast cells, progeny of BL-CFC, express the *Flk1* gene (Choi et al., 1998; Kennedy et al., 1997). These observations would indicate that BL-CFCs express Flk1. To determine if this is the case, day-2.75 EB cells were stained with Flk1 monoclonal antibody (Kabrun et al., 1997) and Flk1^{+} cells were sorted by FACS. Sorted cell populations (Flk1^{+} vs. Flk1^{-}) were replated in blast replating medium. As shown in Figs 5A and 7, Flk1^{+} cells were greatly enriched for BL-CFCs. These results suggest that BL-CFCs express Flk1 and therefore argue that Flk1 can be used as a marker for the hemangioblast.

The increase in BL-CFC frequency by bFGF coincides with that of Flk1 expressing cells

To determine if Flk1-expressing cells also increased in response to bFGF, ES cells differentiated with or without bFGF were stained with monoclonal antibody against Flk1. As shown in Fig. 5B, the percentage of Flk1^{+} cells in EBs increased in the presence of bFGF. As with the gradual increase in blast colony number with increasing bFGF concentrations, the number of Flk1^{+} cells also increased in a bFGF dose-dependent manner (not shown). Together, our results suggested that bFGF not only increased the generation of BL-CFCs, but also Flk1^{+} cells.

To examine if we can detect the increase of Flk1^{+} cells at earlier time points than day 2.75, *Flk1*$^{+/-}$ EB cells were differentiated with or without bFGF and subjected to staining for β-galactosidase. *Flk1*$^{+/-}$ ES cells contain the *lacZ* gene under the transcriptional control of endogenous *Flk1* regulatory elements, thus the β-galactosidase (β-gal, the *lacZ* gene product) activity acts as a histochemical marker of Flk1 expression (Shalaby et al., 1995). As shown in Table 1, the increase in Flk1^{+} cells by bFGF was observed as early as day 1.5 and then throughout EB development (up to day 3.5, the endpoint of the analysis). The increase in Flk1^{+} cells measured by β-galactosidase activity or by Flk1 monoclonal antibody staining was similar, as shown in Table 1.

bFGF confers a proliferative signal to Flk1^{+} cells, but does not stimulate blast colony formation from Flk1^{+} cells

To better elucidate the role of bFGF on hemangioblast development, we determined if Flk1^{+} cells expressed *fgfr1*. Thus, we flow sorted Flk1^{+} cells from day-2.75 EBs and picked individual Flk1^{+} cells into cell lysis buffer by micromanipulation. Single cell samples were then subjected to global amplification of mRNA transcripts, known as single-cell based polymerase chain reaction (PCR) analysis (Brady et al., 1990). The amplified PCR product from single Flk1^{+} cells was analyzed for the expression of *Flk1* and *fgfr1*. As shown in Fig. 6, cells sorted for the surface marker Flk1 all expressed the *Flk1* gene. Furthermore, a majority of these cells (20/26) also expressed *fgfr1*, although the levels of *fgfr1* expression were variable between samples.

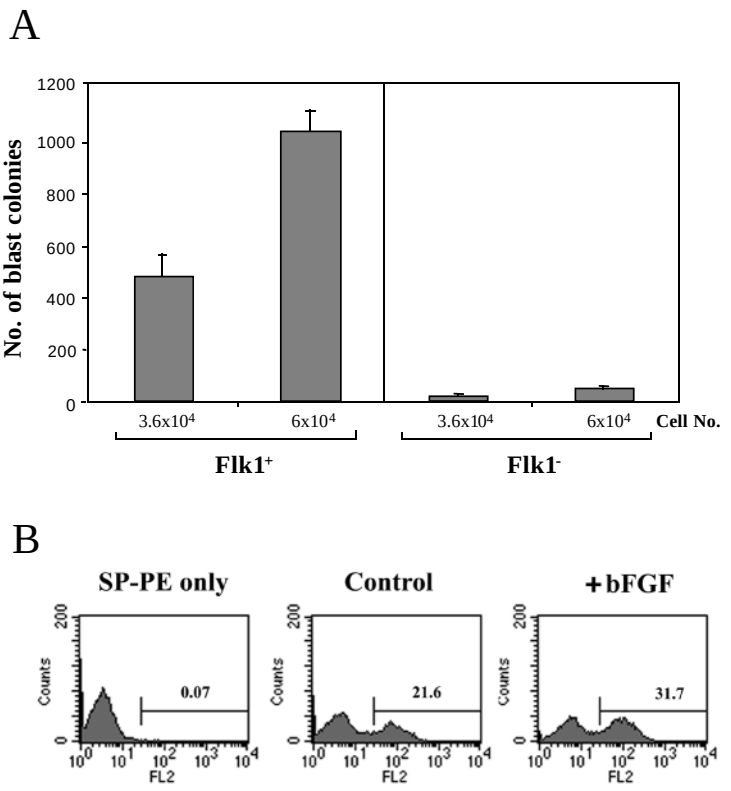


Fig. 5. (A) Flk1^{+} cells are enriched for BL-CFCs. Day-2.75 EBs from R1 ES cells were stained with Flk1 antibody and FACS sorted. Flk1^{+} versus. Flk1^{-} cells were replated in the presence of VEGF and the resulting blast colonies were counted 4 days after the replating. Numbers on the X axis indicate number of Flk1^{+} cells replated. (B) Flk1^{+} cells increase in response to bFGF. The percentage Flk1^{+} cells from day-2.75 R1 EBs differentiated with bFGF (10 ng/ml) is shown. One representative from a minimum of 7 experiments is shown. SP-PE, cells received streptavidin-phycoerythrin only, a secondary reagent. Numbers indicate the percentage of Flk1^{+} cells.

The findings that the increase of Flk1^{+} cells can be detected

Table 1. Flk1^{+} cell analysis

		EB age					
Exp.		1.5	1.75	2.0	2.75	3.0	3.5
1	control	0.95 (0.6)	2.5	2.4		20	49
	+bFGF	1.4	3.9	5.5		40	55
2	control	1.75			22 (24.3)		
	+bFGF	3.3			40 (41.8)		
3	control	1.45	2.8	3	20.5		
	+bFGF	1.5	4.1	5.7	35.8		
4	control	0.88 (0.4)		2.2 (1)	18.5 (21)		42
	+bFGF	1.3 (0.7)		2.5 (2.4)	23.8(32)		47

Flk1^{+} cells from *Flk1*$^{+/-}$ EBs were analyzed by β-galactosidase staining as well as by Flk1 monoclonal antibody staining. The percentage of cells that are positive for Flk1 from β-galactosidase staining is given. Numbers in parentheses indicate the percentage Flk1^{+} cells by Flk1 monoclonal antibody staining. EB age indicates the days on which the EBs were analyzed. bFGF was used at 10 ng/ml. Results from four experiments are shown.

as early as 1.5 days after the initiation of EB differentiation and that a majority of Flk1$^+$ cells expressed *fgfr1* suggested that bFGF conferred a proliferation signal on Flk1$^+$ cells. To further confirm this notion, we next determined if the BL-CFC frequency remains the same among Flk1$^+$ cells that were generated with or without bFGF. Thus, we flow sorted Flk1$^+$ cells from day-2.75 EBs, differentiated with or without bFGF, and replated them for blast colonies. If BL-CFC differentiation among Flk1$^+$ cells could be induced by bFGF, it was expected that the BL-CFC frequency would be higher from Flk1$^+$ cells isolated from EBs differentiated with bFGF. However, if bFGF conferred overall proliferation of Flk1$^+$ cells, the BL-CFC frequency would remain similar. As shown in Fig. 7A and B, the number of blast colonies developed from Flk1$^+$ cells was similar whether the Flk1$^+$ cells were derived with or without bFGF. Together, our data argue for the proliferative role of bFGF on Flk1$^+$ cells.

To investigate if bFGF also plays a role in blast colony formation, we replated sorted Flk1$^+$ cells with VEGF, bFGF, or VEGF and bFGF. As shown in Fig. 7C, the number of blast colonies developed was less when Flk1$^+$ cells were replated with bFGF. The colony size was much smaller and blast cells did not look healthy in the presence of bFGF. Furthermore, bFGF did not show any additive or synergistic effect with VEGF as the number of blast colonies was similar whether the Flk1$^+$ cells were replated in VEGF alone or VEGF and bFGF together. These

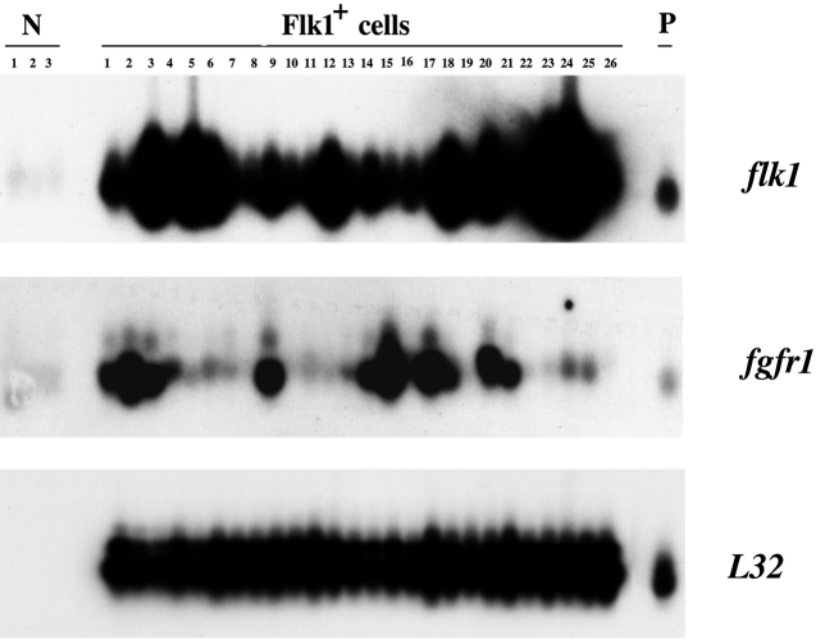
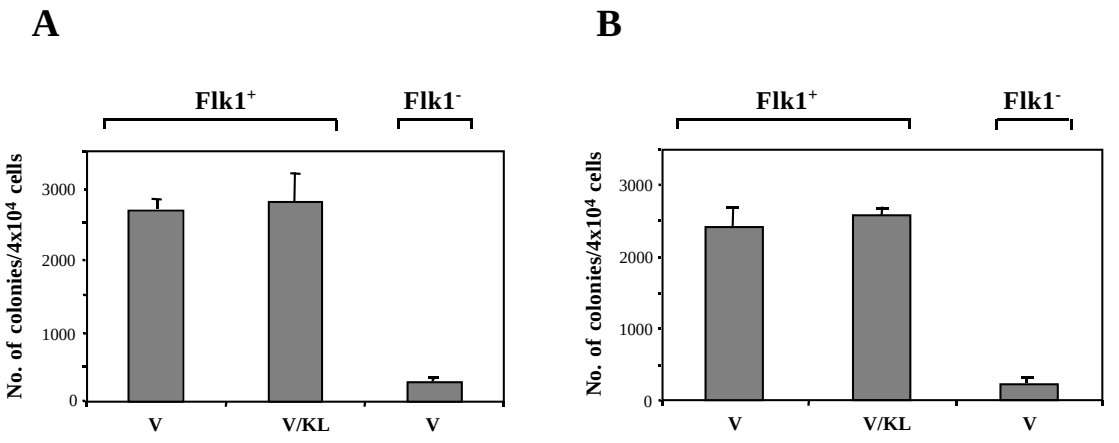


Fig. 6. Flk1$^+$ cells express the *fgfr1* gene. Flk1$^+$ cells were sorted from day-2.75 R1 EBs and subjected to single cell RT-PCR analysis using micromanipulation. N, negative control, which represents RT-PCR reaction received no cells. P, positive control, which represents day-3 EB cells.



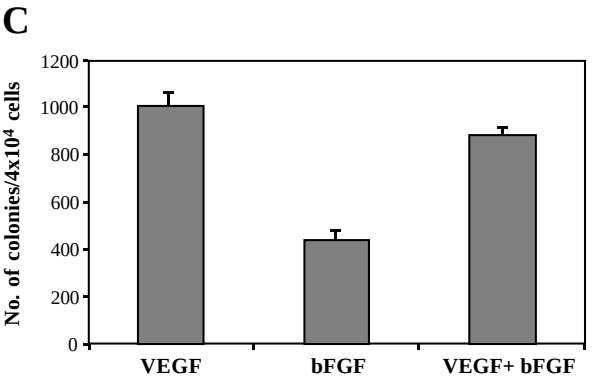


Fig. 7. (A,B) bFGF does not change BL-CFC frequency among Flk1$^+$ cells. Flk1$^+$ cells were sorted from control (A) as well as EBs differentiated with bFGF (B) and replated in methylcellulose culture containing VEGF, KL and D4T conditioned medium (25%). The resulting blast colonies were counted 4 days after. One representative experiment out of three is shown. Error bars indicate standard deviations from triplicate plates. C. bFGF does not enhance blast colony formation. Flow-sorted Flk1$^+$ cells from day-2.75 R1 EBs were replated in the presence of bFGF, VEGF or bFGF and VEGF. All contained D4T conditioned medium at 25%. The resulting blast colonies were counted 4 days later. One representative experiment out of five is shown. Error bars indicate standard deviations from triplicate plates.

Table 2. FACS analysis for cells expressing endothelial cell markers

	EB Age	*scl*	Flk-1	PECAM-1	P-Selectin	CD34	VE-Cadherin
Exp. 1	d3	+/+	29				
		–/–	51				
	d7	+/+	17	40		2	0.8
		–/–	40	55		1.5	1.8
Exp. 2	d10	+/+	15	9	2.6	2	
		–/–	59	28	4.1	5	
	d12	+/+	19	9.4		0.8	
		–/–	45	26.7		1.5	
Exp. 3	d10	+/+	22	8			0.4
		–/–	35	12			1.2
Exp. 4	d10	+/+	22	8.5	13.8	3.6	
		–/–	40	10.2	16.3	4.8	

Wild-type as well as *scl*$^{-/-}$ EB cells were analyzed at various time points for endothelial cell markers. Percentage of positive cells for a given antibody staining is given. Results from four independent experiments are shown.

results indicated that bFGF is important for BL-CFC expansion within EBs but is not critical for the subsequent formation of blast colonies from BL-CFCs in methylcellulose culture.

Flk1$^+$ cells develop in the absence of functional SCL

We have previously demonstrated that both *Flk1* and *scl* are expressed in blast cells (Kennedy et al., 1997). In EBs, *Flk1* gene expression can be detected as early as day 2 and *scl* at day 2.5 (Fig. 2). SCL is a transcription factor, which contains a basic-helix-loop-helix (bHLH) domain (Begley et al., 1989) and functional SCL has been shown to be required for all hematopoietic lineages to develop (Shivdasani et al., 1995; Robb et al., 1995; Porcher et al., 1996; Robb et al., 1996). To further define hemangioblast development, we determined if SCL function is required for BL-CFC/hemangioblast development. Thus, *scl*$^{-/-}$ ES cells were analyzed for their potential to give rise to BL-CFCs and Flk1$^+$ cells. While day-2.75 wild-type EBs gave rise to blast colonies when replated in response to VEGF, *scl*$^{-/-}$ EBs did not generate any blast colonies. However, blast-like colonies, which are characterized by their tighter colony morphology compared to blast colonies, yet looser than EBs, and hence distinct from EBs and blast colonies, were readily detectable when *scl*$^{-/-}$ EBs were replated (Fig. 8A-C). These blast-like colonies were also present in wild-type culture, although at a reduced frequency compared to the frequency of BL-CFCs (not shown). When the blast-like colonies were transferred to medium containing both hematopoietic and endothelial factors, wild-type blast-like colonies gave rise to both hematopoietic and endothelial cells, with a more vigorous growth in the endothelial component (not shown). However, *scl*$^{-/-}$ blast-like colonies gave rise only to endothelial cells (47 out of 47 colonies analyzed). Since these blast-like colonies developed in cultures containing VEGF, it was expected that progenitors giving rise to blast-like colonies expressed Flk1 and thus Flk1-expressing cells still developed in *scl*$^{-/-}$ EBs. Therefore, we analyzed *scl*$^{+/+}$ and *scl*$^{-/-}$ EB cells for the presence of Flk1$^+$ cells. As shown in Fig. 8D, *scl*$^{-/-}$ EBs contained Flk1$^+$ cells, confirming that Flk1$^+$ cells develop in the absence of functional SCL. More importantly, when *scl*$^{-/-}$ ES cells were differentiated in the presence of bFGF, the percentage of Flk1$^+$ cells increased, as is also seen with wild-type ES cells (Fig. 8D).

As *scl*$^{-/-}$ EBs contained a somewhat higher percentage of Flk1$^+$ cells compared to the wild type EBs (Fig. 8D), we determined if the increase in Flk1$^+$ cells within *scl*$^{-/-}$ EBs reflected the increase in endothelial cell generation in the

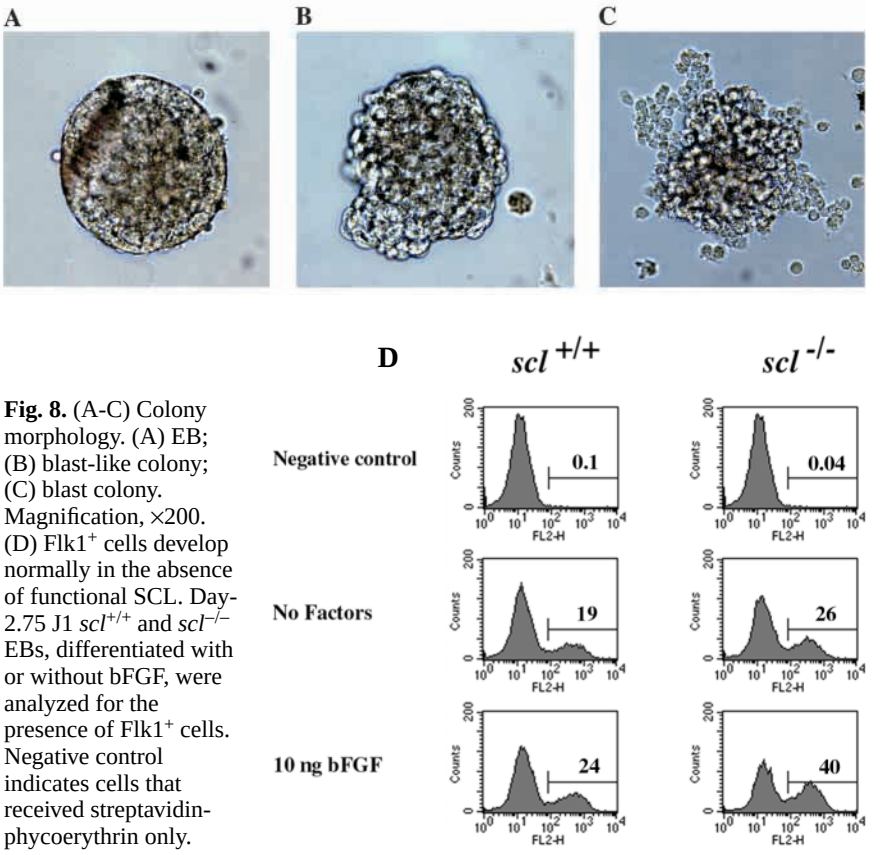


Fig. 8. (A-C) Colony morphology. (A) EB; (B) blast-like colony; (C) blast colony. Magnification, ×200. (D) Flk1$^+$ cells develop normally in the absence of functional SCL. Day-2.75 J1 *scl*$^{+/+}$ and *scl*$^{-/-}$ EBs, differentiated with or without bFGF, were analyzed for the presence of Flk1$^+$ cells. Negative control indicates cells that received streptavidin-phycoerythrin only.

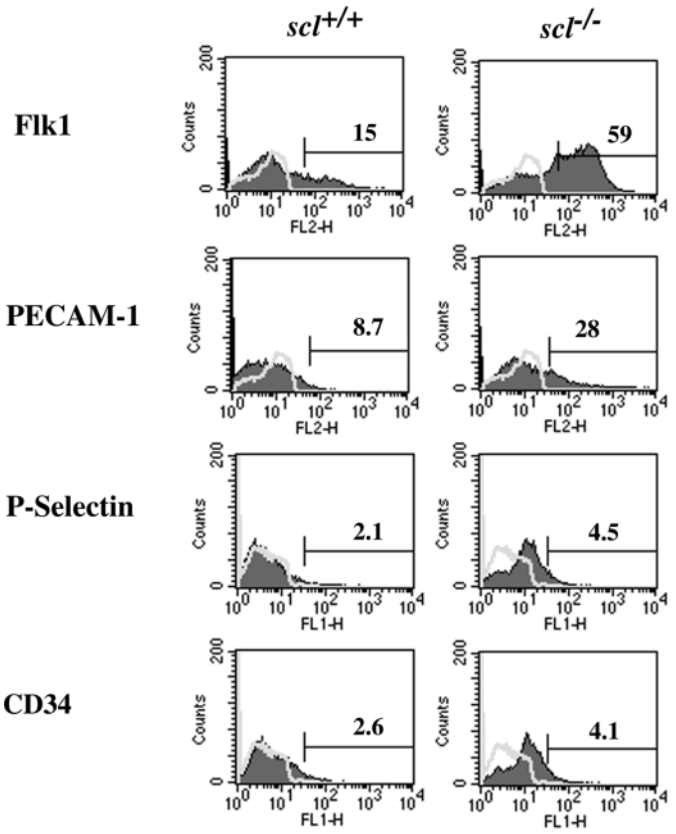


Fig. 9. Cells expressing endothelial cell markers are increased within *scl*$^{-/-}$ EBs. Day-10 *scl*$^{+/+}$ as well as *scl*$^{-/-}$ EBs were analyzed for Flk1, PECAM-1, P-selectin, and CD34. Numbers indicate percentage of cells positive for a given antibody staining. Cell staining patterns and percentages were determined on a two dimensional plot due to the inherent autofluorescent nature of EB-derived cells.

absence of SCL. Thus, *scl*$^{-/-}$ EB cells were subjected to FACS analysis for endothelial markers. As shown in Fig. 9 and Table 2, cells expressing Flk1, PECAM-1, CD34, P-selectin, or VE-cadherin were present at higher levels within *scl*$^{-/-}$ EBs compared to controls throughout EB differentiation (up to day 12). Together, our results suggest that blast-like colonies represent a transient Flk1^{+} population or pre-BL-CFCs, from mesoderm to BL-CFCs and that functional SCL is required for the formation of blast colonies from Flk1^{+} cells. Furthermore, our results suggest that Flk1^{+} cells readily give rise to endothelial cells in the absence of SCL.

DISCUSSION

We have previously demonstrated that BL-CFCs represent the long pursued common progenitor of hematopoietic and endothelial cells, the hemangioblast. As BL-CFCs form blast colonies in the presence of VEGF, a ligand for Flk1, we investigated if Flk1 is expressed on BL-CFCs. By sorting a cell population expressing the receptor tyrosine kinase Flk1 and replating for the generation of blast colonies, we have demonstrated that BL-CFCs indeed express Flk1. The observations that CD45^{+}c-Kit^{+} hematopoietic cells develop from Flk1^{+}VE-cadherin^{+}CD45^{-} cells obtained from in vitro differentiated ES cells or yolk sac cells (Nishikawa et al., 1998) and that CD45^{+} hematopoietic cells develop from VEGF-R2^{+} (Flk1^{+}) cells of the chick intra-aortic region (Jaffredo et al., 1998) are consistent with our notion that the hemangioblast express Flk1.

By measuring the frequency of the BL-CFC and Flk1^{+} cells as experimental readouts, we have assessed the capacity of various mesoderm-inducing factors to promote hematopoietic commitment. Our studies suggest that the generation of BL-CFCs and Flk1^{+} cells is regulated not by a single factor, but by several factors. Of these, bFGF-mediated signals are critical for hematopoietic development. The effect of Activin A was greater at lower concentrations. More importantly, bFGF together with activin A appear to have an additive or synergistic effect on BL-CFC generation and that activin A-mediated BL-CFC generation requires a Fgfr1 signal since the administration of activin A did not rescue the BL-CFC development in *fgfr1*$^{-/-}$ EBs (Fig. 4E). These results are consistent with the findings in *Xenopus* that activin A and bFGF synergize in mesoderm induction and that activin A requires a bFGF-mediated signal (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). Our studies are also consistent with the recent study by Pardanaud and Dieterlen-Lievre (1999) who found that the treatment of somatopleural mesoderm, which otherwise contains angiopoietic potential only, with VEGF, bFGF or TGF-β1 could induce hemangiopoietic potential in this tissue in the quail/chick chimera model.

Previous studies have demonstrated that *fgfr1*$^{-/-}$ embryos show growth arrest at an early stage of gestation and that *fgfr1*$^{-/-}$ embryos die early in gestation and show abnormal mesodermal patterning. However, as *fgfr1*$^{-/-}$ ES cells could differentiate into many different mesodermal cell types, including muscle cells, when teratomas were generated through subcutaneous injection into nude mice, mesodermal

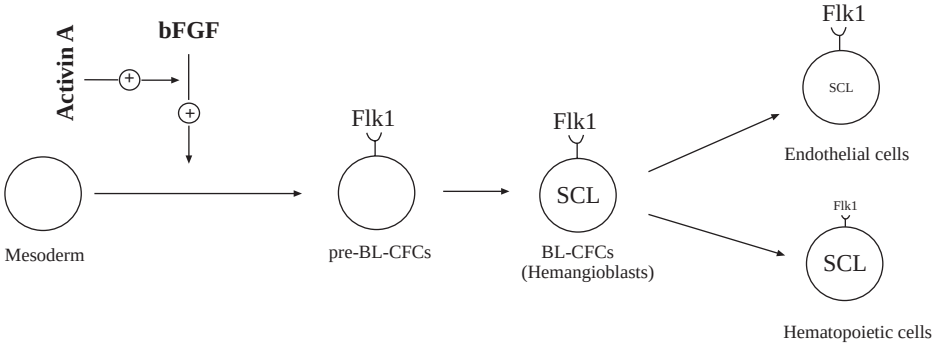


Fig. 10. Schematic view of hematopoietic development from mesoderm. In this scheme, Flk1 single positive cells represent pre-BL-CFCs and Flk1^{+}SCL^{+} double positive cells represent BL-CFCs, hemangioblasts. Activin A likely signals through bFGF signal pathway in positively regulating BL-CFC generation. Mature endothelial progenitors still express *scl*, although at a reduced level, and early hematopoietic progenitors still express Flk1 as demonstrated (Kabrun et al., 1997).

defects observed in *fgfr1*$^{-/-}$ embryos are probably due to abnormal mesodermal patterning, rather than an intrinsic defect of mesoderm differentiation (Deng et al., 1994). Our in vitro analyses of *fgfr1*$^{-/-}$ ES cells for their potential to differentiate and to give rise to hematopoietic progenitors are not only consistent with the in vivo findings but further reveal a role for bFGF on hemangioblast development. One, *fgfr1*$^{-/-}$ ES cells differentiate poorly in vitro judged by the extended expression of the *rex1* gene (Fig. 2) and the reduction in total EB cell numbers throughout EB differentiation. Two, the expression of the brachyury (T) gene, a marker for mesoderm, could be detected in *fgfr1*$^{-/-}$ EBs, consistent with the in vivo findings (Deng et al., 1994; Yamaguchi et al., 1994). In wild-type EBs, T gene expression is temporally up-regulated in the early stage of EB differentiation and down-regulated in the later stage of EB differentiation (Fig. 2). As T gene expression was extended and still observed in day-10 *fgfr1*$^{-/-}$ EBs, with *rex1* being still expressed, unlike the control, it is possible that mesoderm differentiation is delayed in the absence of Fgfr1 or that cells that manage to differentiate to mesoderm are delayed in subsequent differentiation. Three, in vitro differentiated *fgfr1*$^{-/-}$ EBs expressed, although at low levels, genes of both muscle and hematopoietic cells. Considering the fact that *myf5* was up-regulated and that the expression of α-cardiac myosin heavy chain and other hematopoietic genes is greatly down-regulated, it is tempting to speculate that the major role of Fgfr1 signaling, at least in the in vitro differentiation system, is the proliferation of mesodermal progenitors once they are generated. Our data that a majority of Flk1^{+} cells expressed *fgfr1* and expanded in response to bFGF, but that Flk1^{+} cells from day-2.75 EBs did not respond to bFGF in forming blast colonies further support this notion. Clearly VEGF is critical for blast colony formation from BL-CFCs (Choi et al., 1998; Schuh et al., 1999). However, since the addition of VEGF to EB differentiation does not increase the frequency of the BL-CFC or Flk1^{+} cells (not shown), we propose two distinct regulations on hemangioblast development, namely bFGF-mediated hemangioblast proliferation and VEGF-mediated hemangioblast migration and maturation to hematopoietic and endothelial cells (Shalaby et al., 1997; Choi et al., 1998; Schuh et al., 1999).

In zebrafish, *scl* is expressed in the lateral mesoderm, which gives rise to hematopoietic, endothelial, and pronephric lineages (Gering et al., 1998). Several studies might be interpreted to suggest that SCL/Tal-1 lies upstream of Flk1 in zebrafish. First, the zebrafish *cloche* (*clo*) mutation affects both hematopoietic and endothelial differentiation (Stainier et al., 1995; Liao et al., 1998). Furthermore, ectopically expressed SCL partially rescues hematopoietic and endothelial cell defects of *clo* mutants, as evidenced by the development of red cells, expression of hemoglobin, red cell markers (i.e. GATA-1) and endothelial markers (*Flk1* and *tie-2*; Liao et al., 1998). These data suggest that SCL lies downstream of *clo*, yet upstream of Flk1 and GATA-1. However, many studies do not support the positioning of SCL/Tal-1 upstream of Flk1. First, we have demonstrated that expression of the *Flk1* gene is detected prior to that of *scl/tal-1* (day 2 versus day 2.5; Fig. 5). Similarly, while *Flk1* gene expression in mouse embryos is seen in presumptive mesodermal yolk sac blood island progenitors as early as 7.0 dpc (Yamaguchi et al., 1993; Dumont et al., 1995), *scl/tal-1* gene expression can be detected in embryonic and extra-embryonic mesoderm of 7.5 dpc embryos and in blood islands of the yolk sac of 8.5 dpc embryos (Kallianpur et al., 1994; Silver and Palis, 1997). Palis et al. (1999) also showed that *Flk1* gene expression precedes that of *scl* in the developing embryo. Second, gene targeting experiments demonstrate that *Flk1*$^{-/-}$ embryos die early in gestation (between 8.5-9.5) and the phenotype reflects the expression pattern of *Flk1* such that *Flk1*$^{-/-}$ embryos show defective blood islands, with both hematopoietic and endothelial cells being affected (Shalaby et al., 1995). However, *scl*$^{-/-}$ embryos die between 8.5-10.5 days with the major defect being the absence of yolk sac hematopoiesis. In these mutant mice, endothelial cells are present (Robb et al., 1995; Shivdasani et al., 1995). Consistent with this, our studies demonstrate that in vitro differentiated *scl*$^{-/-}$ EBs contained cells with endothelial cell markers. Since cells with endothelial cell markers are present at elevated levels in *scl*$^{-/-}$ EBs, it is tempting to speculate that the fate of hemangioblast in the absence of functional SCL is skewed toward endothelial cell differentiation (Fig. 9 and Table 2). Third, we have demonstrated that Flk1^{+} cells develop in the absence of SCL/Tal1 and that *scl*$^{-/-}$ EB cells respond appropriately to bFGF and give rise to a higher percentage of Flk1^{+} cells, compared to control EBs (Fig. 7D). Consistent with this, *Flk1* expression was not impaired in *scl*$^{-/-}$ EBs (Elefanty et al., 1997). Similarly, *scl* expression is unchanged in *Flk1*$^{-/-}$ EBs compared to *Flk1*$^{+/+}$ EBs (unpublished data). Therefore, these studies suggest that the regulation of *Flk1* and *scl* gene expression is independent, but developmentally coordinate.

The BL-CFC frequency is much lower (0.5-2%, Figure 3 and 4) compared to that of Flk1^{+} cells (15-25%, Fig. 5B and Table 1) in day-2.75 EBs. In addition, percentage change in Flk1^{+} (Fig. 5B and Table 1) cells in response to bFGF does not precisely correlate with the BL-CFC frequency (Figs 3, 4). Furthermore, only a fraction of Flk1^{+} cells give rise to blast colonies when replated (Figs 5A, 7). An explanation for these findings is that the Flk1^{+} cell population is heterogeneous and that only a fraction of Flk1^{+} cells represent true hemangioblasts, although we cannot rule out the possibility that the BL-CFC frequency is underestimated due to sub-optimal culture conditions. Based on the findings that blast colonies are absent, and yet blast-like colonies still develop from *scl*$^{-/-}$ EBs, we postulate that Flk1^{+}SCL^{+} cells represent hemangioblasts or a subset thereof (Fig. 10). According to this model, Flk1 single positive cells represent a transitional pre-BL-CFC cell population from mesoderm to BL-CFCs. Further identification of additional surface markers expressed on hemangioblasts, fractionation of Flk1^{+} cells based on these markers, and studies of their in vitro and in vivo developmental potential should be critical for characterizing Flk1^{+} cells and their contribution to hematopoietic and vascular establishment in the embryo.

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