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Hematopoietic activity of a stromal cell transmembrane protein containing epidermal growth factor-like repeat motifs

(delta-like/preadipocyte factor-1/hematopoietic microenvironment)

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ABSTRACT **Primitive hematopoietic stem cells are closely associated with discrete *in vivo* microenvironments. These "niches" are thought to provide the molecular signals that mediate stem cell differentiation and self-renewal. We have dissected the fetal liver microenvironment into distinct cellular components by establishing an extensive panel of stromal cell lines. One particular cell line maintains repopulating stem cells for prolonged *in vitro* culture periods. A subtraction cloning strategy has yielded a cDNA that encodes a cell surface glycoprotein with a restricted pattern of expression among stromal cell lines. This molecule, previously identified as delta-like/preadipocyte factor-1, contains epidermal growth factor-like repeats that are related to those in the notch/delta/serrate family of proteins. We have investigated the potential role of this molecule in hematopoietic stem/progenitor cell regulation. We show that the delta-like protein displays activity on purified stem cells by promoting the formation of "cobblestone areas" of proliferation. These cobblestone areas contain both primitive high-proliferative potential progenitors and *in vivo* repopulating stem cells.**

The positive and negative regulatory mechanisms that govern the proliferation, self-renewal, and differentiation of primitive hematopoietic stem cells are complex and poorly understood (ref. 1 and references therein). Numerous cytokines have been identified that when used *in vitro* appear to act directly on purified stem cells by promoting proliferation and differentiation. However, attempts to demonstrate the *in vitro* maintenance and/or expansion of transplantable pluripotent stem cells using defined cytokine combinations have been largely unsuccessful (refs. 2 and 3 and references therein). Moreover, it is unclear to what extent any currently identified cytokines reflect mechanisms that are responsible for regulating normal, *in vivo* stem cell behaviors. It is widely accepted that *in vivo* stem cells are intimately associated with discrete microenvironmental "niches" (4). Such niches are likely sources for the molecular signals that collectively mediate the differentiation and self-renewal of stem cells. Indeed, it has long been possible to demonstrate that preestablished stromal cell monolayers derived from hematopoietic tissues can support long-term hematopoiesis *in vitro* (5). The long-term nature of these cultures, together with the continuous production of committed progenitor cells, suggest that both self-renewal and commitment decisions can occur *in vitro*. At the cellular level, the hematopoietic microenvironment consists of numerous distinct cell types. Previous studies have shown that this cellular heterogeneity reflects a similarly broad heterogeneity in terms of hematopoietic supportive abilities (6). Some cloned stromal cell lines can support stem cell activity *in vitro*, while others are ineffective. Similarly, distinct stromal cell types appear to influence the outcomes of stem cell differentiation processes (7). Recent studies have shown that stromal cell lines that efficiently maintain long-term transplantable stem cells *in vitro* for prolonged intervals represent a small fraction of the total stromal cell population (8). We have identified one fetal liver stromal cell line, AFT024, which maintains high levels of transplantable multilineage stem cell activity for extended *in vitro* culture periods (9). The stem cells used to initiate these cultures are highly purified. It is, therefore, likely that in this system the mechanisms that mediate stem cell maintenance do so by acting directly on primitive stem cells. A different fetal liver-derived cell line, 2018, fails to maintain long-term repopulating stem cell activity. A PCR-based RNA expression analysis of 13 cytokines reveals qualitatively identical expression patterns in AFT024 and 2018 (K.A.M., unpublished observations). We, therefore, hypothesized that the hematopoietic supportive ability of AFT024 is, at least in part, mediated by novel gene products not expressed in 2018.

Using a subtractive hybridization approach we have identified a number of cDNA clones specifically expressed in AFT024, but not in 2018 (K.A.M. and I.R.L., unpublished data). The sequence of one cDNA was identical to a molecule that encodes a transmembrane protein that contains six epidermal growth factor (EGF) repeat motifs. The EGF-like repeat sequences of this molecule, variously known as delta-like (dlk) (10), preadipocyte factor-1 (11), and stromal cell protein-1 (GenBank, D16847), are most closely related to those present in the notch/delta/serrate family of signaling proteins. In *Drosophila* and *Caenorhabditis*, these molecules are required for correct cell-fate specification decisions in a variety of tissues (12). Vertebrate homologs of the notch/delta/serrate family have been identified (13–15). While the exact functional relationship of dlk to the activities of this family of molecules is unclear, it has been shown to block adipocyte differentiation (11). No studies have been reported that demonstrate a hematopoietic function for dlk. Expression analyses reported previously and our own observations show a limited temporal pattern of dlk expression during murine fetal development, which coincides with the time period of hematopoietic stem cell expansion (11).

We have undertaken functional studies to determine if dlk can act as a hematopoietic regulator. We show that this molecule affects highly enriched stem cell populations by promoting "cobblestone area" (CSA) colony formation in dexter-type stromal cocultures. These CSA colonies contain an expanded population of primitive, high proliferative potential myeloid-erythroid progenitors. These cultures also contain

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Abbreviations: dlk, delta-like; EGF, epidermal growth factor; BM, bone marrow; CSA, cobblestone area; CFU, colony-forming unit; HPP, high proliferative potential; FBS, fetal bovine serum; RT–PCR, reverse transcription–PCR.

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stem cells capable of *in vivo* engraftment at levels equivalent to those present in parallel AFT024-supported cultures. We propose that dlk represents one molecular component responsible for the hematopoietic supportive ability of AFT024. As such, dlk may define a novel molecular pathway of stem cell regulation by the microenvironment.

MATERIALS AND METHODS

Cell Lines and Culture. The fetal liver stromal cell lines used in this study were derived as previously described (8, 9). Cells were routinely cultured in DMEM containing 10% fetal bovine serum (FBS) and 50 μM 2-mercaptoethanol (2-ME), and maintained at 31°–33°C, 5% CO_2. For long-term cocultures with hematopoietic stem cells, confluent monolayers were irradiated (20 Gy), placed in DMEM (10% FBS, 10% horse serum, 50 μM 2-ME, 0.1 μM hydrocortisone) and maintained at 37°C, 5% CO_2 with weekly media changes. NIH 3T3 cells were obtained from the American Type Culture Collection.

dlk Expression Analysis. Total RNAs from stromal cell lines were poly(A)$^+$ selected, Northern blotted, and hybridized to ^{32}P-labeled probes according to standard protocols (16). A 600-bp dlk-cDNA clone from the AFT024 subtracted library was used as a probe. cDNA templates for reverse transcription–PCR (RT-PCR) were prepared according to manufacturers' protocols (GIBCO/BRL). Oligonucleotide primers were: sense 5′-GACCCAGGCTGCCCC-3′ and antisense 5′-GGTACTCTTGTTGAG-3′. For analysis of dlk expression at the protein level, antisera specific for dlk was generated by immunizing rabbits with a Flag-dlk fusion protein (described below). Resultant antibodies were purified by affinity chromatography. Cell surface expression of dlk in stromal cell lines was accomplished by flow cytometry. Cells were incubated with dlk antibody and a similarly prepared irrelevant control antibody. Specific labeling was developed by donkey anti-rabbit-fluorescein isothiocyanate (Jackson ImmunoResearch). Stained cells were analyzed on a Becton Dickinson FACScan using Cell Quest software.

dlk Fusion Protein Preparation. The expression plasmid pCD4-Ig contains cDNA for the extracellular domain of human CD4 fused to genomic sequences of the human immunoglobin heavy chain (17). cDNA for CH2-CH3 of human IgG_1 (18) was cloned into *Eco*RI and *Not*I sites of pcDNA3 (Invitrogen) to give the plasmid KB52.3.2. cDNA encoding the extracellular domain of dlk was obtained by RT–PCR with primers BP 151 and BP 152 using total RNA from NIH 3T3 cells as template. The resulting PCR fragment was cloned into KB52.3.2 via *Hin*dIII and *Eco*RI sites to obtain the soluble dlk-Ig expression plasmid. pdlk-Ig or pCD4-Ig were transfected into NIH 3T3 cells together with pSVNeo, and stable clones were isolated. Soluble CD4-Ig and dLK-Ig fusion proteins were harvested and then purified by affinity chromatography on Hi Trap Protein G-Sepharose (Pharmacia). Primers: sense BP 151, 5′GAGGGTACCAAGCTTCGTGGTC-CGCAACCAGAAG-3′; anti-sense BP 152, 5′-CTCAGATC-TGAATTCGGCCTGTCCCTCGGTGAGGAG-3′.

Flag-dlk fusion protein was used to immunize rabbits for the production of dlk antiserum. The protein expression plasmid pcDNA3-Flag is a modification of the plasmid pcDNA3 (Invitrogen) and contains the Flag peptide (DYKDDDDKI) (19) and a *Bgl*II restriction site. A cDNA fragment encoding the extracellular domain of dlk was obtained by RT–PCR using RNA from NIH 3T3 cells. Primers: sense BP 155, 5′-GACAAGATCTCAGCTGAATAGCGACCCA-CCCTGTG-3′; antisense BP 154, 5′-GCATCTAGAGCGGC-CGCTCAGGCCTGTCCCTCGGTGAGGAG-3′. The PCR fragment was ligated into pcDNA3-Flag to yield pFlag-dlk. pFlag-dlk was transfected into cos cells. Purification of the Flag-dlk protein from cos-conditioned media was performed according to manufacturer's directions using the Flag monoclonal antibody, M1, immobilized on agarose (International Biotechnologies).

Plasmid Constructs and Stable Transfection. Full-length murine dlk cDNA was obtained by RT–PCR with primers BP 151 (see above) and antisense BP 200: 5′-GCATCTAGAGC-GGCCGCGAACGCTGCTTAGATCTCCT-3′, using total RNA from NIH 3T3 cells as template. The product was subcloned into the vector pCRII (Invitrogen) and then cloned into a retroviral expression vector (20), a kind gift of G. Nolan, Stanford University, via the primer-encoded *Hin*dIII and *Not*I sites. Supercoiled plasmid was transfected into BFC012 stromal cells together with the pZeo (Invitrogen) selectable marker and selected in 50 μg/ml Zeocin (Invitrogen). BFC012 cells also were transfected with pZeo alone and selected as above. Clones from both selected populations were isolated, and all remaining colonies (100–200 per dish) were pooled and expanded as populations.

Hematopoietic Stem Cells and *in Vitro* Hematopoietic Assays. Hematopoietic stem cell populations were derived from wild-type, Ly5.2-C57BL/6J (Jackson Laboratories), day 14 fetal liver, enriched for the AA4.1$^+$, Sca-1$^+$, c-*kit*$^+$, and lin$^{lo/-}$ phenotype, by immunopanning and fluorescence-activated cell sorting as described (21). Adult bone marrow (BM) was used directly after density centrifugation and immunomagnetic bead depletion or was further enriched for Sca-1$^+$, c-*kit*$^+$, lin$^{lo/-}$ cells by flow cytometry as described (22). Cell sorting was accomplished with a Becton Dickinson FACS Vantage using Cell Quest software. Stromal cell/stem cell cocultures were initiated in 12-well trays with 300–1,000 enriched stem cells per well. CSAs were quantitated by inverted-phase microscopy as described (23). Clonogenic progenitor assays were performed with either freshly purified stem cells or cells harvested from the stromal cocultures. These were cultured in cytokine-containing semisolid media according to the manufacturer's recommendations (Stem Cell Technologies, Vancouver, B.C.). Soluble dlk and control fusion proteins were added to semisolid progenitor assays at concentrations of 0.1, 0.5, and 1.0 μg/ml and also to BFC012 stromal cocultures at concentrations of 0.1 μg/ml. Fusion protein was replenished weekly in the stromal cocultures.

Competitive Repopulating Transplantation Assay. Cultured cells were harvested, combined with fresh unfractionated BM obtained from congenic C57BL/6 Ly5.1 mice (National Cancer Institute), and transplanted into lethally irradiated (10 Gy, split dose 3 h apart from a ^{137}Cs source, 1 Gy/min) Ly5.1 recipient mice. Each mouse received 2×10^5 competitor BM cells and a fraction of the cocultured stem cells. Mice were bled by capillary puncture of the orbital venous plexus, and 100 μl was collected; red blood cells were removed by NH_4Cl lysis. The nucleated cells were stained for the Ly5.2 (CD45.2) allelic marker using either fluorescein isothiocyanate-labeled directly conjugated Ly5.2 monoclonal antibody or a biotinylated form developed with streptavidin conjugated to Texas red. Cells also were stained with directly conjugated antibodies to lineage markers. All antibodies and chromogens were obtained from PharMingen. Flow cytometric analysis was done on a Becton Dickinson FACS Vantage using Cell Quest software.

RESULTS

Genes expressed in AFT024, but not in 2018, were identified by a subtractive cloning approach (K.A.M. and I.R.L., unpublished data). Sequence analysis identified one of these AFT024-specific clones as dlk. Expression studies, (Fig. 1*A*) show high levels of dlk in AFT024 and subclones isolated from this line, but undetectable levels in 2018 and BFC012. The latter two stromal cell lines do not support repopulating stem cells. The PA6 stromal cell line and NIH Swiss 3T3 cells both show expression of dlk and were the cell sources for identification of stromal cell protein-1 and dlk, respectively. PA6 cells

have been shown to support *in vitro* hematopoiesis and long-term, *in vivo* repopulating stem cells (24). Swiss 3T3 cells are also capable of supporting multipotent hematopoietic stem cells *in vitro*, promoting CSAs and maintaining *in vivo* spleen colony-forming units (CFU) (25). Interestingly, an additional fetal liver stromal cell line, 2012, which has some degree of stem cell supporting activity (9), and its subclones also express dlk. Furthermore, an RT–PCR analysis (40 cycles) of an additional 10 fetal liver-derived stromal cell lines and several other lines, shows detectable levels of dlk in only two additional lines (Fig. 1*B*). Our previous studies have shown that these two cell lines (CFC032 and CFC008) can maintain some level of long-term transplantable stem cell activity present in whole BM (8). However, they remain to be characterized for an ability to support enriched stem cells. While far from exhaustive, our studies suggest a correlation between a stromal cell line's ability to support stem cells and the expression of dlk. Therefore, we undertook functional studies to ask if dlk can act on hematopoietic stem cells.

As a first approach, soluble dlk protein was added to progenitor cultures in semisolid media. The soluble protein consisted of the dlk extracellular domain fused to the Fc portion of human IgG_1. The stem cell sources in these assays were highly enriched fetal liver cells (AA4.1$^+$, lin$^{lo/-}$, Sca-1$^+$, c-*kit*$^+$). We asked if the addition of soluble dlk could influence hematopoietic progenitor colony-formation. As shown in Table 1, no differences were noted either in the number, sizes, or lineage compositions of colonies. Identical results were obtained at dlk concentrations ranging from 0.1 to 1.0 μg/ml. In addition, no differences were noted in similar studies using enriched BM cells (Sca-1$^+$, c-*kit*$^+$, lin$^{-/lo}$) (data not shown).

The first evidence for a positive effect of the dlk protein on stem/progenitor cells was observed when the soluble form was added to dexter-type cocultures. For these studies we used a stromal cell line (BFC012) that neither expresses endogenous dlk (see Fig. 1) nor maintains significant *in vitro* hematopoiesis. In four experiments, two each using highly enriched adult BM and fetal liver stem cells, we monitored the appearance of CSAs over time. These colonies provide a convenient, quantitative estimate of hematopoietic activity initiated by primitive stromal dependent stem/progenitor cells (23). As shown in Fig. 2, the addition of soluble dlk (0.1 μg/ml) results in an approximately 2-fold increase in the number of CSAs initiated by purified fetal liver or BM stem cells over a 2-week time period ($P = 0.001$ for dlk vs. control and $P = 0.01$ for dlk vs. no additive, Student's t test). There was no difference in the numbers of CSA observed in BFC012/stem cell cultures with or without control fusion protein (mean of no additive/control = 0.96 ± 0.11).

To assess the activity of the normal transmembrane form of dlk, we transfected a full-length dlk cDNA into BFC012 cells. Expression of the introduced dlk was demonstrated at the RNA (Northern blot) and protein levels using both Western blot and flow cytometric analyses with rabbit anti-dlk antibodies. The flow cytometry data are presented in Fig. 3.

Initially, dlk-expressing transfected populations (BFC-dlk) were compared with a negative control "mock" transfected population of BFC012 cells. A 4- to 6-fold increase in the number of CSAs was observed in two separate experiments (data not shown). The maintenance of CSAs was transient, lasting less than 2 weeks. No further hematopoietic activity was observed during an additional 2 weeks of culture. Once dlk-expressing clones had been identified from the transfected populations, they were studied for their ability to support CSAs in experiments designed to more precisely identify the time course of hematopoietic activity. Ficoll-separated, lineage-depleted BM was used in these experiments. Five different negative control, non-dlk expressing BFC012 cell groups (parental BFC012 cells, two "mock" transfected populations, and two "mock" transfected clones) and three dlk-expressing BFC012 cell groups (one transfected population

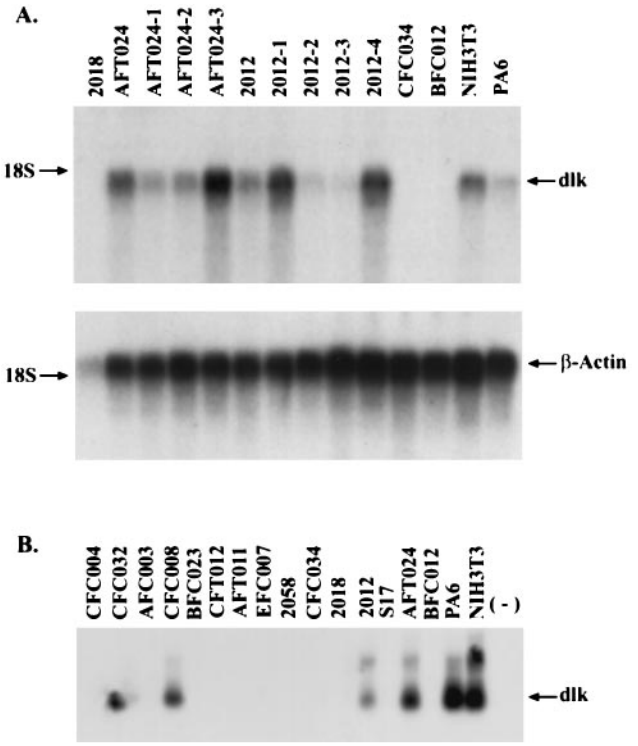

FIG. 1. Dlk expression analysis in stromal cell lines. (*A*) (*Upper*) A 1.6-kb dlk transcript is visualized in the parental AFT024 and 2012 cell lines and their subclones, but not in 2018, CFC034 and BFC012. (*Lower*) The same filter hybridized with a β-actin probe. (*B*) RT-PCR analysis of 14 fetal liver-derived stromal cell lines and other cell lines.

and two clones) were studied. The data are presented in Fig. 4*A*. Neither the negative control BFC populations nor the "mock" transfected BFC clones supported high numbers of CSAs. In contrast, the BFC-dlk populations and the two individual dlk-expressing clones supported significantly greater numbers of CSAs at all time points studied ($P < 0.001$ days 3, 4, and 5; $P < 0.01$ days 6 and 7, Student's t test). As observed previously, all the CSAs were transient. This experiment also indicated that the dlk-promoted hematopoietic activity peaks early, at 4 days, in this culture system. Three additional experiments using purified (AA4.1$^+$, lin$^{lo/-}$, Sca-1$^+$, c-*kit*$^+$) fetal liver stem cells were performed using two individual clones, BFC-dlk-5 and a "mock" transfected negative control BFC-Zeo-1. The results are presented in Fig. 4*B*. There was a dramatic and significant difference in the number of CSAs observed in the BFC-dlk-5 cultures compared with the control line ($P < 0.001$, days 4, 6, and 8,

Table 1. dlk has no affect in clonogenic progenitor assay

Additive	Conc., μg/ml	CFU* Total	G/GM/M	Mix	HPP-Mix
None	—	95.4 (7.93)	50.1 (8.52)	30.5 (2.68)	13.2 (2.58)
dlk	0.1	95.9 (6.86)	59.2 (9.31)	23.9 (3.08)	12.8 (1.82)
	0.5	82, 113	43, 73	26, 22	13, 18
	1.0	99, 110	50, 70	31, 22	18, 18
Control	0.1	95.5 (6.53)	59.1 (9.31)	24.5 (3.82)	11.8 (1.42)
	0.5	78, 121	43, 82	17, 24	18, 14
	1.0	77, 104	41, 67	24, 21	11, 16

Four fetal liver purifications provided the stem cells for the studies with no additive, and 0.1 μg/ml and the data are expressed as the mean of these experiments, with SEM in parentheses. Data from two separate experiments with additive at 0.5 and 1.0 μg/ml are shown.
*CFU are expressed per 1,000 AA4.1$^+$, lin$^{-/lo}$, Sca-1$^+$ and c-kit$^+$ fetal liver cells. G, granulocyte; M, macrophage; GM, granulocyte/macrophage; Mix, contain 2 or more myeloid lineage cells and erythroid bursts, HPP $\geq$ 1 mm in 8 days.

Student's *t* test). As before, the effect was transient, and the CSA declined in number over 2 weeks. AFT024 was included as a positive control and, in each of the three experiments, verified the quality of the input purified stem cells. In the first week of culture the numbers of CSAs observed on AFT024 were similar to the numbers in the BFC-dlk5 cultures (data not shown).

To address the "primitiveness" of the cells that give rise to the CSA observed in the BFC-dlk cocultures, a series of *in vitro* replating experiments were performed. Individual wells were harvested at various time points of coculture, and the cells were plated into semisolid cytokine-containing media. The numbers and lineage compositions of the colonies were scored after 8–12 days. As shown in Fig. 5*A*, the CSAs obtained from day 4 BFC-dlk-5 cocultures contained numerous progenitors capable of extensive proliferation and multilineage differentiation. The total number of progenitors from the dlk-expressing cultures at day 4 was significantly expanded compared with the content in the freshly purified uncultured stem cell population ($P = 0.01$, Student's *t* test). The number and lineage composition of colonies derived from parallel day 4 AFT024 cultures was nearly identical to BFC-dlk-5 derived colonies (data not shown). The content of CSAs replated at day 6 from the BFC-dlk-5 cocultures was devoid of multilineage colonies, although CFU-granulocyte–macrophages were maintained at high levels; the progenitor content in the BFC-dlk-5 cocultures continued to decrease when next sampled at 10 days (data not shown). In contrast, few progenitors could be demonstrated in the BFC-Zeo-1 cultures ($P = 0.001$, BFC-dlk-5 vs. BFC-Zeo-1, Student's *t* test) (Fig. 5*A*). Taken together, the data strongly suggest that dlk acts to promote stromal-dependent colony formation by primitive cells capable of yielding large numbers of committed progenitors, including those endowed with a high proliferative capacity and multilineage differentiation potential. The lack of CSAs and significant progenitor maintenance in the BFC-Zeo-1 cultures argues that expression of dlk in the transfected BFC012 cells is responsible for both their ability to support CSAs and to generate/maintain primitive progenitors.

We next asked if the CSA-containing cultures supported by BFC-dlk-5 contained stem cells capable of *in vivo* engraftment. Accordingly, portions of the same day 4 cocultures that were plated into progenitor assays also were used to transplant mice in competitive repopulation assays. Shown in Fig. 5*B* are the results from two independent experiments analyzed at 10 weeks after transplant. The same BFC-dlk-5 cultures that contain CSAs and primitive CFU-high proliferative potential (HPP)-Mix progenitors also contain repopulating stem cells at levels equal to those maintained in parallel AFT024 cocultures. In addition, a significant difference exists in the levels of repopulating stem cells derived from dlk-expressing cocultures compared with non-dlk expressing BFC012 cells ($P = 0.05$, Student's *t* test). Multicolor flow cytometric analyses also demonstrated that both myeloid and lymphoid Ly5.2 cells are present in these animals (data not shown). A subsequent analysis of these animals at 22 weeks demonstrated lower levels of repopulation with Ly5.2 cells derived from the AFT024 and BFC-dlk-5 supported cultures (data not shown). Most significantly, no repopulation was observed at any time point in mice that received cells cocultured on the non-dlk-expressing monolayers (Fig. 5*B*).

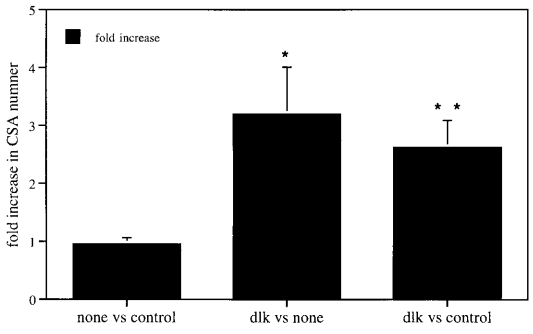

FIG. 2. CSA formation by hematopoietic stem cells in the presence of soluble dlk protein. Data are from four experiments: two each with adult BM cells (Sca-1$^+$, c-*kit*$^+$, lin$^{lo/-}$), and day 14 fetal liver cells (AA4.1$^+$, Sca-1$^+$, c-*kit*$^+$, lin$^{lo/-}$). Results are expressed as the ratio/fold-increase in CSA number for 14 data points each for the four different experiments. Error bars represent SEM. *, $P = 0.01$ comparing dlk vs. none to none vs. control; **, $P = 0.001$ comparing dlk vs. control to none vs. control (Student's *t* test).

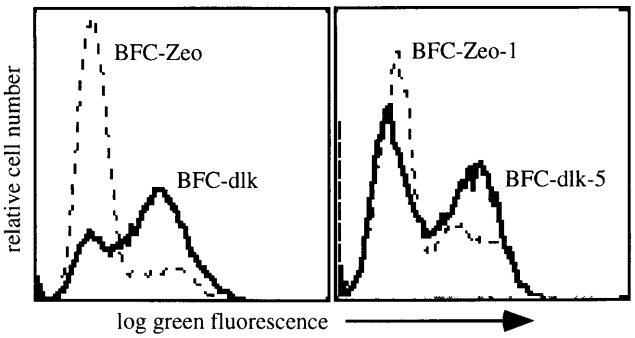

FIG. 3. Membrane-bound dlk expression in transfected BFC012 cells. (*Left*) A flow diagram of dlk expression in transfected BFC012 populations (BFC-dlk) and cells transfected with the selection plasmid alone (BFC-Zeo). (*Right*) Expression of dlk in a cloned line (BFC-dlk-5) derived from the expressing population and a control clone (BFC-Zeo-1).

DISCUSSION

As part of an ongoing effort to understand the biology of the hematopoietic microenvironment, we have established and characterized a panel of stromal cell lines from midgestation fetal liver. Among these cell lines, the AFT024 line has the ability to maintain nearly quantitative levels of transplantable stem cell activity for extended *in vitro* time periods (9). Because these cultures are initiated with highly purified stem cell populations it is likely that the AFT024-derived molecular mechanisms responsible for this ability act directly on the stem cell population. We also have identified other stromal lines that fail to maintain stem cell activity. These observations facilitated a subtractive hybridization approach aimed at identifying potential candidate molecules whose collective actions may be responsible for the AFT024 stem cell maintenance activity. This effort has identified dlk, a transmembrane molecule containing six EGF-like repeat motifs. Although lacking the DSL motif indicative of the notch ligands delta and serrate (26), dlk is most closely homologous to delta/notch/serrate when compared with other EGF-like repeat containing molecules (10, 11). The predominant role of these types of molecules in cell growth and differentiation led us to investigate the potential role of dlk in hematopoiesis. Constitutive expression of translocated human notch (Tan-1) is found in a T-cell leukemia (13). Moreover the expression of Tan-1 in primitive human stem cells has been demonstrated (27). Nevertheless, a functional role in hematopoiesis for the notch ligands jagged (15) and dll-1 (delta-like gene 1) (14) has not been described. We have shown that dlk expression is highly restricted in a panel of stromal cell lines. Two lines, AFT024 and 2012, which maintain repopulating stem cell activity *in vitro*, express dlk, whereas two nonsupportive cell lines, 2018 and BFC012, do not. Interestingly, the S17 stromal cell line, which is considered to be a potent stem cell supporter (28), does not express detectable levels of dlk (Fig. 1*B*). The S17 cell line was derived from adult BM (29). The other lines we have described all are derived from fetal sources (AFT024, 2012, and NIH 3T3 cells) or from newborn calvaria (PA6 cells). It is

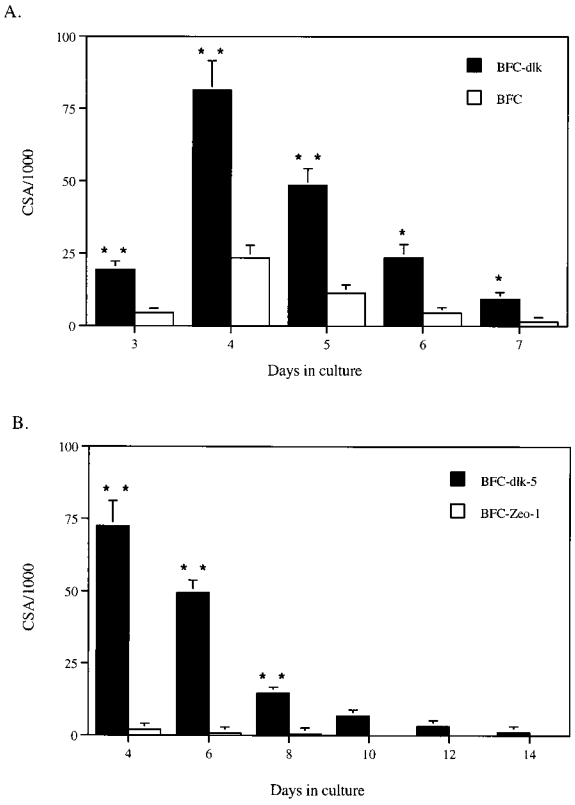

FIG. 4. CSA formation by hematopoietic stem cells in the presence of membrane-bound dlk. (*A*) Bars labeled BFC are from five groups (nontransfected BFC012 cells, two control pZeo-transfected BFC012 populations, and two clones derived from the pZeo-transfected populations). Bars labeled BFC-dlk are from three groups shown to express transfected dlk; one dlk-transfected BFC012 population and two individual transfected clones. Error bars represent SEM. **, $P < 0.001$ days 3, 4, and 5; *, $P < 0.01$ days 6 and 7 (Student's *t* test). (*B*) A clone derived from the dlk-transfected populations of BFC012 cells (BFC-dlk-5) and a clone derived from pZeo-transfected populations (BFC-Zeo-1) were used for CSA assay with purified fetal liver stem cells. CSA/1000 input stem cells are expressed as the mean of three individual experiments, error bars represent the SEM. **, $P < 0.001$ at days 4, 6, and 8 (Student's *t* test).

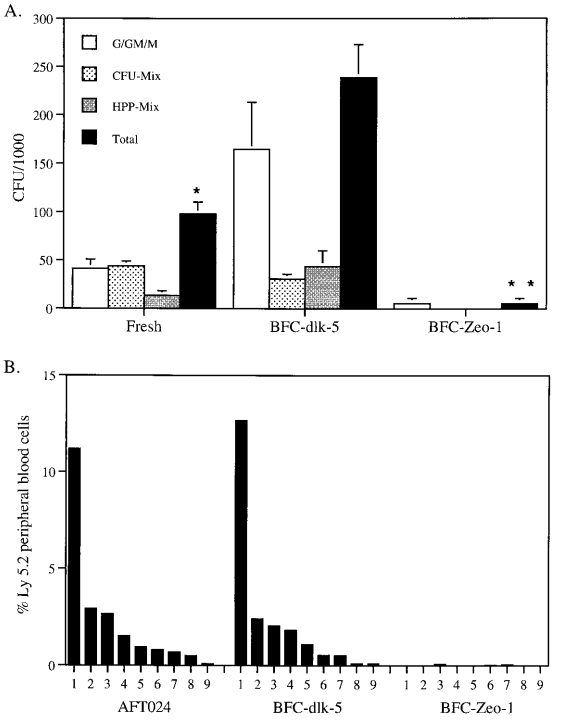

FIG. 5. HPP multilineage clonogenic progenitors and *in vivo* repopulating stem cells are maintained in short-term dlk-expressing cocultures. (*A*) Fetal liver stem cells were purified as described and assayed for their progenitor content immediately after purification and after culture on BFC-dlk-5 and BFC-Zeo-1. At day 4, the cultures were used for clonogenic progenitor (three experiments) and transplantation assay (two experiments). Bars represent data from three experiments with day 0 cells (Fresh) and day 4 cocultured cells (BFC-dlk-5 and BFC-Zeo-1), error bars represent SEM. *, $P = 0.01$ for total CFU-C from fresh stem cells compared with total CFU-C from BFC-dlk-5 cocultures at day 4; **, $P = 0.001$ for total CFU-C from BFC-dlk-5 compared with total CFU-C from BFC-Zeo-1 (Student's *t* test). (*B*) Analysis of *in vivo* repopulating ability of purified fetal liver stem cells cocultured for 4 days on BFC-dlk-5, BFC-Zeo-1, and AFT024 monolayers. Results are from nine individual mice in two experiments (4–5 mice in each experiment) at 10 weeks after transplantation. $P = 0.05$ for BFC-dlk-5 vs. BFC-Zeo-1 (Student's *t* test).

therefore possible that dlk acts in a developmentally regulated fashion. An extensive analysis of dlk expression in adult BM stroma is currently underway. Taken together, our data suggest that, at least in fetal stromal cell types, there exists a correlation between hematopoietic supporting ability and the expression of dlk.

We chose to directly measure the potential activity of both soluble and transmembrane dlk protein, on highly purified stem cell populations using *in vitro* and *in vivo* assays. Initial experiments designed to ask if dlk can enhance colony formation in cytokine-rich semisolid assay systems were negative. These results may indicate that: (*i*) progenitor cells capable of colony formation in semisolid assays do not respond to dlk, (*ii*) the collection of cytokines present in the semisolid cultures may "mask" an effect(s) of added dlk, or (*iii*) the soluble form of dlk requires a stromal monolayer to mediate its effects. The first possibility can be addressed more extensively in delta-type assays, where stem/progenitor cells are first cultured in suspension in serum-free media containing various cytokine combinations, with and without dlk, and then replated into colony assays (30). The second possibility can be addressed by more extensive studies using subsets of the cytokines present in our initial studies. These experiments are underway. As a first step to address the third possibility, we added soluble dlk to preestablished BFC012 monolayers. Using both purified BM and fetal liver stem cell populations, a significant increase in CSA colony formation was observed in the dlk-supplemented cultures (Fig. 2). This was a surprising result, given that dlk is a transmembrane protein; however, before its cDNA cloning, a soluble form of dlk was identified as FA1 or fetal antigen 1 (ref. 31 and references therein). A role in hematopoiesis was not indicated in these studies, but expression was detected in stroma of placental villi, in yolk sac blood islands, and in fetal liver (31). It will be of interest to see if a soluble form is produced by the stromal cell lines that express dlk. An additional explanation, for the effects observed with the soluble form added to stromal/stem cell cocultures, is that they may be facilitated by the Fc portion of the fusion protein. It is possible that Fc receptors expressed by some of the hematopoietic cells in the cultures are able to sequester and present the dlk-Fc fusion protein more effectively. This possibility can be addressed by using a different type of soluble dlk protein. These studies have been initiated. Alternatively, the soluble dlk may be sequestered and thus presented by the stromal cell extracellular matrix. To further address the third possibility, we introduced an intact transmembrane form of dlk into the BFC012 stromal cell line. Initially, dlk-transfected BFC012 cell populations were compared with BFC012 cells transfected with the selectable marker alone. In these studies, the dlk-expressing monolayers were more effective at promot-

ing CSA colonies. As with the soluble dlk experiments, the CSAs appeared early and were transient. When cells were replated from the BFC-dlk supported cocultures onto an AFT024 monolayer a reiteration of the burst of CSAs was seen that was maintained for 3 weeks (data not shown). In addition, replating of these cocultures revealed a high progenitor content (≈1 in 10) that included a high percentage (43%) of multipotential colonies, including HPP-Mix. In the BFC-Zeo control populations neither replatable CSA nor CFU progenitors were maintained. Further experiments with individual clones from the transfected populations confirmed and extended the results obtained with the populations, demonstrating highly significant differences in the numbers of developing CSAs (Fig. 4). However, in two experiments we observed that one dlk-transfected BFC012 clone, which expresses a very high level of dlk, supported fewer CSAs than non-dlk-expressing control cells. These cultures also suggested differentiation phenomena, as indicated by the number of rapidly accumulating nonadherent cells. Experiments using this cell line were not included in our analyses. It is possible that there may be a threshold level of dlk expression necessary in these cultures, and when it is surpassed the cells differentiate and die rapidly in the culture mileau provided by BFC012 cells. In addition, it is possible that an aberrant form of the dlk protein is made by this line. Further studies are necessary to clarify this issue. Nevertheless, in Fig. 4*A*, dlk-transfected BFC012 cells (one population and two clones) show a significant enhancement of CSA formation compared with controls.

The observed low-level maintenance of competitive repopulating stem cells in short-term, dlk-expressing cocultures is of interest even though the activity diminished over time. These studies show that the ectopic expression of a single molecule (dlk) in a previously nonsupporting stromal cell line restores or enables hematopoietic support. This is demonstrated by maintenance of three different stem/progenitor cell compartments: (*i*) CFU-HPP-Mix, (*ii*) CSA, and (*iii*) short-term, *in vivo* repopulating stem cells. It is also of interest that both qualitatively and quantitatively similar stem/progenitor cell compartments are maintained in short-term AFT024 supported cocultures (9).

While it is premature to speculate extensively on the exact mechanism underlying the effects of dlk, two possibilities can be considered. First, it may be that some level of dlk expression is sufficient to retard potent differentiation signals provided by the BFC012 cell line. Second, dlk may provide a proliferative stimulus not normally produced by BFC012. A more direct and perhaps relevant assay will be to eliminate the expression of dlk in AFT024 cocultures, thus maintaining other components that make up the culture "milieu." Studies to evaluate potential neutralizing antibodies and various dlk antisense strategies are underway.

The failure of BFC-dlk-5-supported CSAs to persist for periods longer than 1 to 2 weeks also may suggest the existence of other molecules in AFT024 that facilitate hematopoiesis. In this regard, it is interesting that our subtraction screen has yielded several other clones with expression patterns very similar to dlk. Eventually, with the addition of dlk and other AFT024 specific molecules it may be possible to reconstruct a supportive phenotype. This should lead us toward a further understanding of the *in vivo* hematopoietic microenvironment. In summary, we would like to propose that dlk represents one molecular component responsible for the hematopoietic supportive activities of the AFT024 cell line. As such, dlk may define a novel molecular pathway of stem cell regulation by the hematopoietic microenvironment.

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