[CANCER RESEARCH 56, 1179-1183, March 15, 1996]

Advances in Brief

Double Knockout of the *ALL-1* Gene Blocks Hematopoietic Differentiation *in Vitro*[1]

Vincenzo Fidanza,[2] Paola Melotti,[2] Takahiro Yano, Tatsuya Nakamura, Alan Bradley, Eli Canaani, Bruno Calabretta, and Carlo M. Croce[3]

The Jefferson Cancer Institute and the Jefferson Cancer Center, Jefferson Medical College, Philadelphia, Pennsylvania 19107 [V. F., P. M., T. Y., T. N., B. C., C. M. C.]; The Institute of Molecular Genetics, Baylor College of Medicine, Houston, Texas 77030 [A. B.]; and Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel [E. C.]

Abstract

The *ALL-1* gene is involved in translocations with many partner genes in different types of acute leukemias, but it is not clear whether it acts as an oncogene or whether the fusion proteins resulting from the translocations have dominant negative effects. To distinguish between these two possibilities, we analyzed the ability of wild-type AB2.1 embryonal stem (ES) cells and of single or double *ALL-1* gene knockout cells derived from them to differentiate along hematopoietic lineages after withdrawal of leukemia inhibitory factor, using *in vitro* colony formation assays. *ALL-1* double knockout ES cells formed a significantly greater number of colonies with faster kinetics than wild-type and *ALL-1* single knockout ES cells. Parental ES cells formed lineage-restricted colonies, whereas single and double knockout ES cells developed, at high frequency, immature and/or "biphenotypic" colonies, mimicking the aberrant hematopoiesis typical of leukemic patients. These data are consistent with the possibility that loss of function of the *ALL-1* gene is important in leukemogenesis.

Introduction

Chromosomal translocations involving 11q23 are often observed in acute leukemias, such as acute lymphocytic leukemia, acute myeloblastic leukemia, acute myelomonocytic leukemia, and acute monocytic leukemia (1), cause the fusion of the *ALL-1* gene, also named *MLL* or *Hrx* (2, 3), to one of at least 20–30 different genes mapping to different chromosomal regions (4). Isolation and characterization of the partner genes fusing with *ALL-1* indicate that they have different characteristics (4, 5). This observation, in conjunction with our recent observation that the *ALL-1* gene can fuse with itself in ~50% of acute myeloblastic leukemias with *ALL-1* gene rearrangement (6), suggested that the *ALL-1* gene might be involved in the production of a fused protein with a dominant-negative effect in acute leukemias (6). Thus, the *ALL-1* gene may be a tumor suppressor gene. Mouse ES[4] cells can be maintained *in vitro* for many generations as totipotent cell lines when cultured in the presence of LIF (7). Within 3 to 8 days after LIF removal, ES cells form complex EBs with endoderm, basal lamina, mesoderm, blood islands, and ectoderm (8). Such EBs are morphologically similar to embryos of the 6- to 8-day cylinder stage (9). EBs contain hematopoietic progenitor cells that, under certain culture conditions, can differentiate along distinct hematopoietic lineages (10, 11), following a definite order mimicking that described in the embryo (12). Thus, ES cells provide a unique model to investigate the consequences of ablating the expression of a gene with a postulated hematopoiesis-specific function. Accordingly, *ALL-1* gene expression was knocked out in mouse ES cells, and the consequences of ALL-1 loss of function on hematopoietic cell differentiation and proliferation were determined. We report here that, compared with wild-type cells, ES cells with homozygous disruption of the *ALL-1* locus form a greater number of hematopoietic colonies that are "biphenotypic" and/or more immature. These observations are consistent with the possibility that loss of function of ALL-1 is important in leukemogenesis.

Materials and Methods

Construction of a Targeting Vector. Several overlapping clones were isolated from a λFIXII phage library of a 129SVJ mouse strain (Stratagene) using standard procedures. Restriction mapping and partial sequence analysis were performed to identify exon-intron boundaries. Genomic sequences encompassing exons 2–4 were cloned from an artificial *Sal*I site to a genomic *Aat*II site. The third exon was deleted partially from an intronic 5′ *Sma*I site to an internal (exon 3) *Bam*HI site and substituted by an *Eco*RV-*Bam*HI fragment containing a *neo* gene cassette derived by conventional techniques from the PGK-neo plasmid (13).

In the 5′ segment, the construct also contains a 5′ herpes simplex virus thymidine kinase cassette derived from the *MCI-TK* gene that was derived from the MCI-TK plasmid by a *Sal*I-*Xho*I digestion followed by cloning into the *Sal*I site of the pGEM 5Zf vector (see Fig. 1*a*).

Transfection of ES Cells and Southern Analysis. The targeting construct was linearized with *Sal*I and ES. AB2.1 cells (1×10^7) were electroporated, and clones were selected in G418 and FIAU (14). Two hundred clones were screened sequentially with the 3′ (Fig. 1, *left*) and the 5′ (Fig. 1, *right*) probe, and 7 heterozygous targeted clones were identified and expanded. These heterozygous clones were plated at a concentration of 1×10^5 cells/plate and selected at a high concentration (6 mg/ml) of G418. After 20 days, two clones were obtained (Fig. 1*b*, *Lanes 2* and *3*). Ten μg of genomic DNA was digested with *Eco*RI and electrophoresed on a 0.7 agarose gel. The gel DNA was transferred onto a zeta-probe GT membrane (Bio-Rad, Hercules, CA) and hybridized sequentially to the 3′ and the 5′ probes. Both of these clones were confirmed to be homozygous for the targeted allele.

RNA Extraction and Northern Blot Analysis. Poly(A)$^+$ RNAs were prepared from ES cells by a combination of total RNA extraction using RNAzol (Tel-Test, Inc., Friendswood, TX) and a subsequent purification using mRNA Purification Kit (Pharmacia Biotech, Piscataway, NJ). Fifteen μg of poly(A)$^+$ RNA were electrophoresed in a 1% formaldehyde-agarose gel and transferred onto a Zeta-probe GT membrane. The filter was hybridized with a 0.9-kb mouse cDNA fragment that contained the third exon of the *ALL-1* gene.

Culture, Differentiation, and Hematopoietic Colony Formation of ES Cells. Undifferentiated ES AB2.1 cells were maintained in gelatinized tissue culture dishes in DMEM supplemented with 15% heat-inactivated fetal bovine serum, 2 mM glutamine, 0.1 mM β-mercaptoethanol, and 1500 units of recombinant LIF (Genetics Institute, Inc., Cambridge, MA). Differentiation was allowed to occur in the absence of LIF in suspension culture in 100-mm

Received 1/5/96; accepted 1/31/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by the Falk Medical Research Trust, by Outstanding Investigator Grant CA 39860 from the National Cancer Institute, by National Cancer Institute Grant PO1 CA 50507, and by American Cancer Society Grants DHP-81 and DHP-3D. P. M. was supported in part by a fellowship of the Associazione Italiana per la Ricerca sul Cancro.

[2] The first two authors contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Jefferson Cancer Institute, Thomas Jefferson University, BLSB, Room 1050, 233 South 10th Street, Philadelphia, PA 19107-5799.

[4] The abbreviations used are: ES, embryonal stem; LIF, leukemia inhibitory factor; EB, embryoid body; PGK, phosphoglycerate kinase; PE, phosphatidylethanolamine; RT-PCR, reverse transcription-PCR; MPO, myeloperoxidase; poly(A)$^+$, polyadenylated; RAG-1, recombination activating gene 1; FIAU, 1-(2-deoxy,2-fluoro-β-D-arabinofuranosyl)-5 iodouracil.

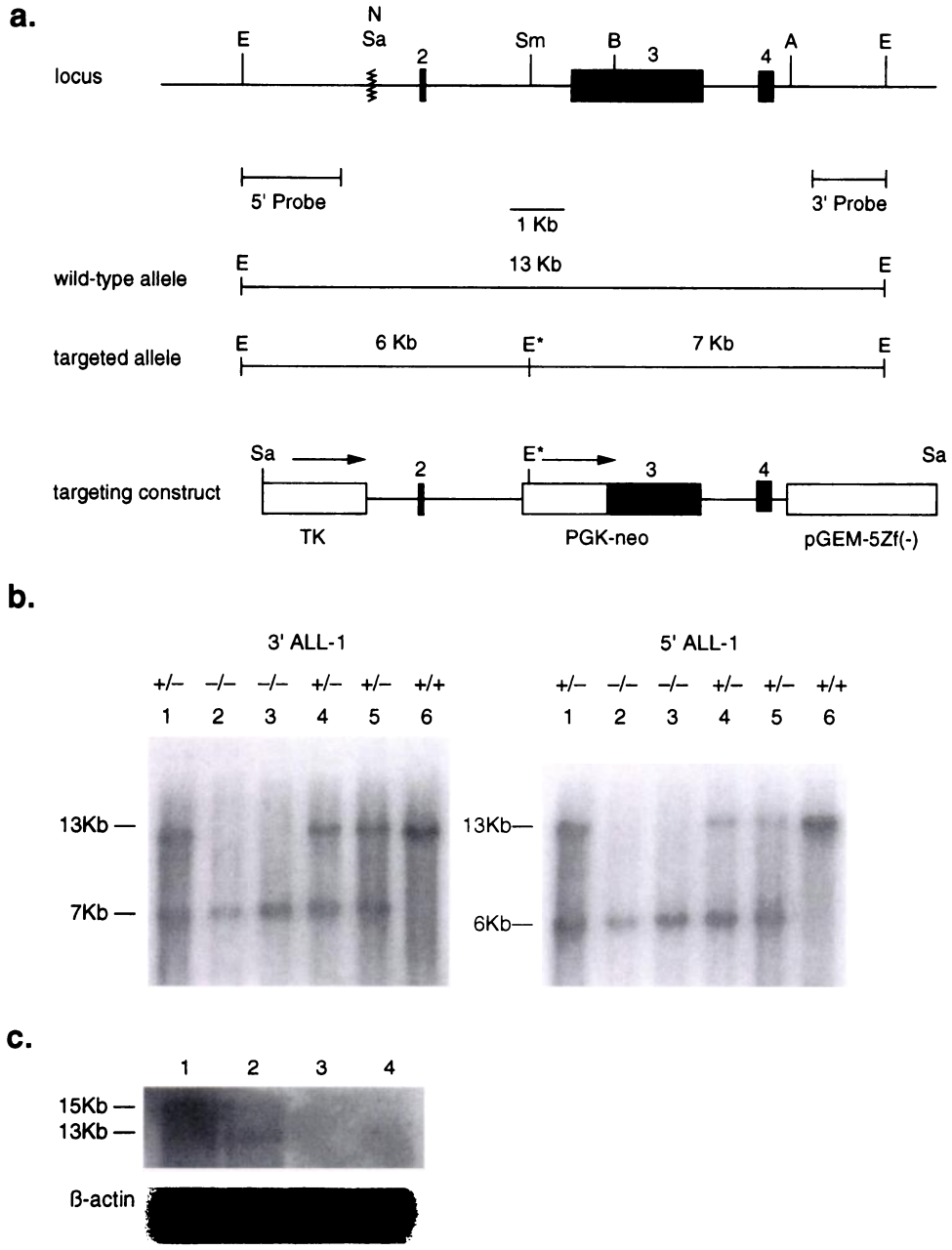

Fig. 1. Targeted disruption of the *ALL-1* locus and ALL-1 expression from single and double knockout AB2.1 ES cells. *a*, partial restriction map of the mouse *ALL-1* locus, wild-type allele, targeted allele, and targeting construct. The *PGK-neo-bpa* cassette gene replaces 5′ sequences of the exon 3 between *Sma*I and *Bam*HI restriction sites. *Eco*RI (*E*), *Bam*HI (*B*), *Sma*I (*SM*), *Aat*II (*A*), *Xba*I (*X*), *Not*I (*N*), and *Sal*I (*Sa*) are artificial sites within the λFixII phage vector E*; *Eco*RI restriction site in the targeted allele indicates the *Eco*RI site introduced with the PGK-neo cassette. The numbers *2*, *3*, and *4* shown above the *dark boxes* indicate, respectively, the second, third, and fourth exons. In the targeting construct, a herpesvirus-thymidine kinase cassette is 5′ of the genomic sequences. This construct was cloned into the pGEM-5ZF( ) vector (Promega). The 5′ probe was isolated from an *ALL-1* overlapping phage, and the 3′ probe was isolated from the same clone used to prepare the targeting construct. *b*, Southern blot analysis of the *ALL-1* locus in AB2.1 ES cells. *Lanes 1*, *4*, and *5*, single knockout; *Lanes 2* and *3*, double knockout; *Lane 6*, AB2.1 wild-type cells. The blot was hybridized sequentially with the 3′ (*left*) and 5′ (*right*) probes. The wild-type band is a 13-kb *Eco*RI fragment. The length of the disrupted alleles is 6 or 7 kb, depending on the probe used. *c*, Northern blot analysis of ALL-1 transcripts in AB2.1 cells. Total RNA was extracted from wild-type, *ALL-1* single and double knockout AB2.1 ES cells maintained in culture in the presence of LIF. For Northern blot analysis, 10 μg poly(A)$^+$ RNA were used in each lane and hybridized sequentially to a 0.9-kb exon 3 fragment and to a 2.0-kb β-actin insert ^{32}P-labeled by the random-priming method. *Lane 1*, AB2.1 cells; *Lane 2*, AB2.1 48$^{all+/-}$ cells; *Lane 3*, 48N$^{all-/-}$ AB2.1 cells; *Lane 4*, 48S$^{all-/-}$ *AB2.1* cells.

bacterial Petri dishes in DMEM supplemented with 15% heat-inactivated fetal bovine serum and 2 mM glutamine.

At various times during differentiation, EBs were dissociated, and cells (10^4/35-mm bacterial-grade dish) were cultured in 0.9% methylcellulose as described (12) with or without recombinant murine c-kit ligand (25 ng/ml; Immunex Corp., Seattle, WA), and recombinant FLAG human flt-3 ligand (50 ng/ml; Immunex Corp.). Colonies (>125 μM) were scored 14 days later.

Cell Surface Marker Expression Analysis. Staining of cells harvested from 14-day methylcellulose colonies from parental and *ALL-1* knockout ES cells was performed with FITC-conjugated antimouse Ly-6G (Gr-1); FITC-labeled antimouse c-kit; FITC-conjugated antimouse H-2K^b; PE-conjugated antimouse TER119/erythroid cells; and PE-labeled antimouse H-2D^b, irrelevant PE, or FITC-labeled mouse immunoglobulin G, as negative control (PharMingen, San Diego, CA). Cells were analyzed by flow cytometry on an EPICS Profile Analyzer (Coulter, Hialeah, FL).

Lineage Specificity of ES-derived Colonies. Individual colonies (20/experiment) were aspirated randomly 12 days after methylcellulose plating of AB2.1 cells derived from EBs after 3 or 7 days of growth in suspension culture in the absence of LIF. After RNA extraction (15), cDNA was synthesized using random hexamers in the reverse transcriptase reaction carried out at 37°C for 60 min. PCR amplifications were performed under standard conditions for 40 cycles using 10% of the first-strand reaction product and synthetic primers specific for the murine sequence of the following genes: *CD34* (16); c-*kit* (5′ primer corresponding to nucleotides 1750–1774 and 3′ primer to nucleotides 2018–2042 of the published murine c-*kit* sequence; Ref. 17); *MPO* (5′ primer corresponding to nucleotides 824–847 and 3′ primer to nucleotides 1476–1500 of the published murine *MPO* sequence; Ref. 18); c-*fms* (5′ primer corresponding to nucleotides 1441–1465 and 3′ primer to nucleotides 1865–1889 of the published murine c-*fms* sequence; Ref. 19); embryonic β-*globin* (βH1-globin; Ref. 12); and *RAG-1* (5′ primer corresponding to nucleotides 372–389 and 3′ primer to nucleotides 588–605 of the published murine *RAG-1* sequence; Ref. 20); and *Ikaros* (5′ primer corresponding to nucleotides 100–117 and 3′ primer to nucleotides 356–373 of the murine *Ikaros* published sequence; Ref. 21). Endogenous β-actin mRNA levels were also measured using synthetic primers as described (16) to ensure that similar amounts of RNA were utilized for mRNA expression analysis (data not shown). As a negative control, RT-PCR amplifications were performed in the absence of RNA. Amplified DNA was subjected to electrophoresis, transferred

to Zetabind nylon filters (Cuno, Inc., Meriden, CT), and detected by Southern hybridization with a [γ-^{32}P]ATP end-labeled oligoprobe.

Results and Discussion

Targeted Disruption of the *ALL-1* Locus and *ALL-1* Expression from Single and Double Knockout AB2.1 ES Cells. To inactivate the mouse *ALL-1* gene, we cloned the *ALL-1* gene from the 129 mouse strain and constructed a targeting vector (Fig. 1*a*), which was used to transfect AB2.1 ES cells (13). After selection in G418 and FIAU (14), DNAs of resistant clones were screened for *ALL-1* gene rearrangement by Southern blot hybridization with 3′ and 5′ *ALL-1* probes (Fig. 1*b*). Seven positive clones with a single knocked-out *ALL-1* gene were obtained and expanded. After 20-day selection in high concentrations of G418 (6 mg/ml), two clones with knockout of both copies of the *ALL-1* gene were obtained (Fig. 1*b*). The two homozygous cell lines, 48N$^{all1-/-}$ and 48S$^{all1-/-}$, did not express ALL-1 transcripts (Fig. 1*c*). Two ALL-1 transcripts were detected in parental AB2.1 cells and, at lower levels, in the heterozygous cell line 48$^{all1+/-}$ (Fig. 1*c*).

Hematopoietic Colony Formation from Parental and *ALL-1* Knockout ES Cells. ES cells undergo commitment to different hematopoietic lineages, thereby providing a unique cellular model to assess the phenotype of ALL-1-deficient cells. Undifferentiated wild-type AB2.1, 48$^{all1+/-}$ heterozygotes, and 48N$^{all1-/-}$ and 48S$^{all1-/-}$ double knockout AB2.1 cells did not form hematopoietic colonies when plated in methylcellulose (Fig. 2). At 3, 7, 10, and 14 days of liquid culture after LIF removal, parental, 48$^{all1+/-}$, 48N$^{all1-/-}$, and 48S$^{all1-/-}$ AB2.1 cells were disaggregated and plated in methylcellulose. After 3 days, colonies developed from 48$^{all1+/-}$, 48N$^{all1-/-}$, and 48S$^{all1-/-}$ AB2.1 cells but not from the parental AB2.1 wild-type ES cells (Fig. 2). At the later times, parental, single knockout, and double knockout ES cells all formed hematopoietic colonies; however, colonies from double knockout cells were consistently more numerous than those derived from parental and single knockout ES cells (Fig. 2*A*). Culture of ES cells in the presence of c-kit and flt-3 ligands resulted in increased colony formation, which was ~2-fold higher from *ALL-1* double knockout ES cells (Fig. 2*B*). In these experiments, we also used as control a clone of *ALL-1* single knockout ES cells (48T$^{all1+/-}$) that remained heterozygous after selection in a high concentration of G418. This selection procedure did not modify the colony formation properties of heterozygous *ALL-1*-deficient ES cells (Fig. 2*B*). Together, these data are consistent with the possibility that inactivation of the *ALL-1* gene accelerates stem cell commitment and/or progenitor cell expansion and differentiation; conversely, *ALL-1* may act as a negative regulator of hematopoietic development from ES cells, perhaps reflecting a similar role for it in bone marrow hematopoiesis. By inverted light microscopy, the morphology and size of the colonies derived from parental, 48$^{all1+/-}$, 48N$^{all1-/-}$, and 48S$^{all1-/-}$ AB2.1 cells were undistinguishable. However, their molecular phenotype might be different, reflecting the absence of *ALL-1* expression.

Lineage Specificity of ES-derived Colonies. Expression of *CD34* and c-*kit* (early hematopoietic progenitor markers), *RAG-1* and *Ikaros* (lymphoid precursor markers), *MPO* (granulocytic marker), c-*fms* (macrophage marker), and embryonic β-*globin* (βH1-globin; erythroid marker) mRNA was analyzed by RT-PCR (Table 1) in individual colonies derived from ES cells plated in semisolid medium at days 3 and 7 after LIF removal. *CD34* and c-*kit* mRNA were detected only in colonies which had developed from 48$^{all1+/-}$, 48N$^{all1-/-}$, and 48S$^{all1-/-}$ AB2.1 cells (Table 1). The erythroid-specific βH1-globin mRNA was expressed in 55% of the colonies derived from parental AB2.1 cells but only in 10, 5, and 10% of those which had developed from 48$^{all1+/-}$, 48N$^{all1-/-}$, and 48S$^{all1-/-}$ clones, respectively (Table 1). Colonies coexpressing *CD34*, c-*kit*, *MPO*, and *RAG-1* were

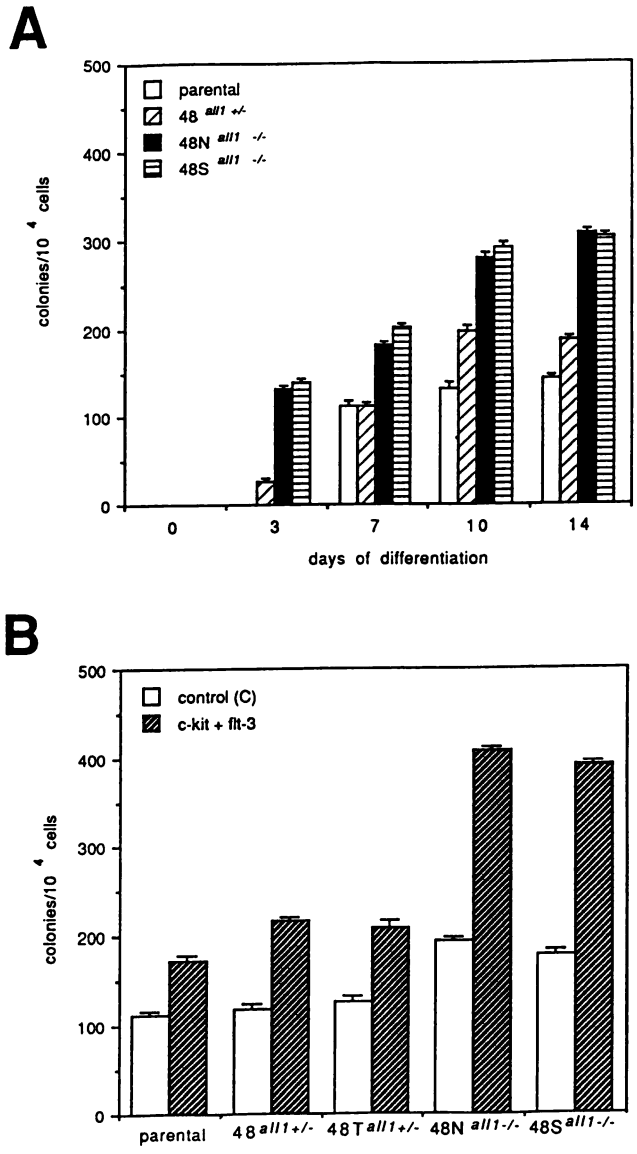

Fig. 2. Development of hematopoietic colonies from ES cells. Wild-type AB2.1, 48$^{all1+/-}$ heterozygotes, and 48N$^{all1-/-}$ and 48S$^{all1-/-}$ double knockout AB2.1 ES cells were tested for colony formation in methylcellulose (*A*). Days of differentiation are indicated at the *bottom*. Data are the means of three independent experiments; *bars*, SD. Parental AB2.1, 48$^{all+/-}$ heterozygotes, 48T$^{all1+/-}$, 48N$^{all1-/-}$, and 48S$^{all1-/-}$ double knockout AB2.1 ES cells were tested for colony formation in methylcellulose (*B*). The colony assay was performed in the presence (c-kit and flt-3) or absence (control) of recombinant murine kit ligand (25 ng/ml) and recombinant FLAG human flt-3 ligand (50 ng/ml) 7 days after LIF removal. Data are the means of three independent experiments; *bars*, SD.

derived from 48$^{all1+/-}$ (5% of the colonies derived from this clone) but at higher frequency, from 48N$^{all1-/-}$ and 48S$^{all1-/-}$ AB2.1 cells (25% of the colonies developed from each clone; Table 1). In contrast, colonies derived from parental AB2.1 ES cells and expressing lymphoid-specific markers (*RAG-1* and *Ikaros*) did not coexpress early hematopoietic progenitors (*CD34* and c-*kit*) or myeloid (*MPO*) markers (Table 1). This lymphoid-specific phenotype was detected in ~10% of parental AB2.1 cell-derived colonies and in a similar percentage of 48$^{all1+/-}$, 48N$^{all1-/-}$, and 48S$^{all1-/-}$ cells (Table 1), whereas colonies expressing only the myeloid marker formed at higher frequency from parental and 48$^{all1+/-}$ AB2.1 cells (20%) compared with 48N$^{all1-/-}$ and 48S$^{all1-/-}$ double knockout AB2.1 cells (0 and 5%, respectively; Table 1). None of the colonies analyzed coexpressed early hematopoietic markers and myeloid markers. These

Table 1 *Lineage specificity of ES-derived colonies*

Individual colonies (20/experiment) were tested by RT-PCR analysis for lineage-specific marker expression. Figures are the number of individual colonies expressing the corresponding gene, as indicated. Each colony tested positive for β-actin expression. Results are representative of two independent experiments with identical results.

	Day 3			Day 7			
	+/−	−/−N	−/−S	+/+	+/−	−/−N	−/−S
MPO	7	8	7	4	8	7	8
c-*fms*	4	2	3	3	4	3	3
βH1-globin	2	1	2	11	2	3	2
CD34	7	10	11	0	7	10	10
c-*kit*	8	12	11	0	7	10	11
RAG-1	4	8	8	2	4	8	8
Ikaros	4	8	8	2	4	8	8
c-*kit* + *CD34* only	4	4	2	0	4	2	3
MPO only	4	1	0	4	5	0	1
Ikaros + *RAG-1* only	1	3	2	2	1	0	1
CD34 + c-*kit* + *RAG-1* + *Ikaros* + *MPO*	1	5	5	0	1	5	5

data indicate that, as expected, parental AB2.1 cells, 7 days after LIF removal, develop hematopoietic precursors that generate either erythroid, myeloid, or lymphoid colonies after a process resembling that occurring physiologically during hematopoietic differentiation. In contrast, $48N^{all1-/-}$ and $48S^{all1-/-}$ AB2.1 cells, plated in methylcellulose after 3 or 7 days of differentiation, generate significant numbers of immature and/or biphenotypic colonies. In agreement with the RT-PCR data, c-kit surface expression was detected on cells from 12-day methylcellulose colonies derived from $48N^{all1-/-}$ and $48S^{all1-/-}$ AB2.1 double knockout cells at day 7 of differentiation but not from parental cells (Fig. 3). In contrast, expression of late myeloid and erythroid differentiation markers (Gr-1 and TER119, respectively) was detected primarily in parental cells (Fig. 3). The block in differentiation is particularly evident from the comparison of c-kit and TER119 antigen expression; $c\text{-}kit^+$ cells account for up to 70% in the double knockout ALL-1 cells, whereas the proportion of $TER119^+$ cells is ~5%. In marked contrast, up to 50% of parental cells express the TER119 erythroid antigen. The phenotype of hematopoietic colonies from ALL-1 homozygous-deficient ES cells was also investigated from cultures grown in the presence of c-kit and flt-3 ligands. As suggested by RT-PCR analysis of individual colonies assessed for c-kit, MPO, and β-globin expression (Fig. 4), the block in differentiation of *ALL-1*-deficient cells also persisted in the presence of these recombinant factors. It is striking that c-kit expression was found in ~50% of the *ALL-1*-deficient colonies, whereas none of these colonies was positive for βH1-globin expression. The number of colonies positive for MPO expression was similar in cultures of parental and *ALL-1*-deficient cells, which suggests that these latter cells reach a differentiation stage at which MPO is expressed.

Using the two-step assay of the hematopoietic differentiation of ES cells, we determined that the loss of a functional *ALL-1* gene results in the earlier appearance and increased numbers of hematopoietic colonies. It is interesting that, whereas most of the colonies derived from the wild-type parental ES cells differentiate along a single lineage, a large fraction of immature and/or biphenotypic colonies was formed from the double and single knockout cells (Table 1). The inability of ALL-1-deficient ES cells to differentiate normally suggests that ALL-11 is involved in the regulation of early stages of hematopoietic development, either directly or indirectly, by interfering with other known regulators of such a process (22–24). The observation that ALL-1-deficient ES cells develop biphenotypic colonies is consistent with the existence of multilineage progenitors (25,26); importantly, such a phenotype is suggestive of the stage of hematopoiesis influenced by ALL-1 activity and is consistent with the mixed-lineage phenotype observed in acute leukemias with chromosomal translocations or rearrangements involving 11q23. Thus, it seems reasonable to speculate that the *ALL-1* gene plays an important role in normal hematopoiesis and that its loss of function, either through the creation of a fused protein with a dominant-negative effect or by knockout, might be involved in leukemogenesis as a consequence of the inability of hematopoietic progenitor cells to differentiate along the different hematopoietic lineages.

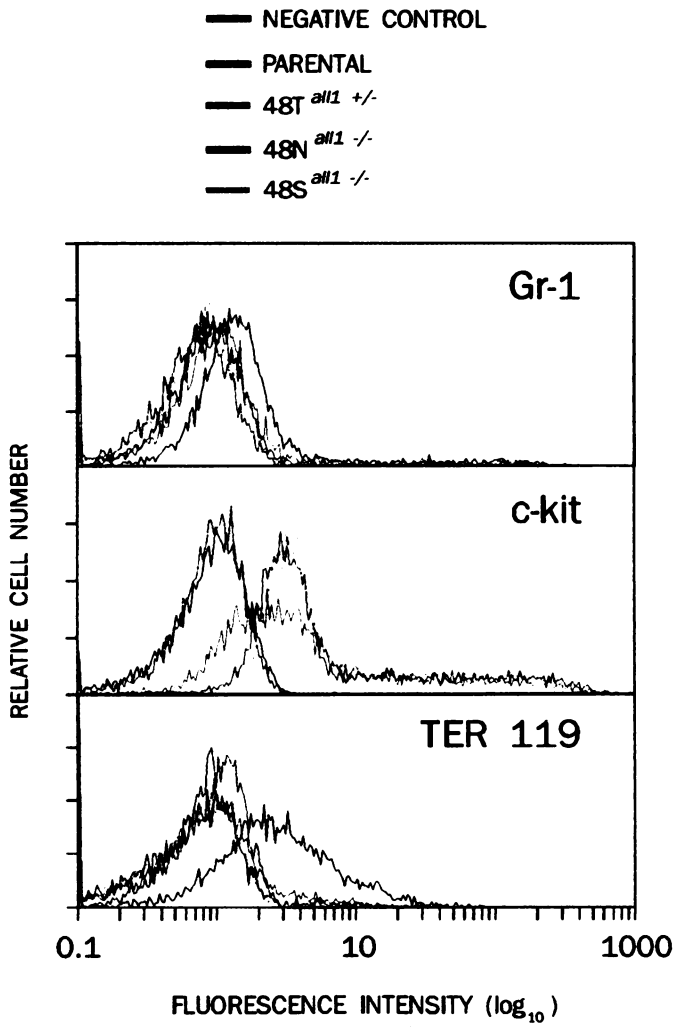

Fig. 3. Surface expression of GR-1, c-kit, and TER119/erythroid marker in colonies derived from parental, $48T^{all1+/-}$, $48N^{all1-/-}$, and $48S^{all1-/-}$ double knockout AB2.1 ES cells. Expression of c-kit, Gr-1 (myeloid), and the erythroid marker recognized by TER-119 monoclonal antibody was determined by flow cytometry analysis on parental, $48T^{all1+/-}$ heterozygote, $48N^{all1+/-}$ heterozygote, $48N^{all1-/-}$, and $48S^{all1-/-}$ double knockout AB2.1 ES cells. Cells were harvested from colonies 14 days after plating in methylcellulose ES cells at day 3 of differentiation. The analysis was performed twice with identical results.

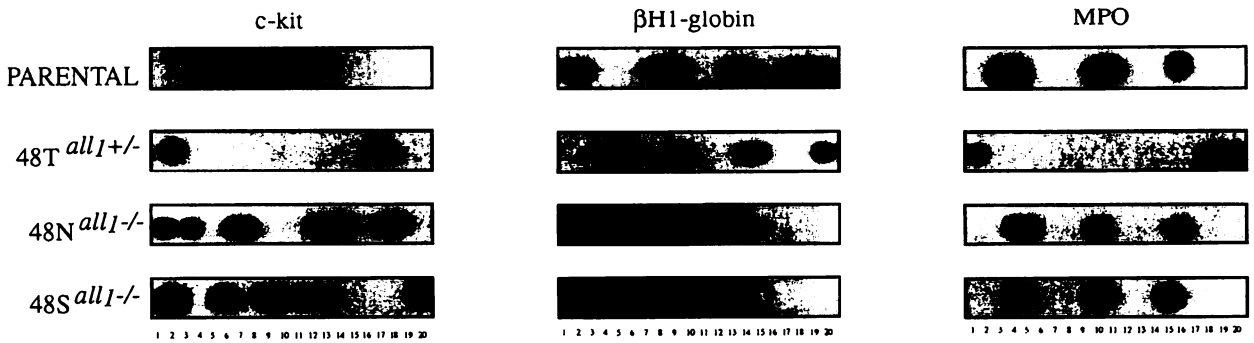

Fig. 4. RT-PCR phenotype of ES-derived colonies grown in the presence of growth factors. Individual colonies (20/experiment) were tested by RT-PCR analysis for c-kit, β-globin, and MPO expression. β-actin expression used as control of RNA integrity was essentially identical in each colony (data not shown). Colony assay was performed in the presence of growth factors by plating in methylcellulose parental, $48T^{all1+/-}$ heterozygote, and $48N^{all1-/-}$ and $48S^{all1-/-}$ double knockout AB2.1 ES cells at day 7 of differentiation.

The maintenance of the undifferentiated phenotype in hematopoietic colonies derived from ALL-1 homozygous-deficient ES cells grown in the presence of c-kit and flt-3 ligands (Fig. 4) suggests that the defect in differentiation ability caused by the loss of ALL-1 function might be rather severe. However, MPO expression was detected in a comparable number of colonies derived from parental and ALL-1-deficient ES cells (Fig. 4). This suggests that, upon c-kit and flt-3 ligand stimulation, the ALL-1-deficient phenotype is permissive for a partial differentiation. Thus, it will be necessary to stimulate ES cells with cytokines acting on more differentiated precursor cells (*i.e.*, granulocyte-colony-stimulating factor, macrophage-colony-stimulating factor, and erythropoietin) for a better assessment of the impairment on the differentiation potential of homozygous-deficient ES cells. The *ALL-1* gene might also be involved in the control of differentiation of nonhematopoietic cells, and its loss of function might contribute to the development of solid malignancies, as suggested by our recent observation that self-fusion of the *ALL-1* gene occurred in a case of gastric carcinoma (27). In solid malignancies, however, the function of the *ALL-1* gene may be knocked out by mechanisms, *e.g.*, point mutations distinct from those operating in hematopoietic cells. Analysis of the mechanism(s) of *ALL-1* gene alterations in carcinomas is likely to provide additional insights on its role in neoplastic transformation.

Acknowledgments

We thank Dr. Bice Perussia for critical reading of the manuscript.

References

1. Raimondi, S. C. Current status of cytogenetic research in childhood acute lymphoblastic leukemia. Blood, *81:* 2237–2251, 1993.
2. Ziemin-von der Poel, S., McCabe, N. R., Gill, H. J., Epinosa, R., Patel, Y., Harden, A., Rubinelli, P., Smith, S. D., LeBeau, M., Rowley, J. D., and Diaz, M. O. Identification of a gene, *MLL*, that spans the breakpoint in 11q23 translocations associated with human leukemias. Proc. Natl. Acad. Sci. USA, *88:* 10735–10739, 1991.
3. Tkachuk, D. C., Kohler, S., and Cleary, M. L. Involvement of a homolog of *Drosophila* trithorax by 11q23 chromosomal translocations in acute leukemias. Cell, *71:* 691–700, 1992.
4. Schichman, S. A., Canaani, E., and Croce, C. M. Self-fusion of the *ALL-1* gene: a new genetic mechanism for acute leukemia. J. Am. Med. Assoc., *273:* 571–576, 1995.
5. Prasad, R., Leshkowitz, D., Gu, Y., Alder, H., Nakamura, T., Saito, H., Huebner, K., Berger, R., Croce, C. M., and Canaani, E. Leucine-zipper dimerization motif encoded by the *AF 17* gene fused to ALL-1 partial duplication in acute leukemia. Proc. Natl. Acad. Sci. USA, *91:* 8107–8111, 1994.
6. Schichman, S. A., Caligiuri, M. A., Gu, Y., Strout, M. P., Canaani, E. Bloomfield, C. D., and Croce, C. M. ALL-1 partial duplication in acute leukemia. Proc. Natl. Acad. Sci. USA, *91:* 6236–6239, 1994.
7. Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N., and Gough, N. M. Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature (Lond.), *336:* 684–687, 1988.
8. Schmitt, R. M., Bruyns, E., and Snodgrass, H. R. Hematopoietic development of embryonic stem cells *in vitro*: cytokine and receptor gene expressing. Genes & Dev., *5:* 728–740, 1991.
9. Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. J. The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J. Embryol. Exp. Morphol., *87:* 27–45, 1985.
10. Wiles, M. V., and Keller, G. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development (Camb.), *111:* 259–267, 1991.
11. Chen, U., Kosco, M., and Staerz, U. Establishment and characterization of lymphoid and myeloid mixed-cell populations from mouse late embryoid bodies, "embryonic-stem-cell fetuses." Proc. Natl. Acad. Sci. USA, *89:* 2541–2545, 1992.
12. Keller, G., Kennedy, M., Papayannopoulou, T., and Wiles, M. V. Hematopoietic commitment during embryonic stem cell differentiation in culture. Mol. Cell. Biol., *13:* 473–486, 1993.
13. Soriano, P., Montgomery, C., Geske, R., and Bradley, A. Targeted disruption of the c-*src* proto-oncogene leads to osteopetrosis in mice. Cell, *64:* 693–702, 1991.
14. McMahon, A. P., and Bradley, A. The *Wnt-1* proto-oncogene is required for development of a large region of the mouse brain. Cell *62:* 1073–1085, 1990.
15. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolations by acid guanidium thiocyanate-phenol chloroform extraction. Anal. Biochem., *162:* 156–161, 1987.
16. Melotti, P., Ku, D-H., and Calabretta, B. Regulation of the expression of the hematopoietic stem cell antigene CD34: role of c-myb. J. Exp. Med., *179:* 1023–1028, 1994.
17. Qiu, F. R. P., Brown, K., Barker, P. E., Jhanwar, S., and Ruddle, F. H. Primary structure of c-kit: relationship with the CSF-1/PDGF receptor kinase family-oncogenic activation of v-kit involves deletion of extracellular domain and C-terminus. EMBO J., *7:* 1003–1008, 1988.
18. Venturelli, D., Shirsat, N., Gemperlein, I., Bittenbender, S., Hudson, S., and Rovera, G. Nucleotide sequence of cDNA for murine myeloperoxidase. Nucleic Acids Res., *17:* 5852, 1989.
19. Rothwell, V. M., and Rohrschneider, L. R. Murine c-*fms* DNA: cloning, sequence analysis and retroviral expression. Oncogene Res., *1:* 311–316, 1987.
20. Schatz, D. G., Oettinger, M. A., and Baltimore, D. The VC(D)J recombination activating gene, *RAG-1*. Cell, *59:* 1035–1048, 1989.
21. Georgopulos, K., Moore, D. D., and Derfler, B. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. Science (Washington DC), *258:* 808–812, 1992.
22. Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreener, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J., and Potter, S. S. A functional c-*myb* gene is required for normal murine fetal hepatic hematopoiesis. Cell, *65:* 677–689, 1991.
23. Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W., and Orkin, S. H. An early hematopoietic defect in mice lacking the transcription factor GATA-2. Nature (Lond.), *371:* 221–226, 1994.
24. Scott, E. W., Simon, M. C., Anastasi, J., and Singh, H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science (Washington DC), *265:* 1573–1577, 1994.
25. Cumano, A., Parge, C. J., Iscove, N. N., and Brady, G. Bipotential precursors of B cells and macrophages in murine fetal liver. Nature (Lond.), *356:* 612–615, 1992.
26. Palacios, R., and Samandis, T. Bone marrow clones representing an intermediate stage of development between hematopoietic stem cells and pro-T-lymphocytes or pro-B-lymphocyte progenitors. Blood, *81:* 1222–1233, 1993.
27. Baffa, R., Negrini, M., Schichman, S. A., Huebner, K., and Croce, C. M. Involvement of the *ALL-1* gene in a solid tumor. Proc. Natl. Acad. Sci. USA, *92:* 4922–4926, 1995.