Absence of the transcription factor CCAAT enhancer binding protein α results in loss of myeloid identity in bcr/abl-induced malignancy

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The lineage-determining transcription factor CCAAT enhancer binding protein α (C/EBPα) is required for myeloid differentiation. Decreased function or expression of C/EBPα is often found in human acute myeloid leukemia. However, the precise impact of C/EBPα deficiency on the maturation arrest in leukemogenesis is not well understood. To address this question, we used a murine transplantation model of a bcr/abl-induced myeloproliferative disease. The expression of bcr/abl in C/EBPα^{pos} fetal liver cells led to a chronic myeloid leukemia-like disease. Surprisingly, bcr/abl-expressing C/EBP$\alpha^{-/-}$ fetal liver cells failed to induce a myeloid disease in transplanted mice, but caused a fatal, transplantable erythroleukemia instead. Accordingly, increased expression of the transcription factors SCL and GATA-1 in hematopoietic precursor cells of C/EBP$\alpha^{-/-}$ fetal livers was found. The mechanism for the lineage shift from myeloid to erythroid leukemia was studied in a bcr/abl-positive cell line. Consistent with findings of the transplant model, expression of C/EBPα and GATA-1 was inversely correlated. Id1, an inhibitor of erythroid differentiation, was identified as a critical direct target of C/EBPα. Down-regulation of Id1 by RNA interference impaired C/EBPα-induced granulocytic differentiation. Taken together, our study provides evidence that myeloid lineage identity of malignant hematopoietic progenitor cells requires the residual expression of C/EBPα.

differentiation | leukemia | lineage commitment

Current concepts for leukemogenesis suggest that at least two genetic events are necessary for the development of acute myeloid leukemia (AML). Some are required to interrupt normal myeloid differentiation and frequently involve transcription factors. Others confer a proliferative or antiapoptotic signal. However, although several loss-of-function mutations of transcription factors have been described in AML (1), little is known about the precise role of transcription factor deficiency in leukemogenesis. To further address this issue, we combined the deficiency of a transcription factor required for myeloid differentiation with the expression of an activated tyrosine kinase in a murine transplantation model.

As the target transcription factor, CCAAT enhancer binding protein α (C/EBPα) was chosen. C/EBPα is essential for normal differentiation of myeloid progenitors. C/EBPα-deficient mice demonstrate a lack of mature granulocytes (2). Analysis of hematopoietic precursors of C/EBPα-deleted mice showed that granulopoiesis is blocked at the transition from the common myeloid progenitor (CMP) to the granulocyte macrophage precursor (GMP) (3). The lack of mature granulocytes together with the accumulation of immature cells in the bone marrow (BM) is reminiscent of AML. Accordingly, dominant negative mutations in the C/EBPα gene have been described in human AML (4). Moreover, it has been shown that AML1/ETO, the fusion protein resulting from the $t(8,21)$ translocation, leads to down-regulation of C/EBPα RNA levels (5).

As a proliferative and/or antiapoptotic signal, bcr/abl was selected. Bcr/abl is a constitutively activated tyrosine kinase resulting from the $t(9,22)$ chromosomal translocation in chronic myelogenous leukemia (CML). In human CML, bcr/abl causes a chronic phase characterized by an increased production of granulocytic cells with normal maturation. Retroviral expression of bcr/abl in mouse BM transplantation assays leads to increased proliferation of myeloid progenitors and accumulation of mature myeloid cells, resembling the chronic phase of human disease (reviewed in ref. 6). In most cases of human CML, the disease ultimately undergoes a transition to blast crisis. The majority of blast crises have a myeloid (65%) or lymphoid (30%) phenotype, but erythroid blast crisis has also been described (7). This transformation occurs mostly after several years and has been attributed to secondary genetic events (6). In myeloid blast crisis, granulocytic differentiation is blocked, and BM and blood demonstrate immature myeloid blasts similar to those observed in AML. Down-regulation of C/EBPα protein has been implicated in this transition (8).

In this study, we asked whether C/EBPα deficiency in the context of bcr/abl-induced murine leukemia would promote the differentiation block observed in human AML. We show that in the absence of C/EBPα bcr/abl induces an immature erythroleukemia, and no myeloid cells are detected. These results indicate that in malignant hematopoiesis residual C/EBPα function is also required for myeloid lineage commitment.

Results

Mice Transplanted with bcr/abl-Expressing C/EBP$\alpha^{-/-}$ Cells Develop Erythroid Leukemia. Fetal liver hematopoietic cells from C/EBPα^{pos} (C/EBP$\alpha^{+/+}$ and C/EBP$\alpha^{+/-}$) and C/EBP$\alpha^{-/-}$ embryos were transduced with bcr/abl internal ribosome entry site-GFP or GFP alone and transplanted into sublethally irradiated mice. Mice transplanted with bcr/abl-expressing cells in the context of C/EBPα^{pos} and C/EBP$\alpha^{-/-}$ genetic backgrounds had similar numbers of GFP$^+$ cells and developed a lethal hematologic disease with a short latency (Table 1). Mice transplanted with cells expressing only GFP did not show any signs of disease (data not shown).

No difference was detected between mice transplanted with

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Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; C/EBP, CCAAT enhancer binding protein; ChIP, chromatin immunoprecipitation; CML, chronic myelogenous leukemia; CMP, common myeloid progenitor; GMP, granulocyte macrophage precursor; MPO, myeloperoxidase; 3PRE, 3 prime regulatory element.

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Table 1. Clinical course of recipient mice after transplantation

Characteristic	C/EBPα^{pos} + bcr/abl	C/EBP$\alpha^{-/-}$ + bcr/abl
Survival, days (median)*	22–169 (26)	20–157 (26)
Spleen weight, mg (median)*	330–750 (517)	100–1000 (465)
WBC per μl (median)*	18,000–230,000 (82,000)	7,300–71,000 (24,500)
Hemoglobin, g/dl (median)*	11–14.8 (13.2)	10.2–18.5 (16.6)
Blasts†	0.4 ± 0.4	8.6 ± 3.8§
Myelocytes†‡	2.5 ± 0.7	0.3 ± 0.5§
Neutrophils†	64.7 ± 16.2	4.8 ± 5.7§
Eosinophils†	0.7 ± 0.7	0.0 ± 0.0
Lymphocytes†	26 ± 16.2	17 ± 21.5
Monocytes†	4.2 ± 1.6	1.6 ± 0.9§
Normoblasts, erythroblasts†	1.4 ± 1.5	66.5 ± 31.4§

More than 80% of the mice were killed within 60 days after transplantation. The mouse with acute lymphocytic leukemia (recipient of C/EBP$\alpha^{+/-}$ + bcr/abl) is not included. For the differential counts, the percentage of each type of the nucleated blood cells ± SD is given.
*$n = 7$ for C/EBPα^{pos} and $n = 9$ for C/EBP$\alpha^{-/-}$ transplants.
†$n = 4$ for C/EBPα^{pos} and $n = 5$ for C/EBP$\alpha^{-/-}$ transplants.
‡Myelocytes include promyelocytes, myelocytes, and metamyelocytes.
§$P < 0.05$.

bcr/abl-expressing C/EBP$\alpha^{+/+}$ or C/EBP$\alpha^{+/-}$ cells. In those mice we observed highly elevated leukocytes, a pronounced increase in granulocytes together with the appearance of less mature myeloid forms and a reduction in the percentage of lymphocytes (Table 1 and Fig. 1*A1*). In mice transplanted with bcr/abl-expressing C/EBP$\alpha^{-/-}$ cells the blood smears showed erythroid precursor elements (Fig. 1*A2*). These normoblasts and erythroblasts comprised two-thirds of the nucleated cells, and a significant number of proerythroblasts were detected. The lymphocytic and granulocytic compartments were reduced (Table 1).

In BM and spleen the same phenotype of the malignant cells was detected (Table 2, which is published as supporting information on the PNAS web site). Bcr/abl protein was demonstrated in the leukemic cells (Fig. 1*B*). Cytologic and histologic analysis of mice transplanted with bcr/abl-expressing C/EBPα^{pos} cells displayed a cellular composition consistent with chronic-phase CML. An accumulation of mature granulocytes, variably increased numbers of late nucleated erythroid precursors, and a reduction in lymphocytes were detected (Fig. 1 *C1* and *D1*). The preponderance of cells stained positive for myeloperoxidase (MPO) (Fig. 1*D2*). In contrast, an accumulation of immature erythroid cells was observed after transplantation with bcr/abl-expressing C/EBP$\alpha^{-/-}$ cells (Fig. 1 *C2*, *D3*, and *D4*). MPO-positive cells were markedly reduced (Fig. 1*D5*). In

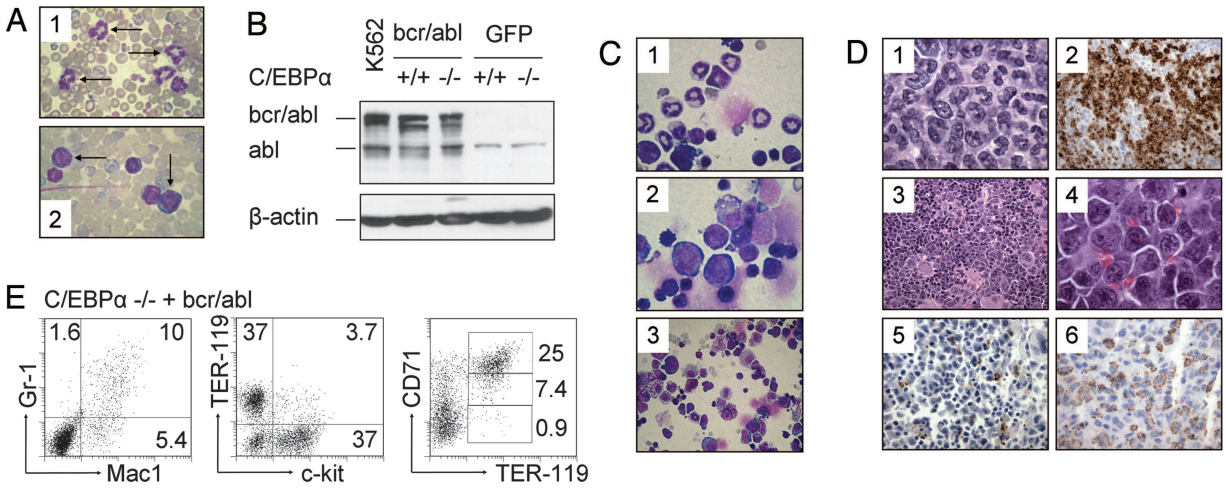

Fig. 1. Development of erythroleukemia after transplantation with bcr/abl-expressing C/EBP$\alpha^{-/-}$ cells. (*A*) Wright-Giemsa staining of blood smears from mice transplanted with bcr/abl-expressing C/EBPα^{pos} cells (panel 1), showing an increase in granulocytes (arrows), and bcr/abl-expressing C/EBP$\alpha^{-/-}$ cells (panel 2), showing the appearance of erythroblasts (arrows). (Magnification: ×1,000.) (*B*) Expression of bcr/abl protein in 5×10^5 BM cells from mice transplanted with cells as indicated. The membrane was probed with an anti-c-abl antibody. (*C*) Cytospin of BM from mice after transplantation with bcr/abl-expressing cells. Note the predominance of granulocytic cells in the presence of C/EBPα (panel 1) and the accumulation of immature erythroid cells in the absence of C/EBPα (panel 2). Some mice also had an increase in mast cells (panel 3). (Magnifications: ×1,000, panels 1 and 2; ×400, panel 3.) (*D*) Histology of BM and spleen from mice after transplantation with bcr/abl-expressing cells demonstrating disruption of normal architecture. Hematoxylin and eosin (H&E) stain (panel 1) and MPO stain (panel 2) show extensive granulocytic, MPO-positive infiltration after transplantation of C/EBPα^{pos} cells. Hematoxylin and eosin stain of a spleen from a mouse transplanted with C/EBP$\alpha^{-/-}$cells demonstrates the predominance of immature erythropoiesis (panels 3 and 4). Complete absence of mature myeloid elements in BM of these mice was confirmed by MPO immunostaining (panel 5). Accumulation of mast cells was detected by immunhistochemistry for mast cell tryptase (panel 6). (Magnifications: ×1,000, panels 1 and 4; ×400, panels 5 and 6; ×200, panels 2 and 3.) (*E*) Flow cytometric analysis of BM from bcr/abl-expressing C/EBP$\alpha^{-/-}$ cells. The immunophenotype of the GFP$^+$ cells is shown. The percentage of cells in each quadrant is indicated. (*Left*) In the absence of C/EBPα, no mature myeloid elements were detected. (*Center*) Instead, an accumulation of TER-119$^+$ erythropoietic precursors was observed. (*Right*) Analysis of differentiation status of erythroid cells by staining with CD71 and TER-119 (9) demonstrated an increase in erythroid precursors (TER-119^{high}CD71 ^{high}).

addition to the expansion of erythroblasts, an increase of mast cells, confirmed by immunohistochemistry for mast cell tryptase and touloudine blue staining, was found in some of the mice (Fig. 1 *C3* and *D6*). Flow cytometry supported the histologic and cytologic observations. Whereas a predominance of Gr-1$^+$/Mac1$^+$ cells was detected after transplantation with bcr/abl-expressing C/EBPα^{pos} cells (data not shown), an increase in expression of the erythroid marker TER-119 and c-*kit*, a marker for hematopoietic progenitors, was observed after transplantation with bcr/abl-expressing C/EBP$\alpha^{-/-}$ cells (Fig. 1*E*). Erythroid maturation is accompanied by a decrease of CD71 surface expression in TER-119-positive cells (9). The majority of erythroblasts after transplantation with bcr/abl-expressing C/EBP$\alpha^{-/-}$ cells exhibited high expression of CD71 (Fig. 1*E*), indicating the presence of immature erythropoiesis. In contrast, in control mice lower CD71 expression was observed (data not shown).

In summary, mice transplanted with bcr/abl-expressing C/EBPα^{pos} fetal liver cells displayed CML as described after transduction and transplantation of BM cells. One recipient mouse developed a B-lineage acute lymphoblastic leukemia (data not shown). However, mice transplanted with bcr/abl-expressing C/EBP$\alpha^{-/-}$ cells demonstrated a complete absence of cells with myeloid identity.

Myeloid Differentiation Capacity Is Absent in C/EBP$\alpha^{-/-}$ Leukemic Repopulating Cells. Histologic analysis of extrahematopoietic tissues supported a malignant phenotype of the bcr/abl-expressing cells. In mice transplanted with C/EBPα^{pos} cells, prominent granulocytic infiltrates and extramedullary hematopoiesis, including maturing erythroid elements and megakaryocytes, were observed in the liver (Fig. 6*A*, which is published as supporting information on the PNAS web site). In mice transplanted with C/EBP$\alpha^{-/-}$ cells, extramedullary erythropoiesis was also appreciated. However, in contrast to the wild-type cells, C/EBP$\alpha^{-/-}$ cells showed increased immature forms with a blast-like appearance and large nuclei consistent with erythroblasts (Fig. 6*B*).

To assess the differentiation capacity of the leukemic stem cell, bcr/abl-expressing C/EBP$\alpha^{-/-}$ cells from a moribund animal were transplanted into secondary recipients. Five of six mice died of malignant hematologic disease with a latency of 110–215 days. Infiltrates of malignant erythropoiesis and/or mast cells led to suppression of normal hematopoiesis in the BM and disruption of splenic archicture (Fig. 6 *C* and *D*). The spleen weights were increased (median 540 mg, range 280–1,300 mg). In summary, recipients of bcr/abl-expressing C/EBP$\alpha^{-/-}$ cells develop an infiltrative, transplantable disease with accumulation of immature erythroid cells, fulfilling the Bethesda Criteria of erythroleukemia in mice (10). The absence of myeloid donor cells in the secondary recipients indicates that the differentiation capacity of the leukemic stem cell is shifted from myeloid to erythroid lineage in the absence of C/EBPα.

Expression of Transcription Factors Associated with Erythropoiesis Is Increased in C/EBP$\alpha^{-/-}$ Fetal Liver Hematopoietic Cells. To evaluate the mechanism responsible for erythroid lineage commitment in the bcr/abl-transformed C/EBP$\alpha^{-/-}$ cells, expression of genes associated with erythropoiesis was analyzed. Expression of SCL, a transcription factor essential for the commitment to the erythroid lineage (11), was increased compared with C/EBPα^{pos} cells (Fig. 2*A*). Moreover, GATA-1, which is tightly regulated during erythropoiesis (12), was up-regulated, along with the precursor marker c-*kit* (Fig. 2*B* and data not shown). This expression pattern might be caused by either an elevated number of erythropoietic progenitors or an altered gene expression profile in precursor cells of the C/EBP$\alpha^{-/-}$ fetal livers. Therefore, we analyzed SCL expression in CMPs. This population represents the branching point between the granulocytic-

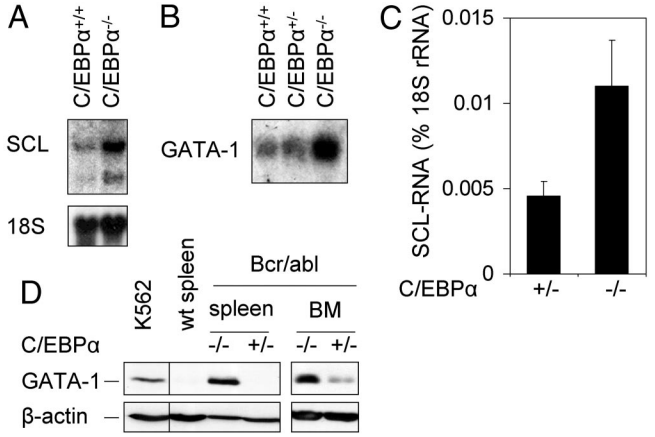

Fig. 2. Increased expression of transcription factors associated with erythropoiesis in C/EBP$\alpha^{-/-}$ fetal livers. (*A* and *B*) Northern blot of SCL (*A*) and GATA-1 (*B*) RNA in fetal livers of C/EBP$\alpha^{+/+}$, C/EBP$\alpha^{+/-}$, and C/EBP$\alpha^{-/-}$ mice. (*C*) Real-time RT-PCR analysis of SCL RNA in CMP isolated from fetal liver hematopoietic cells of C/EBP$\alpha^{+/-}$ and C/EBP$\alpha^{-/-}$ mice. Cells from four embryos were pooled. (*D*) Western blot of extracts derived from spleen or BM as indicated were analyzed for GATA-1 expression. Note the strong expression of GATA-1 in the bcr/abl-expressing C/EBP$\alpha^{-/-}$ infiltrates.

monocytic and the megakaryocytic-erythroid pathway (13). SCL expression was increased in CMPs of C/EBP$\alpha^{-/-}$ fetal livers in comparison with the C/EBPα^{pos} counterpart (Fig. 2*C*). In addition, we did not observe an increase in the number of *in vitro* erythroid colony-forming units derived from C/EBP$\alpha^{-/-}$ fetal liver cells compared with wild type (2). Therefore, in the absence of C/EBPα an alteration in the expression pattern of important erythroid genes was observed. Consistent with these findings, increased expression of GATA-1 was found in infiltrates of mice transplanted with bcr/abl-expressing C/EBP$\alpha^{-/-}$ cells (Fig. 2*D*).

Expression of C/EBPα and GATA-1 Is Inversely Correlated in bcr/abl-Positive Cells. To further investigate the role of the differential expression of myeloid and erythroid transcription factors in the determination of the cell fate of bcr/abl-transformed cells, we tested the effect of C/EBPα function on GATA-1 expression in K562 cells. This erythroleukemic cell line is an ideal *in vitro* surrogate for our mouse model, because it is bcr/abl-positive and expresses GATA-1. Moreover, there is no endogenous expression of C/EBPα (14). Myeloid differentiation can be forced by expression of C/EBPα in K562 and other bcr/abl-positive cell lines (15). For these experiments, the K562αER cell line was used. Upon treatment with β-estradiol, the C/EBPα-ER fusion protein translocates to the nucleus, allowing C/EBPα to be transcriptionally active. After induction with β-estradiol, a decrease in GATA-1 RNA was observed. C/EBPε RNA, a marker of granulocytic differentiation, was increased (Fig. 3*A*). Consistently, induction of C/EBPα transcriptional activity led to down-regulation of GATA-1 protein and up-regulation of C/EBPε protein (Fig. 3*B*). Therefore, restoration of C/EBPα into a bcr/abl-expressing line can inhibit erythroid gene expression and induce granulocytic genes.

Id1, a Direct Target of C/EBPα, Is Essential for C/EBPα-Induced Myeloid Differentiation. Id1 expression is up-regulated by C/EBPα in CD34$^+$ cells (16). Because Id1 inhibits erythroid differentiation (17) and enhances neutrophil development (18), it might play a role for the lineage shift from erythroid to myeloid differentiation upon C/EBPα expression. In K562αER cells, Id1 expression was strongly up-regulated as early as 6 h upon induction of C/EBPα (Fig. 3*B*). To assess whether Id1 is a direct

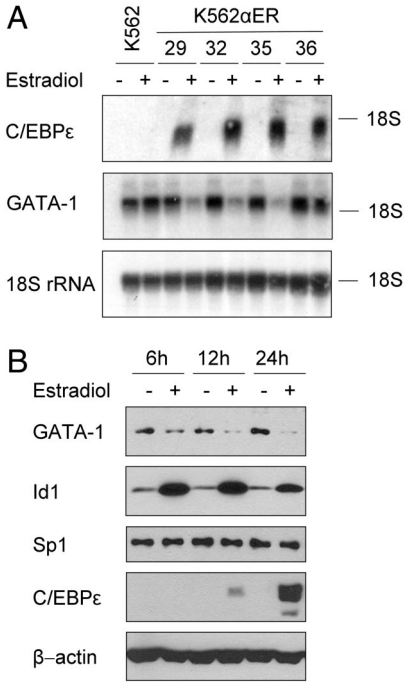

Fig. 3. Inverse correlation of myeloid and erythroid transcription factors upon restoration of C/EBPα expression in bcr/abl-expressing cells. (*A*) Northern blot of four independent clones of K562αER cells (14) treated with vehicle alone (−) or with 1 μM β-estradiol (+) for 48 h. The blots were hybridized to probes detecting the granulocytic marker C/EBPε and the erythroid marker GATA-1. 18S rRNA was used as a loading control for GATA-1. (*B*) Western blot of K562αER cells at various time points after treatment with 1 μM β-estradiol. GATA-1 and Id1 were detected by using nuclear extracts. C/EBPε was detected by using trichloroacetic acid extracts. A rapid and strong up-regulation of Id1 was detected.

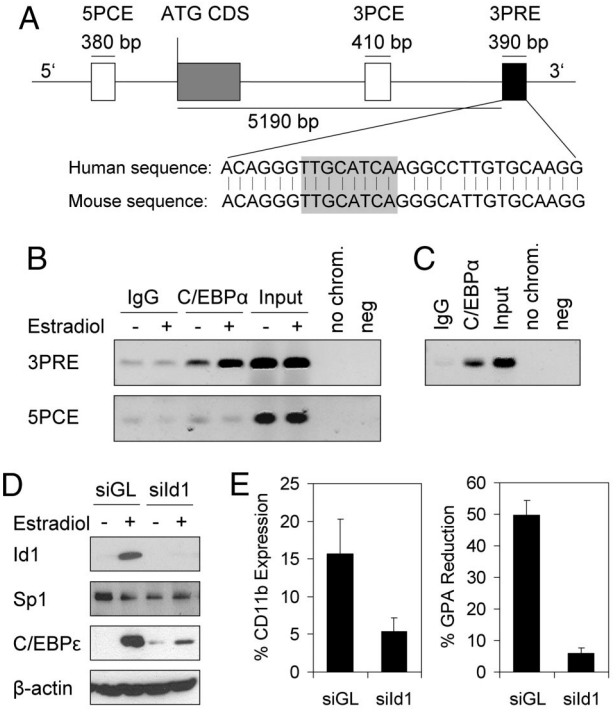

Fig. 4. *Id1* is a direct target gene of C/EBPα and important for myeloid differentiation. (*A*) Genomic locus of the *Id1* gene. The gray box represents the coding sequence including one intron, and the white and black boxes represent elements conserved between mouse and human. Within the black box C/EBPs have been shown to bind to an 8-bp sequence (highlighted in gray) (19). 5PCE, 5 prime conserved element; ATG CDS, ATG coding sequence; 3PCE, 3 prime conserved element. (*B*) ChIP analysis of K562αER cells treated with vehicle alone or with 1 μM β-estradiol for 24 h. Note the increased signal for C/EBPα binding to the 3PRE after induction. The PCR product observed in cells without C/EBPα induction probably is caused by spontaneous translocation of a small amount of C/EBPα-ER in the absence of β-estradiol. This hypothesis is supported by the observation that no binding was detected in parental K562 cells (data not shown). No binding to the 5 prime conserved element (5PCE) was detected. (*C*) ChIP analysis of mouse BM demonstrated C/EBPα binding to the 3PRE *in vivo*. no chrom., no chromatin; neg., negative. (*D*) Western blot of K562αER cells expressing short hairpin RNA (shRNA) against Id1 (sild1) or a control short hairpin RNA (siGL). Cells were treated with 20 nM β-estradiol. This concentration was used, because up-regulation of C/EBPε and Id1 induction was comparable to treatment with 1 μM β-estradiol, but the cells were more viable. Id1 induction upon C/EBPα activation was suppressed and accompanied by severely impaired C/EBPε induction. (*E*) Flow cytometry showed decreased expression of CD11b ($P < 0.05$) and impaired down-regulation of glycophorin A (GPA) ($P < 0.05$) upon suppression of Id1 after 24 h. For this analysis, cells with a high GFP expression were gated.

target gene of C/EBPα, chromatin immunoprecipitation (ChIP) was performed. By sequence analysis between mouse and human three homology regions of ≈400 bp were defined in the Id1 genomic locus (Fig. 4*A*). One of these regions, referred to as 3 prime regulatory element (3PRE), comprises an 8-bp site that was initially described as a pro-B-enhancer in B cells (19). Binding of C/EBP proteins to the 3PRE has been described (20). In induced K562αER cells strong binding of C/EBPα to the 3PRE was detected (Fig. 4*B*). No binding was detected to the putative C/EBP binding sites of the two other homology regions (Fig. 4*B* and data not shown). Analysis of mouse BM demonstrated binding of C/EBPα to the 3PRE *in vivo*, indicating that C/EBPα-mediated regulation of Id1 expression plays a role in normal myeloid development (Fig. 4*C*). To analyze whether Id1 is an essential C/EBPα target for myeloid cell fate, myeloid differentiation was assessed after inhibition of Id1 expression by RNA interference. Using a lentiviral short hairpin RNA against Id1, C/EBPα-induced Id1 induction was suppressed (Fig. 4*D*). This suppression was accompanied by a markedly impaired C/EBPε induction (Fig. 4*D*) and decreased CD11b surface expression (Fig. 4*E*). Moreover, glycophorin A surface expression as a marker for erythroid differentiation was maintained despite C/EBPα function (Fig. 4*E*). These data corroborate the hypothesis that Id1 is an important target of C/EBPα for myeloid differentiation.

Discussion

Suppression of C/EBPα expression or function is associated with myeloid leukemia in humans (4, 5, 8). We analyzed the consequences of loss of C/EBPα expression in the context of a murine transplantation model of bcr/abl-induced myeloproliferative disease. We show that bcr/abl-expressing C/EBPα$^{-/-}$ cells induce an immature, lethal hematologic disease that fulfills the Bethesda Criteria for acute erythroleukemia in mice (10). Unexpectedly, none of the mice demonstrated an accumulation of myeloblasts. An effect of bcr/abl itself was unlikely, because erythroid disease occurred only in C/EBPα$^{-/-}$ cells, whereas recipients of C/EBPα^{pos}-transduced cells consistently developed a chronic-phase CML-like disease.

Most likely the absence of C/EBPα is responsible for the development of erythroleukemia. C/EBPα$^{-/-}$ fetal livers might have more erythroid precursors than wild-type fetal livers. Alternatively, the lack of the C/EBPα gene might alter the differentiation capacity of transformed precursors, leading to a complete block of myeloid differentiation and resulting in preferential erythroid development (Fig. 5). This hypothesis

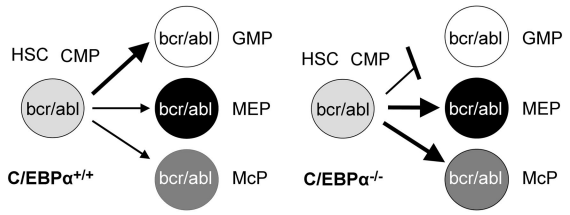

Fig. 5. Model for the development of erythroleukemia in the absence of C/EBPα. Mouse studies support that bcr/abl transforms the hematopoietic stem cell (HSC) (6, 35). (*Left*) In the presence of C/EBPα, the majority of cells differentiate toward the granulocytic lineage, derived from the GMP. (*Right*) C/EBPα deficiency leads to a block from the CMP to the GMP stage and thus, the myeloproliferative effect of bcr/abl results in a predominance of early erythroid cells and mast cells. Our data show that C/EBPα retains its essential role for myeloid cell fate decision in malignant hematopoiesis. MEP, megakaryocyte/erythrocyte progenitor; McP, presumptive mast cell precursor.

would be consistent with data showing that C/EBPα expression in CD34$^+$ cells leads to a reduction of erythroid differentiation (16). Analysis of C/EBP$\alpha^{-/-}$-sorted precursors and experiments with the K562 cell line favor the second hypothesis over the first. Expression of transcription factors essential for erythropoiesis is increased in cells that completely lack C/EBPα. C/EBPα is required to direct cell fate from the CMP to GMP stage. Our data show that this central role of C/EBPα for lineage commitment is preserved in malignant hematopoiesis. In the complete absence of C/EBPα the transition from CMP to GMP is blocked and the transformed cells differentiate toward the megakaryocyte/erythrocyte progenitor (MEP) pathway. Thus, an erythroid phenotype is observed. The observation of an increase in mast cells supports that hypothesis, because both mast cells and MEPs are believed to derive from CMP (21) (Fig. 5). In summary, our findings show that the isolated deficiency of C/EBPα is not sufficient to confer the differentiation block essential for an AML phenotype. Moreover, our data indicate that *Id1* is an important direct target gene of C/EBPα for myeloid lineage commitment.

We hypothesize that a relative reduction of C/EBPα as opposed to its complete absence might be necessary for the development of AML in transformed hematopoietic precursors rather than the erythroleukemia observed in transformed C/EBP$\alpha^{-/-}$ fetal liver cells. Analysis of the leukemic stem cell has recently demonstrated the hierarchical system of leukemic stem cell classes (22), implying that differentiation is a process not only of normal hematopoiesis but also of leukemic cells. In addition, it has been shown that transduction of purified hematopoietic stem cell, CMP, and GMP populations with the MLL-ENL fusion leads to a myeloid leukemia with a phenotype resembling GMP (23). The same GMP phenotype was detected in a model for myeloid blast crisis induced by expression of bcr/abl and bcl-2 (24). Taken together, these findings demonstrate that leukemias with an immature myeloid phenotype, such as AML, or myeloid blast crisis of CML, require a certain level of differentiation capacity. The data in our study indicate that without C/EBPα the cells are not capable of committing to a myeloid cell fate. This finding is consistent with the observations of dominant negative C/EBPα mutations (4) or down-regulation of C/EBPα RNA (5) in human AML patients, either of which would lead to decreased, but not to completely absent, C/EBPα activity. We propose that the remaining C/EBPα activity is not sufficient to sustain normal differentiation, but might be necessary to allow the leukemic cells to differentiate to a GMP phenotype. This hypothesis is consistent with the recent observation that PU.1 hypomorphic mice, which express 20% of wild-type PU.1 levels, develop a lethal AML, whereas conditional and nonconditional PU.1 knockout mice do not (25, 26). Such residual activity of lineage-determining transcription factors may be necessary for allowing hematopoietic precursors to commit along a differentiation pathway, but not be sufficient for full differentiation (27). The concept of reduced rather than loss of transcription factor expression might be important for future attempts at modeling leukemia.

Materials and Methods

Retroviral Transduction and Transplantation Assay. The bcr/abl cDNA (p210) was cloned into a murine stem cell virus retroviral vector plasmid containing an internal ribosome entry site (IRES)-GFP cassette (28). For generation of virus, Bosc 23 cells were transfected with the retroviral vector and a plasmid expressing ecotropic env and gag polymerase by Lipofectamine reagent (GIBCO/BRL) (29). C/EBP$\alpha^{+/-}$ mice were crossed at least five breeding cycles into the C57BL/6 background. Fetal livers were harvested at days 14.5–16.5 of gestation. Genotype was determined by touch smears and confirmed by Southern blot analysis (30). Fetal liver cells were placed on fibronectin-coated plates (Sigma) with bcr/abl IRES-GFP virus, 4 ng/ml of polybrene (Sigma), 10 ng/ml of IL-3 (R & D Systems), and 10 ng/ml of stem cell factor (R & D Systems). Two spinoculations were done, and 0.25 million to 1 million cells were injected into the tail vein of sublethally irradiated (850 rad) congeneic recipients. Secondary recipients were irradiated with 500 rad, and 5 million cells were injected.

Cell Culture and Lentiviral Transduction. K562αER cells were maintained in RPMI medium 1640 supplemented with 10% charcoal-stripped FCS at 37°C and 5% CO_2 (14). Short hairpin RNA against Id1 (target sequence: AAGGTGAGCAAGGTGGAGATT) was cloned into a lentiviral construct, virus was prepared, and cells were transduced as described (31). siGL2 was used as a control short hairpin RNA.

Western Blot Analysis. Whole-cell lysates were obtained either by radioimmunoprecipitation assay or trichloroacetic acid extraction (32). Nuclear extracts were performed according to Andrews and Faller (33). Proteins were separated on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with a 1:1,000 dilution of an antibody for c-abl or C/EBPε, a 1:500 dilution for Id1, or a 1:250 dilution of an antibody against GATA-1 (all Santa Cruz Biotechnology). An antibody against β-actin (Sigma) or Sp1 (Upstate, Charlottesville, VA) was used as control for loading and protein integrity for whole-cell lysates and nuclear extracts, respectively.

Flow Cytometry. After lysis of erythrocytes BM and spleen cells were labeled with biotin-, phycoerythrin (PE)-, or allophycocyanin (APC)-conjugated antibodies against the following molecules: c-*kit* (2B8), Gr-1 (RB6-8C5), B220 (RA3-6B2), CD3 (17A2), TER-119, CD71 (Pharmingen) or Mac-1/CD11b (M1/70.15) (Caltag, Burlingame, CA) followed by incubation with APC-conjugated streptavidin if necessary. K562 cells were labeled with a PE-conjugated antibody against glycophorin A or CD11b (Becton Dickinson). Dead cells were excluded by propidium iodide staining. Cells were analyzed on a Cytomics FC500 (Beckman Coulter) or a FACScalibur (Becton Dickinson). For analysis of SCL expression in purified progenitors, the CMP population defined as lin$^-$, Sca1$^-$, c-*kit*$^+$, CD34$^+$, and FcγRIII/II^{low} was sorted by using a double laser (488-nm/350-nm Enterprise II + 647-nm Spectrum) high-speed cell sorter (Moflo-MLS; Cytomation, Fort Collins, CO) (13).

Hematology and Histology. Blood counts were performed on a Hemavet Analyzer (CDC Technologies, Oxford, CT). Differential counts were done manually on 300 nucleated blood cells per

smear after staining with Diff–Quik (Dade Behring, Newark, DE). The median values for normal C57BL/6 mice in the facility at the Harvard Institutes of Medicine are 9,900 WBC per μl, 14.2 g/dl Hb, and spleen weight of 85 mg. The normal differential count is 85% lymphocytes, 9% neutrophils, 5% monocytes, and 1% eosinophils.

Tissues were fixed in 10% phosphate-buffered formalin, processed and embedded in paraffin, and stained with hematoxylin and eosin, MPO, or mast cell tryptase by standard techniques. Statistical analysis was performed with the Wilcoxon test.

Northern Blot Analysis and Quantitative Real-Time RT-PCR. Total RNA was isolated by using TriReagent (Molecular Research Center, Cincinnati, OH). Fifteen micrograms of RNA was separated by agarose formaldehyde gel electrophoresis and transferred to biotrans nylon membranes (ICN Biomedicals, Irvine, CA). The blots were hybridized to the following radiolabeled probes: a 1.3-kb XbaI fragment from murine SCL cDNA, a 2-kb XhoI fragment from murine GATA-1 cDNA, a 1.8-kb EcoRI fragment from human GATA-1, and a PstI fragment for C/EBPε, and the following oligonucleotide probe for 18S rRNA: TCGGGCCTGCTTTGAACA.

Quantitative real-time RT-PCR with 100 ng of RNA per sample was performed on an ABI 7700 sequence detector (Applied Biosystems). The expression of SCL RNA was normalized to 18S rRNA. SCL was detected with the following primer/probe set: forward primer, 5′-AGCGCTGCTCTATAGCCTTAGC-3′; reverse primer, 5′-CTCTTCACCCGGTTGTTGTTG-3′; probe, FAM-5′-AACCGGATGCCTTCCCCATGTTCA-3′-TAMRA. A 18S rRNA primer/probe set (VIC-labeled) was purchased from Applied Biosystems.

ChIP Analysis. ChIP was done as described (34). Briefly, cross-link was performed by incubation in 0.37% formaldehyde. Nuclear extracts were prepared, and DNA length was reduced by sonification. Chromatin of 4×10^6 cells (20% input) was removed, and for each ChIP, 2×10^7 cells were used. Immunoprecipitation was done with 5 μg of normal rabbit IgG (Santa Cruz Biotechnology) or 5 μg of rabbit polyclonal anti C/EBPα antibody (sc-61; Santa Cruz Biotechnology). Immune complexes were collected by incubation with protein A-agarose beads. Cross-links of the samples were reversed by incubation with RNAseA at 67°C followed by incubation with proteinase K at 45°C. PCR analysis for C/EBPα binding was done with the following primers: 3PRE forward primer, 5′-GCCCTTATCTCCCTGGACCT-3′, reverse primer, 5′-CCACTGTCCTCCCTTTAACCC-3′; 5PCE forward primer, 5′-TTGTCGTCTCCATGGCGAC-3′, reverse primer 5′-CTGCGGAGCTACAGTCTCCC-3′.

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