The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia

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Summary

CREB is a transcription factor that functions in glucose homeostasis, growth factor-dependent cell survival, and memory. In this study, we describe a role of CREB in human cancer. CREB overexpression is associated with increased risk of relapse and decreased event-free survival. CREB levels are elevated in blast cells from patients with acute myeloid leukemia. To understand the role of CREB in leukemogenesis, we studied the biological consequences of CREB overexpression in primary human leukemia cells, leukemia cell lines, and transgenic mice. Our results demonstrate that CREB promotes abnormal proliferation and survival of myeloid cells in vitro and in vivo through upregulation of specific target genes. Thus, we report that CREB is implicated in myeloid cell transformation.

Introduction

Leukemia is defined as uncontrolled proliferation of cells of the hematopoietic lineage. The disease results from the accumulation of mutations in oncogenes and tumor suppressor genes, and the loss of equilibrium between proliferation and differentiation of the hematopoietic progenitor cells (Dash and Gilliland, 2001; Haywitz and Greenberg, 1999; Kelly and Gilliland, 2002; Tenen et al., 1997). Recent evidence suggests that overexpression of chromosomal translocations specific to leukemias, such as PML-RARα or AML-ETO in mice, results in a prolonged latency or myeloproliferative syndrome without development of acute leukemia (Rego and Pandolfi, 2002; Yuan et al., 2001). Therefore, existence of additional genetic mutations is necessary for the development of acute leukemia.

To this end, we sought to understand the signaling pathways that regulate aberrant myelopoiesis. Myeloid leukemia cells express functional, high-affinity receptors for growth factors such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3). We previously demonstrated that the cyclic adenosine monophosphate response element binding protein (CREB) is activated through phosphorylation at serine 133 in response to GM-CSF stimulation (Sakamoto et al., 1994). Furthermore, activation of CREB is downstream of mitogen-activated protein kinase kinase (MEKK)- and pp90 ribosomal S6 kinase (RSK)-dependent pathways in myeloid leukemic cells (Kwon et al., 2000; Lee et al., 1995). Since we identified CREB as a downstream target of GM-CSF signaling pathways, we examined the status of CREB expression in primary myeloid leukemia cells in bone marrow samples from patients with acute leukemia (Crans-Vargas et al., 2002). Bone marrow samples from patients with acute myeloid or lymphoid leukemias expressed higher detectable levels of CREB compared to nonleukemic, remission, or control bone marrow (Crans and Sakamoto, 2001; Crans-Vargas et al., 2002). Therefore, our results demonstrated that CREB overexpression is found in a subset of patients, and that CREB may play a role in leukemogenesis.

CREB is a 43 kDa-basic/leucine zipper (bZip) transcription factor that regulates gene expression through the activation of cAMP-dependent or -independent signal transduction pathways. CREB binds to an octanucleotide cAMP response ele-

SIGNIFICANCE

Acute myeloid leukemia (AML) in adults has a 20% 5-year disease-free survival, despite treatment with aggressive cytotoxic chemotherapy. In this study, we show that CREB is overexpressed in primary myeloid leukemia cells in some AML patients and is associated with poor initial outcome of clinical disease. CREB overexpression results in increased survival and proliferation of myeloid cells and blast transformation of bone marrow progenitor cells from transgenic mice expressing CREB in the myeloid lineage. CREB transgenic mice also develop myeloproliferative disease after one year. Thus, CREB acts as a proto-oncogene to regulate hematopoiesis and contributes to the leukemia phenotype. Our results suggest that CREB-dependent pathways may serve as targets for directed therapies in leukemia.

Table 1. Immunophenotype, AML subtype, and CREB expression in leukemia cells from AML patients

Diagnosis	CREB	CD34	Other markers
Not leukemia			
Non-Hodgkin's lymphoma, off therapy, (11-year-old male)	–	–	CD19 (13%), CD10 (16%), CD22 (0), CD34 (0)
Hodgkin's lymphoma (13-year-old female)	–	–	CD19 (12%), CD20 (7%), CD22 (14%), CD34 (0)
AML diagnosis			
AML-M1 (25-year-old female)	+	+	CD13 (97%), CD14 (6%), CD15 (27%), CD33 (84%), CD34 (79%), CD117 (76%)
AML-M4, relapse (14-year-old male)	+	–	CD13 (83%), CD33 (46%), CD14 (36%), CD34 (100%), CD14 (81%), CD56 (16%)
AML-M4, diagnosis (17-year-old male)	+	–	CD13 (56%), CD33 (99%), CD14 (32%), CD45 (100%), CD15 (95%), CD117 (14%)
AML-M5a, diagnosis (33-year-old female)	+	–	CD13 (11%), CD14 (0%), CD15 (93%), CD33 (98%), CD56 (8%), CD45 (100%)
AML-M4, diagnosis (50-year-old male)	+	+	CD15 (36%), CD16 (3%), CD33 (90%), CD34 (47%), CD45 (low), CD117 (53%)

ment (CRE) consensus sequence in promoters of target genes as a homodimer or heterodimer with other members of the CREB/ATF superfamily. Phosphorylation of CREB at serine 133 is essential for CREB-mediated transcription (Haywitz and Greenberg, 1999; Kuo and Leiden, 1999; Lee et al., 1995; Sakamoto et al., 1994; Wong and Sakamoto, 1995). CREB enhances both proliferation and survival in neuronal cells (Haywitz and Greenberg, 1999; Kuo and Leiden, 1999; Riccio et al., 1999; Riccio and Ginty, 2002; Riccio et al., 1997).

To understand the role of CREB in the development of leukemia, we studied the molecular and biological consequences of CREB overexpression in primary leukemia cells, leukemia cell lines, and transgenic mice. We also examined whether CREB is critical for leukemia cell growth and survival using RNA interference. Our results demonstrated that CREB promotes abnormal proliferation, cell cycle progression, and survival of myeloid cells in vitro and in vivo through induction of specific target genes. Furthermore, CREB overexpression in AML patients is associated with relapse or persistent disease and may predict a poor outcome in patients with AML.

Results

Elevated CREB expression and activation in bone marrow cells from AML patients

To examine CREB expression and activation in primary AML cells, Western blot analysis was performed with cell lysates from five AML patients who were previously classified as CREB-positive and two patients without leukemia (Table 1; Crans-Vargas et al., 2002). We observed a 2- to 3-fold increase in CREB expression and activation, compared to no expression or low levels of CREB expression in the nonleukemic control samples (Figure 1A and data not shown).

To determine whether increased CREB expression in primary AML cells was due to increased transcription or posttranslational modification, we performed quantitative real-time reverse transcription PCR with mRNA obtained from the bone marrow of eight AML patients that were CREB-positive by Western blot analysis, as well as four normal samples. The mean expression of *CREB* mRNA from AML patient samples was 2- to 3-fold higher than control patients ($p < 0.01$; Figure 1B), which correlated with protein levels quantitated using densitometry. Therefore, both CREB mRNA and protein levels are increased in CREB-positive AML cells.

To investigate whether the leukemic blast cells in the bone marrow specifically express CREB, we performed immunohistochemistry with anti-CREB antisera on bone marrow biopsy touch preparations obtained from CREB-positive and -negative patients (as determined by Western blot analysis) or normal bone marrow. Leukemic blasts from CREB-positive but not controls had nuclear staining of CREB (Figure 1C). Our results indicate that overexpression of CREB occurs in the blast cells from CREB-positive patients, but not those from CREB-negative patients or normal bone marrow.

There was also no association between CD34, CD33, and CREB expression on leukemic blast cells from patients who were CREB-positive or -negative (Supplemental Table S1). All of the blast cells from AML patients expressed CD33. Quantitative real-time PCR with RNA from normal CD34+CD33–, CD34+CD33+, and CD34–CD33+ cells and blast cells from CREB-positive AML patients demonstrated a 2-fold increase in *CREB* expression in AML CD34+CD33+ cells ($p < 0.003$) and a 1.4-fold increase in CD34–CD33+ cells ($p < 0.006$) compared to controls, but no difference in CD34+CD33– cells ($p = 0.23$, Supplemental Figure S1). These results suggest that *CREB* expression is higher in CREB-positive leukemia stem cells compared to normal cells.

Since both CREB protein and mRNA levels were increased in CREB-positive primary AML cells, we postulated that CREB could be amplified as with other oncogenes, e.g., myc. We performed fluorescence in situ hybridization (FISH) on blast cells from four CREB-overexpressing patients using a *CREB*-specific BAC clone. *CREB* has been previously localized to chromosome 2q32.3-q34 (Taylor et al., 1990). In normal control lymphocytes in metaphase and CREB nonamplified (negative) bone marrow cells in interphase, the CREB BAC showed two clear and unambiguous signals (Figure 1D, upper panels). However, in the blast cells from three out of four AML patients that overexpress CREB (CREB+), we detected three to four signals from one homolog and one signal from the other in over 250 interphase nuclei analyzed (Figure 1D, lower panels). Three out of the four CREB-positive patients analyzed had a normal karyotype with no evidence of aneuploidy or hyperdiploidy. These results indicate that in certain patients, there are more

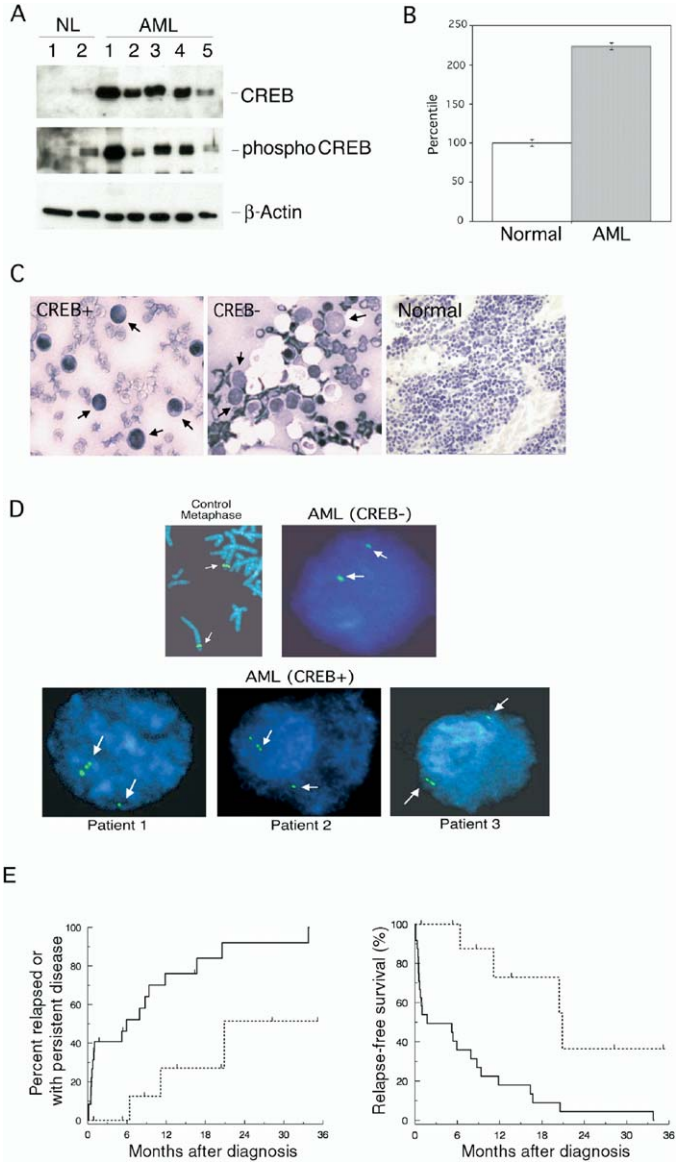


Figure 1. CREB overexpression in patients with AML

A: Western blot analysis with lysates from five representative patients with CREB-positive AML, and two individuals who were normal/without leukemia (NL). The blots were probed with anti-CREB or phospho-CREB antisera (UBI, Inc.).

B: Real-time RT-PCR analysis of CREB expression. The cDNAs from patients with AML and patients without leukemia were subjected to real-time RT-PCR with CREB-specific primers and probe. The results are expressed in percentage, with expression of CREB mRNA in AML relative to that observed in nonleukemia samples (which have been assigned the value of 100%). Error bars denote the standard deviation of each sample measured in triplicate.

C: Immunohistochemistry performed on bone marrow biopsy touch preps using anti-CREB antisera. Increased expression of CREB in the nucleus of AML blasts was seen in CREB-positive patients, but not in AML patients without CREB overexpression (CREB-negative) or normal neonatal bone marrow.

D: FISH on normal metaphase from PHA-stimulated lymphocytes (upper left) and interphase cells from CREB nonoverexpressing blast cells (upper right) showing two distinct signals. Interphase cells from three different AML patient samples with CREB overexpression (CREB+) show three signals from one homolog and one from another (bottom panels; see arrows). CREB-negative (CREB−) represents AML patients without CREB overexpression by Western blot analysis.

E: Risk of relapse in 34 adult AML patients (left). Cumulative percentage of patients relapsing or showing persistent disease after first induction chemotherapy was greater in those patients whose initial bone marrow overexpressed CREB (solid line, n = 24) compared to those not expressing CREB (dashed line, n = 10); p = 0.001 by exact log rank test. The difference remained significant (p = 0.01) after stratification on cytogenetic prognosis. Relapse-free survival curves (right) show that the percentage remaining alive without relapse or persistent disease was lower in those patients overexpressing CREB; p = 0.0003 without stratification and p = 0.003 after stratification on cytogenetics. For both figures, a vertical tick mark indicates a censored observation. The differences in outcome between the CREB groups were also statistically significant (p < 0.005 by likelihood ratio tests) in Cox regression models that included age, gender, initial percent blasts, WBC, and CD34+ percentage as potential covariates, and stratified on cytogenetic prognosis.

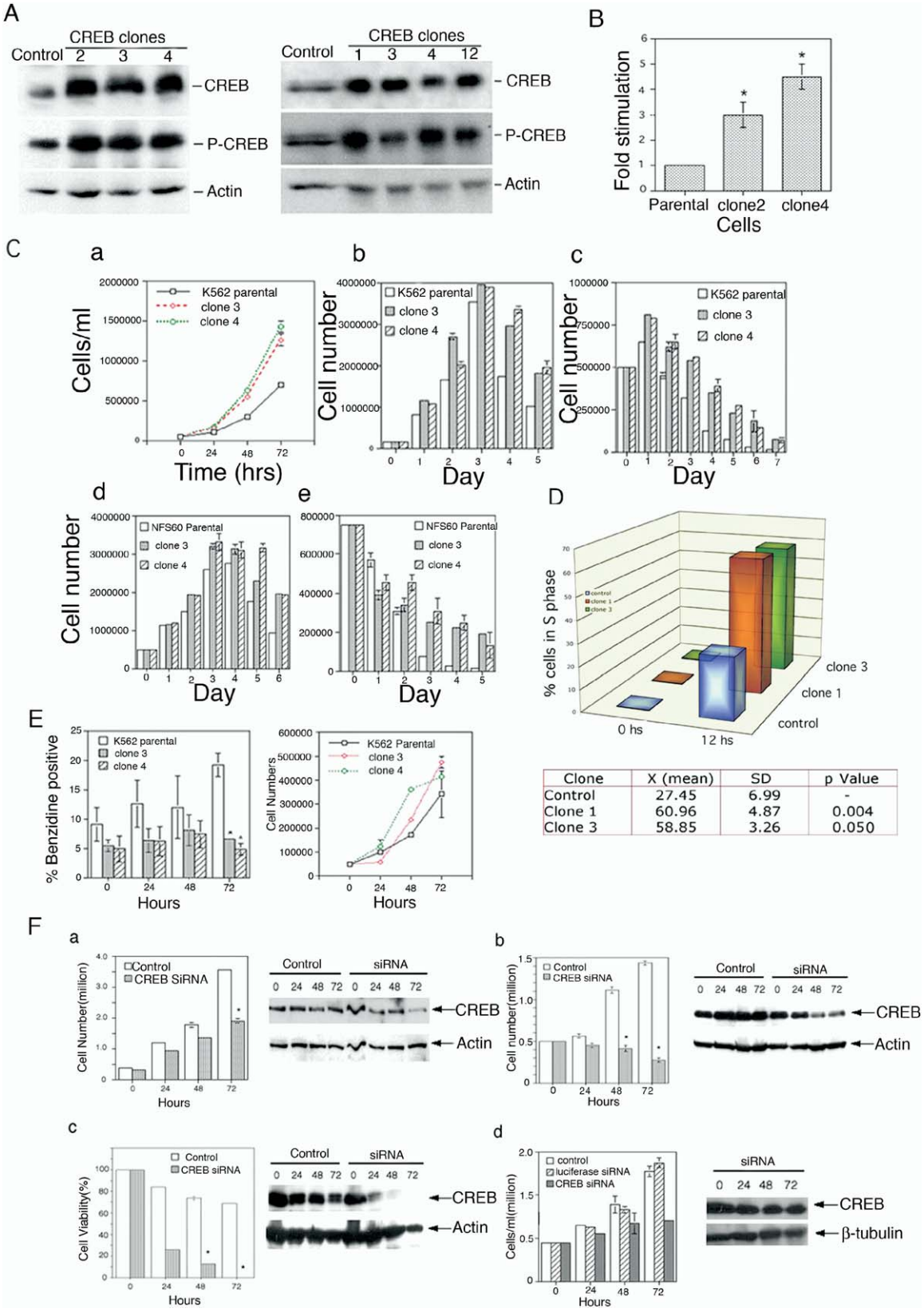
than the normal two copies of *CREB*, which may be one potential mechanism by which increased CREB expression occurs.

To evaluate whether CREB expression predicts outcome in patients with AML, Kaplan-Meier analysis was performed on data from 34 adult patients (median age 48 years, range 19–82; Supplemental Table S1). Persistent disease and shorter time to relapse (Figure 1E, left) was observed in the 24 CREB-positive patients compared to the 10 not expressing CREB, both before and after stratification on cytogenetics (p = 0.001 and p = 0.01, respectively). Likewise, event-free survival (Figure 1E, right) was worse in the CREB-positive group (p < 0.01 after stratification on cytogenetics). Interestingly, all eight patients who showed persistent disease after initial induction therapy were CREB-positive. No variable other than CREB overexpression had a significant association with risk of relapse or event-free survival. Initial treatment, cytogenetics, AML subtype, age, gender, initial white blood count, percent blast count, and percent CD33+ and CD34+ cells were similar between the two CREB groups. There was no strong evidence for an association of overall survival with CREB expression (p = 0.11 after stratification on cytogenetics). Our results suggest that CREB expression is associated with an increased risk of relapse or persistent disease and decreased event-free survival.

CREB promotes growth and survival in leukemia cells

To study the biological significance of CREB overexpression in vitro, we constructed an expression vector that contains the hMRP8 promoter, which drives the expression of *CREB* cDNA in cells of the myeloid lineage (Frantzen et al., 1993; Lagasse and Weissman, 1992; Lagasse and Weissman, 1996; Lagasse and Weissman, 1997; Roth et al., 1993; Yuan et al., 2001). The advantage of using an endogenous promoter is to overexpress CREB in vitro and in vivo at levels similar to those found in CREB-positive primary leukemia blast cells. The hMRP8-CREB construct was stably transfected into the myeloid leukemic cell lines NFS60 and K562. Clones expressing the highest levels of CREB protein were isolated (Figure 2A; top panel). We then confirmed that CREB was activated in these clones using anti-phospho CREB antisera (Figure 2A; middle panel).

To test the transcriptional activity of overexpressed CREB, we transfected K562 clones or control cells with a reporter construct containing 600 nucleotides (nt) of the human early growth response gene-1 (egr-1) promoter, which contains a CRE consensus sequence fused to the chloramphenicol acetyl transferase (CAT) reporter gene. A 3- to 4-fold increase in CAT activity was observed in the CREB-overexpressing cells,



Clone	X (mean)	SD	p Value
Control	27.45	6.99	-
Clone 1	60.96	4.87	0.004
Clone 3	58.85	3.26	0.050

Figure 2. Overexpression of CREB in vitro

A: NFS60 (right) and K562 cells express CREB under the control of the myeloid-specific HMRP8 promoter.

B: Increased transcriptional activity in CREB-expressing cells. A statistically significant increase in fold stimulation was observed with CREB-overexpressing cells compared to control cells (represented by *).

compared to control cells ($p < 0.05$, Figure 2B). These results indicate that overexpression of CREB results in increased transcriptional activity of promoters that contain the CREB recognition sequence.

The growth and survival of hMRP8-CREB overexpression in myeloid cell lines was assessed. Our results demonstrated that both K562 and NFS-60 cells expressing CREB grew faster in exponentially growing cultures and survived longer (after reaching saturation density) under normal growth conditions and in serum-free conditions ($p \leq 0.05$, Figure 2C). Cells that overexpressed CREB also had a 40% increase in numbers of cells in S phase by Brdu incorporation ($p < 0.05$, Figure 2D).

To test if overexpression of CREB perturbs differentiation pathways in myeloid cells, we induced K562 cells to differentiate along the erythroid lineage using sodium butyrate (Pace et al., 2003; Yu et al., 2003). Approximately 25% of parental K562 cells stained positive for benzidine after 72 hr (Figure 2E, left). In contrast, 5%–8% of clones that overexpressed CREB stained positive for benzidine ($p < 0.04$). Cell growth was not affected by sodium butyrate treatment (Figure 2E, right). These results indicate that CREB overexpression inhibits differentiation.

CREB downregulation suppresses myeloid cell proliferation and survival

Our previous results demonstrated that ectopic expression of CREB resulted in enhanced cell proliferation and survival in myeloid cell lines. Next, we used small interfering RNA (siRNA) to downregulate expression of endogenous CREB in K562 and TF-1 human myeloid leukemia cell lines. Addition of CREB siRNA, but not control siRNA, at a concentration of 100 nM significantly inhibited the growth of K562 at 72 hr (Figure 2Fa; asterisk represents $p = 0.038$) and TF-1 cells at both 48 and 72 hr (Figure 2Fb; asterisk represents $p = 0.0001$). CREB expression decreased by 60% to 90% at 72 hr following siRNA treatment in regular growth media and following serum deprivation (Figure 2F). Inhibition in survival was observed at 48 and 72 hr ($p < 0.0001$). In contrast, inhibition of nonspecific siRNA to luciferase did not inhibit the growth of K562 cells after 72 hr (Figure 2Fd). Levels of CREB were not affected by the addition of luciferase or Jab1 siRNA (data not shown). These results suggest that CREB regulates growth and survival of myeloid leukemia cells.

CREB overexpression in vivo leads to aberrant myelopoiesis

To study CREB overexpression in vivo, we generated transgenic mice in which overexpression of CREB was specifically targeted to the macrophage/monocyte lineage. The transgene contains the CREB cDNA under the control of the human MRP8 promoter. MRP8 is expressed in immature myeloid cells of the bone marrow and myeloid cells in the splenic red pulp and marginal zone, in addition to monocytes and blood neutrophils (Lagasse and Weissman, 1992; Lagasse and Weissman, 1996; Lagasse and Weissman, 1997).

CREB transgenic mice were identified by Southern blot analysis of tail DNA (Supplemental Figure S2A). Three independent lines with germline transmission were established from five founders. Tissue specific expression was confirmed with a 3- to 4-fold increase in CREB protein levels (Supplemental Figures S2B and S2C). Peripheral blood and bone marrow from the transgenic mice at 3.5 and 13 months of age showed increased white blood cell count compared to age-matched littermate controls, the most consistent being in the monocyte lineage (Figures 3A and 3B and Table 2). The monocyte count was higher in double transgenic mice, i.e., CREB transgenic mice bred with other CREB transgenic mice, compared to single transgenic mice. No differences were observed in the hematocrit or platelet count (data not shown). In mice less than 1 year of age, the histopathologic analyses of spleen, bone marrow, lymph nodes, and liver were normal. However, 6/7 CREB transgenic mice and 0/11 normal age-matched littermate controls between the ages of 14 and 20 months developed myeloproliferative syndrome with splenomegaly and aberrant myeloid proliferation in the spleen ($p = 0.0004$ by Fisher's exact test; Figure 3B). Interestingly, an increase in peripheral blood lymphocytes in addition to myeloid cells was observed, which was confirmed by FACs analysis (Table 2 and Figure 3C). Work by others showed that increased numbers of activated monocytes that express hMRP8 resulted in stimulation of T helper cell production due to cytokines that are released in mice (Roth et al., 1993). Several of the CREB transgenic mice but not control mice developed dermatitis, as previously reported (Frantzen et al., 1993; Roth et al., 1993).

To look for alterations in the myeloid progenitor cells of CREB transgenic mice, we performed colony assays in methyl-

C: Increased cell growth and survival in CREB-overexpressing cells. NFS60 and K562 cells stably transfected with wild-type CREB. K562 cell numbers were analyzed in exponentially growing cultures (y axis = cells/ml) (**a**). For other growth curves (**b** and **c**), viable cells were counted for six days in growth media. For survival experiments (**d** and **e**), cells were plated in BSA. CREB overexpression resulted in increased cell growth once maximum density was reached (**b** and **d**), and increased cell survival (**c** and **e**).

D: CREB overexpression results in increased proliferation. NFS60 parental and CREB clones were synchronized by serum starvation and pulsed with BrdU. CREB overexpression resulted in a statistically significant increase in the numbers of cells in S phase (graph and table). These experiments were performed in triplicate on three separate days.

E: CREB overexpression inhibits differentiation. K562 parental and CREB-overexpressing cells were induced to differentiate by sodium butyrate. The left graph represents the percentage of cells that were benzidine-positive. A statistically significant difference in the number of differentiated cells between the control and the CREB-expressing clones was seen (indicated by *). Growth curves show that butyrate does not inhibit proliferation or increase cell death (right).

F: CREB siRNA inhibits growth and survival of myeloid leukemia cells. **a:** K562 growth curve. K562 cells were transfected with 100 nM 3′ CREB siRNA or control at 0, 24, and 48 hr. Western blot analysis shows 75% and 90% inhibition of CREB with siRNA at 48 and 72 hr (* represents $p < 0.038$), respectively. **b:** TF-1 growth curve. TF-1 cells were treated with 100 nM 3′ CREB siRNA. Western blot analysis demonstrates greater than 50% and 90% inhibition at 24 and 48 (* represents $p < 0.0001$) or 72 (* represents $p < 0.0001$) hours, respectively. **c:** K562 survival curve. K562 cells were treated in the absence of serum. The Western blot analysis shows 100% inhibition of CREB expression with siRNA at 48 (* represents $p < 0.0001$) and 72 (* represents $p < 0.0001$) hours. **d:** K562 cells were transfected with TKO reagent alone, 100 nM luciferase siRNA, or 100 nM 3′ CREB siRNA at 0, 24, and 48 hr. Western blot analysis shows no CREB inhibition with luciferase siRNA, confirming the specificity of 3′ CREB siRNA. Experiments were performed in triplicate and on three separate days.
Error bars represent the standard deviation for each sample measured in triplicate.

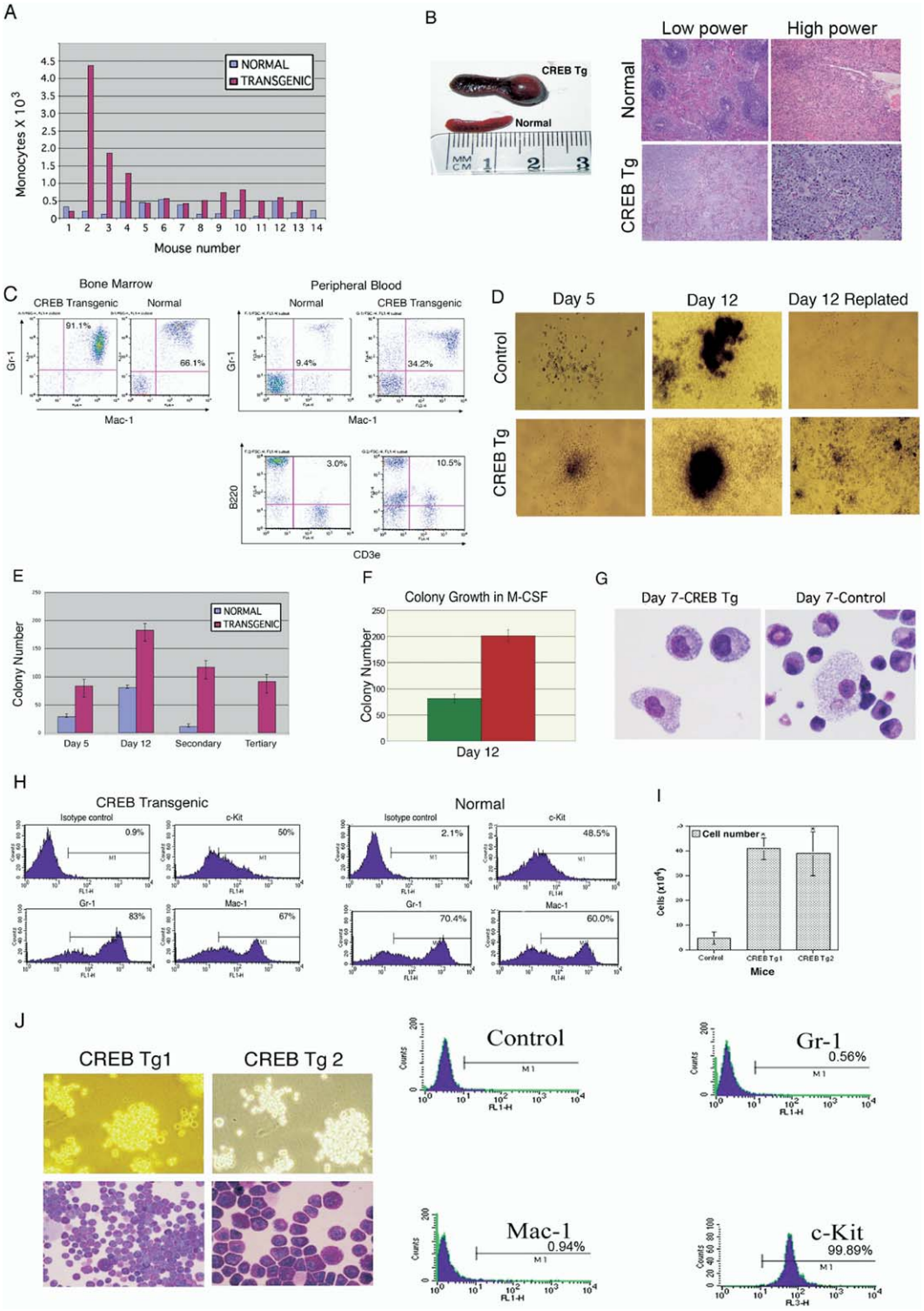


Figure 3. Peripheral blood counts from CREB transgenic mice

A: Monocyte counts were determined from orbital bleeds taken from transgenic (red) or age-matched control littermates (blue) at different ages (mice 1–12 are 4 months of age, mice 12–14 are 6 months of age).

B: Spleens from a CREB transgenic mouse and normal age-matched, littermate control. The splenic architecture is abnormal (low power); aberrant myeloid cells are also present (high power).

C: FACS staining of bone marrow and peripheral blood from CREB transgenic mice or normal controls.

D: Left: CREB overexpression results in myeloid proliferation and immortalization of hematopoietic progenitor cells. A photomicrograph of colonies in methylcellulose at day 5 after culture of control (upper panel) and transgenic bone marrow (lower panel) is shown. Middle: colonies at day 12 after plating showing fully differentiated CFU-GMs in the normal marrow in contrast to the more diffuse proliferating immature colonies in the CREB transgenics.

Table 2. Summary of the peripheral blood counts from two founder lines at different ages

Genotype	WBC (×10³/ml) [median]	Segmented neutrophils (×10³/μl) [median]	Lymphocytes (×10³/μl) [median]	Monocytes (×10³/μl) [median]	Eosinophils (×10³/μl) [median]	Basophils (×10³/μl) [median]
Wild-type (3.5 months), n = 14	2.3–9.3 [4.47]	0.35–2.0 [1.0]	1.82–6.71 [3.06]	0.05–0.75 [0.22]	0.04–0.9 [0.21]	0–0.2 [0.05]
Transgenic (3.5 months), n =11	4.62–27.28 [6.28]	0.41–2.59 [0.94]	2.5–22.0 [4.56]	0.42–4.3 [0.5]	0.02–0.83 [0.13]	0–0.09 [0.03]
Wild-type (13 months), n = 10	2.5–8.1 [4.0]	0.25–1.9 [0.6]	1.5–7.01 [3.1]	0.05–0.48 [0.25]	0.04–0.5 [0.21]	0–0.1 [0.04]
Transgenic (13 months), n = 12	4.9–18.6 [7.61]	0.7–4.5 [1.74]	2.6–11.7 [4.79]	0.23–1.92 [0.5]	0–1.2 [0.13]	0–0.08 [0.04]

cellulose containing SCF, IL-6, and IL-3 or the lineage-specific growth factor, M-CSF. Bone marrow cells from transgenic mice formed robust colonies earlier and formed significant colony-forming units (CFU-GM or CFU-M) when compared to bone marrow from age-matched, nontransgenic littermates ($p < 0.05$; Figures 3D–3F). A significant increase in colony-forming units was also observed ($p < 0.001$; Figures 3E and 3F). Cells from colony assays also overexpressed CREB (Supplemental Figure S2D). To assess self-renewal of progenitors from CREB transgenic mice, we replated bone marrow cells (10^4) in methylcellulose that contained growth factors SCF, IL-6, and IL-3, and counted the colonies after 12 days. Bone marrow cells from transgenic mice were highly successful in repopulating the methylcellulose (Figure 3D, right panel, and Figure 3E). In contrast, bone marrow cells from control mice were unable to grow after serial replating.

Cytospins of cells from colonies at day 7 showed the presence of more immature myeloid cells compared to the control cells (Figure 3G). FACs analysis showed that the colonies consisted of cells that were 50% c-Kit-positive, 83% Gr-1-positive, and 67% Mac-1-positive (Figure 3H). These results suggest that the bone marrow from transgenic mice contains myeloid progenitor cells with an increased proliferative potential when grown within the proper environment.

To determine whether increased CREB expression confers growth factor independence in hematopoietic progenitor cells, we plated bone marrow cells (10^4) from CREB transgenic or normal mice in methylcellulose without GM-CSF, IL-3, or IL-6. We observed a 10-fold increase in the number of cells cultured in the absence of cytokines in all founder lines compared to normal bone marrow (for Tg mouse1, $p = 0.0012$; for Tg mouse 2, $p = 0.0050$). The phenotype was similar to cells grown in the presence of cytokines. Cells from transgenic mice survived longer in liquid culture containing IL-3, but did not grow (data not shown). Therefore, CREB overexpression promotes growth factor-independent proliferation and survival (Figure 3I).

Among the hallmarks of leukemia are immortalization and the continued proliferation of immature blast cells without differentiation. After tertiary replating of CREB transgenic mouse bone marrow in methylcellulose containing SCF, IL-6, and IL-3, we observed the emergence of colonies (mean of 96 ± 3.5) that consisted of larger, homogeneous-appearing, immature blast cells that were >99% c-Kit-positive and <1% Gr-1, Mac-1-positive (Figure 3J). Therefore, persistent expression of CREB leads to a blast-like phenotype in the absence of differentiation.

Cyclin A1 is upregulated in myeloid leukemia cells that overexpress CREB

We previously demonstrated that CREB increased cell proliferation, cell cycle progression, and survival (Figures 2C and 2D). To investigate possible mechanisms by which CREB causes this phenotype, we examined known target genes that are induced in response to CREB activation in other systems (Mayr

Right: a photomicrograph showing the colonies after serial replating is shown. Experiments were performed in triplicate with bone marrow from five transgenic mice derived from two independent lines. These results indicate that the myeloid progenitor cells from CREB transgenic mice have increased proliferative potential.

E: Increased numbers of colonies from CREB transgenic mouse bone marrow at days 5 and 12, and after replating. These results were reproducible in three experiments, and experiments were performed in triplicate. The difference was statistically significant ($p < 0.05$). Error bars represent the standard deviation for each sample measured in triplicate.

F: Colony assays with M-CSF. Progenitor cells were grown in methylcellulose containing M-CSF (10 ng/ml). Red (right bar) represents bone marrow from CREB transgenic mice; green (left bar) represents age-matched littermate control. Error bars represent the standard deviation for each sample measured in triplicate.

There is a statistically significant increase in the number of CFU-M colonies from transgenic mice ($p < 0.05$).

G: Morphology of cells in the methylcellulose colonies. Cytospin preparations of cells from transgenic and normal colonies stained with Wright-Giemsa. The cells from the transgenic marrow show an increased number of immature cells, in contrast to the differentiated monocytic cells of the normal marrow.

H: Immunophenotypic analysis of cells from colony assays demonstrated expression of c-Kit, Gr-1, and Mac-1.

I: Growth factor-independent colony-forming activity of CREB transgenic bone marrow cells was demonstrated in two independent experiments in two different transgenic lines. The cells from all the colonies in the methylcellulose cultures were pooled and counted. The values shown are the mean of duplicate cultures. Bone marrow cells from CREB transgenic mice have a statistically significant higher cell number compared to control mice (indicated by *) in the absence of growth factors.

J: CREB overexpression leads to blast-like colonies. Microscopic appearance (top left), cytospins (bottom left), and FACS (right) following tertiary replating of bone marrow progenitor cells in methylcellulose containing SCF, IL-3, and IL-6. CREB Tg1 and 2 represent two different founder lines. FACS analysis shows >99% cells were c-kit-positive and <1% were GR-1- and MAC-1-negative. These experiments were performed in triplicate on three separate days.

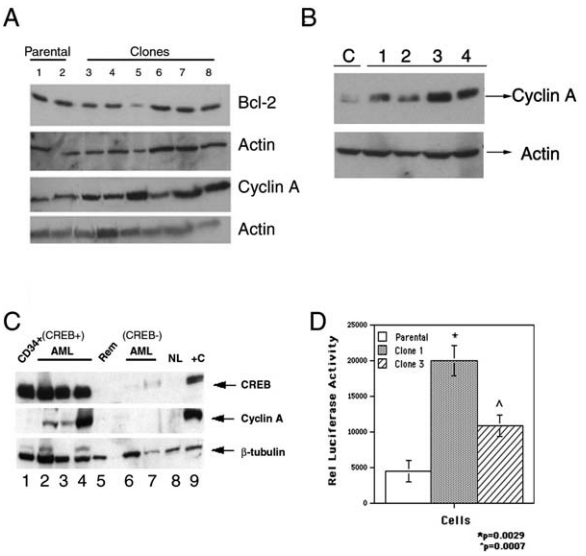


Figure 4. Analysis of potential CREB target genes

A and B: Western blot analysis was performed with CREB-overexpressing K562 cells (**A**), NFS60 cells (**B**), or controls. Cyclin A1 but not Bcl-2 protein levels were increased in most CREB-overexpressing clones compared to controls.

C: Western blot analysis of protein lysates from CREB-positive and CREB-negative AML patients using cyclin A1 antisera. Cyclin A1 expression was observed in CREB-positive, but not CREB-negative or nonleukemic, samples.

D: CREB-overexpressing clones demonstrated increased cyclin A1 promoter activity. NFS60 cells were transiently transfected with a cyclin A1 promoter luciferase construct. Experiments were performed in triplicate on three separate days. Error bars denote the standard deviation for each sample. A statistically significant increase in cyclin A1 transactivation was observed (indicated by * and ^).

and Montminy, 2001). Cyclin A1 has been reported to be a CREB target gene and regulates G1 to S transition through activation of CDK2 (Woo and Poon, 2003). CREB directly binds the CRE in the cyclin A1 promoter and increases transcription of cyclin A1 upon activation of CREB (Desdouets et al., 1995; Kothapalli et al., 2003; Mayr and Montminy, 2001). In our experiments, cyclin A1 protein levels were elevated 3-fold in the myeloid cells that overexpress CREB, but not in control cells (Figures 4A and 4B). However, Bcl-2 levels remained the same in CREB-positive and control cell lines (Figure 4A). To study cyclin A1 levels in primary cells, we looked at expression by Western blot analysis using lysates from CREB-positive and -negative AML patients, and nonleukemic controls. Our results showed that in the AML samples that overexpressed CREB, a corresponding increase in the cyclin A levels was observed (Figure 4C). However, we did not see cyclin A1 expression in the bone marrow of AML patients who were CREB-negative or in normal samples; this suggests that CREB expression and cyclin A1 are coregulated. These results were confirmed with mouse bone marrow cells from colony assays (Supplemental Figure S2D).

To study the possibility of cyclin A1 upregulation downstream of CREB, we examined transcriptional activation of cyclin A1 in CREB-overexpressing NFS60 cells. A luciferase reporter construct containing the cyclin A1 promoter with the CRE was transfected into NFS60 cells that overexpressed CREB. A significant increase in relative luciferase activity was observed in the CREB-overexpressing cells compared to control cells ($p < 0.05$; Figure 4D). Our results suggest that CREB overexpression leads to upregulation of a known CREB target gene, cyclin A1, thereby promoting aberrant cell cycle progression and increased proliferation.

Discussion

Novel role of CREB in cell transformation

CREB has been shown to be a critical regulator of proliferation and survival in normal neuronal development (Haywitz and Greenberg, 1999; Mayr and Montminy, 2001). CREB also induces proliferation of T cells in response to a variety of stimuli (Kuo and Leiden, 1999). Transgenic mice expressing dominant negative CREB (serine133 to alanine) had normal T cell development but displayed a profound defect in T cell proliferation following stimulation with α-CD3 and PMA. Unlike myc, which appears to be sufficient for malignant transformation to cause leukemia (Langenau et al., 2003), metastatic prostate cancer (Ellwood-Yen et al., 2003), and osteogenic sarcoma (Fukunaga et al., 1993), CREB appears to be in a different class of preoncogenic proteins that contribute to the malignant phenotype. We previously demonstrated that CREB is overexpressed in a subset of AML patients. In this paper, we report that aberrant expression of CREB leads to enhanced survival and growth properties in vitro and in vivo.

The precise mechanism of CREB overexpression is not known. Our results suggest that CREB is upregulated at both the protein and mRNA levels. FISH analysis of CREB-positive AML blasts suggests that an increased copy number of the *CREB* gene is one possible mechanism for CREB overexpression in a subset of AML patients.

CREB overexpression results in a blast-like phenotype of hematopoietic progenitor cells

CREB has been shown to promote survival of neuronal cells. We report that CREB overexpression is sufficient for immortalization, growth factor-independent proliferation, and blast-like phenotype. Recently, retroviral transduction of bone marrow progenitor cells with a dimerizable fusion protein, MLL-FKBP, was shown to result in immortalization of hematopoietic progenitor in methylcellulose replating assays (Martin et al., 2003). In addition, mouse bone marrow cells transduced with MLL-AF9, a fusion protein associated with AML, transformed cells to generate myeloblastic cell lines capable of growing in liquid culture containing only IL-3. Bone marrow from our hMRP8-CREB transgenic mice demonstrated increased hematopoietic colony size and numbers in colony assays compared to nontransgenic control mice. Furthermore, these myeloid progenitor cells were capable of sustained survival in liquid culture containing only IL-3, although cells did not proliferate to a significant degree (data not shown). Taken together, these results suggest that CREB alone is sufficient to promote survival of myeloid progenitor cells and contributes to proliferative potential and immortalization of these cells in culture.

Potential mechanism: Overexpression of CREB leads to inappropriate expression of cyclin A

We propose that elevated expression of CREB leads to upregulation of CREB target genes that control cell proliferation and

survival as one potential mechanism for the phenotypes observed. We hypothesize that CREB overexpression results in inappropriate expression of cyclin A1, leading to cell cycle progression. This is further confirmed in primary leukemia blast cells from patients with AML, in which CREB-positive AML blasts also overexpress cyclin A1. Recent work has shown that overexpression of cyclin A1 under the control of the myeloid-specific cathepsin G promoter in mice leads to myeloid leukemia at a low frequency after a prolonged latency (Liao et al., 2001). Furthermore, cyclin A1 overexpression has been detected in leukemia samples from a subset of AML patients as well as in normal CD34+ cells (Muller-Tidow et al., 2001; Yang et al., 1999). These results are consistent with our observations. The fact that CREB induces cyclin A1 explains enhanced proliferation and increased numbers of cells in S phase, but not increased survival. It is likely that additional target genes that are induced by CREB activate downstream signaling pathways that promote survival.

CREB is not sufficient to induce leukemia

In vitro, CREB-overexpressing cells acquire a blast-like phenotype. Although CREB is not sufficient to induce acute leukemia in vivo, overexpression does lead to a myeloproliferative disorder. Similar observations have been made with translocations such as AML1-ETO, which requires additional mutations for leukemia to develop in mice (Yuan et al., 2001). Oncogenic K-ras expression in myeloid progenitor cells has been shown to result in myeloproliferative disease, but not AML (Braun et al., 2004; Chan et al., 2004). We conclude that CREB contributes to the positive growth phenotype of myeloid cells, but not to the point of overt transformation in a mouse model. Additional "hits" or steps are required for leukemia to develop.

CREB overexpression is associated with increased relapse or persistent disease

Our results suggest that CREB may be associated with high risk AML, and that its expression can be used to follow certain patients during therapy for evidence of relapse or resistant disease. Recently, overexpression of cyclin A1 was found to be associated with improved survival in AML patients (Nakamaki et al., 2003). We hypothesize that in a subset of myeloid leukemia blast cells, CREB overexpression occurs, resulting in additional cell cycle abnormalities and increased genomic instability (see model, Figure 5). Clearly, additional target genes upregulated by CREB and multiple signaling pathways are involved in clonal selection and leukemic progression in AML.

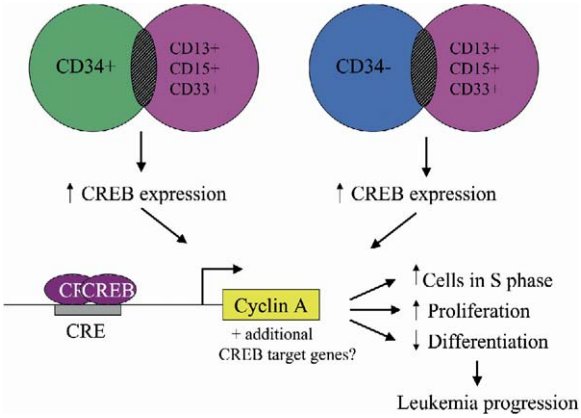


Figure 5. Model summarizing the role of CREB in myeloid cell transformation
CREB is expressed at higher detectable levels in a subset of AML blast cells, which results in upregulation of CREB target genes and increased cell cycle progression and proliferation and decreased differentiation.

Experimental procedures

Cell lines and transfection

K562, TF-1 cells were obtained from the American Type Culture Collection (ATCC) and maintained in Iscoves medium (Invitrogen) with 10% fetal bovine serum (FBS), and RPMI with 10% FBS and rhGM-CSF (Amgen), respectively. NFS 60 cells were a gift from Dr. Jim Ihle, and were maintained in RPMI (Invitrogen) supplemented with 10% FBS and IL3. The hMRP8-CREB construct was transfected into the various cell lines as previously described (Mora-Garcia and Sakamoto, 2000). PUC-19 neo plasmid (5 μg) was cotransfected with the CREB plasmid. After transfection, 100 cells were plated in 24 cell wells and selected with G418 (Invitrogen). Several clones were thus generated and tested for CREB expression by Western blot analysis.

Patient selection and bone marrow isolation

Isolation of mononuclear cells from the bone marrow from AML patients was as previously described (Crans-Vargas et al., 2002).

Protein extraction and Western blot analysis

Cell lysates were made from 1 × 10^6 cells (cell culture and primary cells), and Western blot analysis was performed as previously described (Crans-Vargas et al., 2002; Lee et al., 1995; Lin et al., 1998; Lin and Sakamoto, 2001). The following antibodies were used: Polyclonal anti-CREB and anti-phospho-CREB (UBI, NY), anti-Actin, Bcl2, cyclin A1, and β-tubulin (Santa Cruz, CA).

Immunohistochemical studies

Bone marrow was obtained from both pediatric and adult patients with AML and ALL following informed consent. Bone marrow aspirate slides were fixed in 4% paraformaldehyde for 20 min, and then rinsed in PBS and Triton-X 100 for 10 min each. To eliminate endogenous peroxidase activity, slides were incubated in 0.3% hydrogen peroxide for four min, followed by blocking with 5% BSA/PBS for 45 min to reduce background staining. After removing the blocking solution, 50 μl of the primary antibody (anti-CREB rabbit polyclonal IgG, UBI, Lake Placid, NY) was added in a dilution of 1/50 in 0.1% Triton X-100. The slides were then incubated overnight at 4°. After two 10 min washes in 1× TBS, 50 μl of the secondary antibody (Biotinylated Goat Anti-Rabbit) was applied in a 1/300 dilution in 0.1% Triton X-100 for 30 min. After a quick wash with 1× TBS-T, 50 μl of peroxidase-conjugated streptavidin/HRP in a 1/300 dilution in 0.1% Triton X-100 was applied for 30 min. The slides were then washed in water for 10 min, and 100 μl of hematoxylin counterstain was applied for 30 s. Slides were then examined under a microscope for CREB staining.

RNA isolation and real-time RT-PCR

Total RNA was isolated from bone marrow tissue (2 × 10^6 to 10^7 cells per sample) by the acid guanidium thiocyanate-phenol-chloroform (TRIzol; Gibco BRL, Gaithersburg, MD, USA) method (Chomczynski and Sacchi, 1987) and quantified using UV absorbance at 260 nm on a spectrophotometer (DU640; Beckman Coulter, Fullerton, CA, USA.) cDNA was synthesized using the SuperScript III system for RT-PCR (Invitrogen), as per manufacturer's instructions. Reactions were reproduced three times and performed in 20 μl using 5 μg of total bone marrow RNA and oligo (dT) primer. cDNA was precipitated with 3 M NaOAc (pH 5.0) and 100% EtOH and resuspended in 200 μl of water. Target primers and probe sequences for CREB amplification were designed using Primer Express Software. Forward primer: 5′-AAGCT GAAAGTCAACAAATGACAGTT-3′ and reverse primer: 5′-TGGACTGTCTG CCCATTGG-3′. The TaqMan probe sequence (5′-CTCATGCGACGTCAT CTGCTCCCA-3′) was labeled with fluorescent reporter dye VIC. Each 25

μl of reaction mixture contained 12.5 μl of TaqMan Master Mix (2×) (Applied Biosystems), 7.2 μM primers, and 2 μM probe. For cell sorting experiments, total RNA was extracted from the different sorted subpopulations using RNA easy kit (Qiagen Inc, CA) and reverse transcribed using superscript III reverse transcriptase (Invitrogen Corp., CA) according to manufacturer's instructions. Real-time RT-PCR analysis was performed with an automated sequence detection system (ABI 7700) with TaqMan dual-label fluorogenic detection. The GAPDH gene was used as the internal control to test the integrity of the RNA and for standardization. The data were analyzed using the comparative Ct method (Delta Ct), and results are shown as fold induction of mRNA. All experiments were performed in triplicate.

Fluorescence in situ hybridization (FISH)

BAC RP11-17I6, which includes the *creb1* gene (http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?gnl=NT_005403&MAPS=cntg-r,loc,clone&query=RP11-17I6&ABS_ZOOM=3M), was purchased from Invitrogen and labeled with 11-dUTP-digoxigenin (Roche Molecular). The labeled probe was hybridized to metaphase chromosomes prepared from normal controls (fresh blood) and patient interphase nuclei (frozen blood lymphocytes) following standard cytogenetic protocols (Rao et al., 1992). Briefly, 40–60 ng of the labeled probe was hybridized to cells overnight. The probe was first hybridized to normal control metaphases to determine and confirm its localization on chromosome 2q. At least 25 metaphases were examined per hybridization. The hybridized probe was detected by FITC conjugated anti-digoxygenin antibodies, and successive layers of fluorescence-conjugated antibody were used to obtain easily detectable signals. About 250 interphase nuclei from the patient samples were analyzed. The slides were counterstained with DAPI in an antifade solution and analyzed using a Zeiss Axiophot microscope equipped with epifluorescence filters for simultaneous FITC and DAPI observations. The images were captured on an Applied Imaging Image analysis system.

Relapse and survival analysis

With Medical Institutional Review Board approval, medical records were reviewed on 34 AML patients over age 18 whose initial marrows had been analyzed for CREB expression (two diagnosed in years 1993–1997, 18 from 1998–2000, and 14 from 2001–2003). All patients had received Ara-C/Daunomycin-based chemotherapy generally within 1–2 days following diagnosis. Date of diagnosis, age, gender, initial WBC, percent blasts, percentage CD33+ and CD34+ cells, FAB classification of AML subtype, and cytogenetics were recorded. The first date upon which peripheral blood showed evidence of persistent disease was recorded for any patient not in remission after initial induction chemotherapy. Otherwise, the date of first relapse (if any) was recorded. Total followup time was based on the last known date of patient contact. Comparisons of categorical or continuous variables between CREB groups were tested by Fisher's exact test or rank sum test, respectively. Kaplan-Meier analysis was used to estimate time until first relapse or persistent disease, event-free survival, and overall survival. Exact logrank tests, with and without stratification, were used to compare curves between CREB expression groups. Cox proportional hazard regression models were also used to test the significance of CREB expression after controlling for other covariates and stratifying on cytogenetic prognosis. Prognostic stratification of marrow cytogenetics was by classification as high risk, intermediate or unknown, and low risk (Newland, 2002; Rohatiner and Lister, 2002).

BrdU incorporation

NFS60 and K562 cells were cultured in serum-free conditions for 12 and 24 hr, respectively, to synchronize the cells. The cells were then washed and resuspended in regular medium containing 10% FBS, and BrdU (BD Biosciences) at a final concentration of 10 μM was added to the cells. The cells were then harvested at 0, 1, 6, 12, and 24 hr after labeling for analysis by flow cytometry. The BrdU-pulsed cells were fixed and stained with FITC-anti-BrdU antibody using manufacturer's protocol (BD Pharmingen BrdU Flow Kit). Total DNA content was measured by staining with 7AAD, and flow cytometric analysis was performed using the BD FACScan and CellQuest software.

Differentiation of K562

CREB-overexpressing clones or parental K562 cells (4×10^5 cells/ml) were cultured with sodium butyrate (1 mM final concentration) or control in Iscoves media with 10% FBS. At 0, 24, 48, and 72 hr, cells were harvested, stained with benzidine (Sigma Inc., St. Louis, MO), and then counted. Five microliters of 30% hydrogen peroxide was added per milliliter of cells, and the cells were then incubated at 37°C for 5 min. One milliliter of phosphate-buffered saline (PBS) was added to stop the reaction. The total number of cells and number of positively stained cells was counted and the percentage calculated. Statistical analysis was performed using the JmpIn software program.

CAT and luciferase reporter assays

The preparation of the –600 egr-1 CAT and β-galactosidase constructs was as previously described (Mora-Garcia and Sakamoto, 2000; Sakamoto et al., 1994). Parental K562 and CREB clones were transiently transfected with the CAT and β-galactosidase constructs (Mora-Garcia and Sakamoto, 2000; Sakamoto et al., 1994). Fold stimulation was determined, and statistical analysis was done using JmpIn software. The –1299 to +145 cyclin A1 luciferase construct was obtained from Dr. Carsten Muller-Tidow, Germany. The NFS60 parental and clones were transfected with the cyclin A reporter construct (15 μg) and β-galactosidase reporter plasmid (5 μg) by electroporation. After 24 hr, cells were lysed in luciferase cell lysis buffer, and luciferase assay performed according to manufacturer's instructions (Promega).

siRNA experiments

A 19-nucleotide (nt), CREB-specific, double-stranded siRNA sequence 5′-GAGAGAGGUCCGUCUAAUG-3′ was identified and synthesized by Dharmacon Industries (Lafayette, CO). The selection criteria for our CREB siRNA included: $AAN_{19}TT$ format, G-C content 30%–50%, >2 nt unique by GenBank Blast comparison, and confirmation of no sequence polymorphisms against the NCBI SNP database. Transient transfections were performed with the Trans-it-TKO reagent from Mirus Inc. Cells (0.5×10^6 cells/ml) were placed in 1 ml of Iscoves media with heat-inactivated 10% FBS. CREB siRNA was added at a final concentration of 100 nM and incubated for 20 min. The complex was then added to the cells in 6-well plates and gently mixed. Media changes were performed every 24 hr with repeat addition of siRNA complex. Cells were counted for viability every 24 hr and lysates were prepared with boiling SDS-Laemmli buffer. To assess cell survival, cells were placed in media containing 0.5% bovine serum albumin (BSA) instead of fetal calf serum. The statistical analyses for p values were obtained using the JmpIn program.

Pathology

Serial blood counts were performed on the transgenic mice. Peripheral blood (100–500 μl) was collected in EDTA-coated Microvet (Becton-Dickinson) tubes from orbital eye bleeds. Complete blood counts and differential were analyzed at the MU-RADIL facility of University of Missouri using Hemavet 850 and stained blood smears, respectively. Bone marrow was obtained from the transgenic and normal mice after euthanasia. Femurs and tibia from mice were dissected and bone marrow was flushed out in 1–2 ml of Iscoves medium with 2% fetal bovine serum. The cells were resuspended to make a single cell suspension, and nucleated cells were counted after dilution in 3% acetic acid using a hemocytometer.

Colony assays

Bone marrow cells at a dilution of 0.3 ml at 2×10^5 cells/ml were suspended in 3 ml of methyl cellulose-containing growth factors (Methocult 3534, Stem Cell Technology, Vancouver, Canada). The mixture was plated in 1.1 ml duplicate cultures. The colonies were observed every day and counted on day 5 and day 12. For self-renewal experiments, the colonies were harvested and replated on fresh methylcellulose containing growth factors, and colony formation was observed. Triplicate platings were performed on day 12. Cellular morphology of the colonies was evaluated with cytospin preparations and Wright-Giemsa staining.

Flow cytometric analysis

Cells from the colonies were harvested and washed, and single cell suspensions were prepared. Approximately 1×10^6 cells were stained for 30 min on ice with monoclonal antibodies, washed in staining buffer, and analyzed. The antibodies used were FITC-conjugated Gr-1, Mac-1, and c-Kit (Pharmingen, CA). Flow cytometry was performed with a FACScan cytometer

(Becton-Dickinson, CA). A minimum of 10,000 events were acquired and analyzed with CellQuest software.

Supplemental data

Supplemental data for this article can be found at can be found at http://www.cancercell.org/cgi/content/full/7/4/351/DC1/.

Acknowledgments

We would like to thank Rich Pan, Kenny Kuwahara, Jun Wang, and Violetta Headley for technical assistance. We thank Rob Lane for his help with real-time PCR, and Athena Countouriotis (Cell Therapeutics, Inc.) and George Sandusky (Eli Lilly, Inc.) for help with immunohistochemistry. We are grateful to Mike Teitell, Charles Sawyers, and Steve Feig for helpful suggestions. This work was funded by NIH (CA68221, CA16042, and HL 75826), American Cancer Society (RSG-99-081-04-LIB), Diamond-Blackfan Anemia Foundation, and Sean's Hope Foundation (K.M.S). K.M.S. is a Scholar of the Leukemia and Lymphoma Society. D.B.S. is supported by the Hamburger endowment from the UCLA Jonsson Comprehensive Cancer Center. J.C.C. is a recipient of the Resident Research Award from the American Academy of Pediatrics.

Received: April 5, 2004
Revised: November 24, 2004
Accepted: February 24, 2005
Published: April 18, 2005

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