Loss of C/EBPα cell cycle control increases myeloid progenitor proliferation and transforms the neutrophil granulocyte lineage

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CCAAT/enhancer binding protein (C/EBP)α is a myeloid-specific transcription factor that couples lineage commitment to terminal differentiation and cell cycle arrest, and is found mutated in 9% of patients who have acute myeloid leukemia (AML). We previously showed that mutations which dissociate the ability of C/EBPα to block cell cycle progression through E2F inhibition from its function as a transcriptional activator impair the in vivo development of the neutrophil granulocyte and adipose lineages. We now show that such mutations increase the capacity of bone marrow (BM) myeloid progenitors to proliferate, and predispose mice to a granulocytic myeloproliferative disorder and transformation of the myeloid compartment of the BM. Both of these phenotypes were transplantable into lethally irradiated recipients. BM transformation was characterized by a block in granulocyte differentiation, accumulation of myeloblasts and promyelocytes, and expansion of myeloid progenitor populations—all characteristics of AML. Circulating myeloblasts and hepatic leukocyte infiltration were observed, but thrombocytopenia, anemia, and elevated leukocyte count—normally associated with AML—were absent. These results show that disrupting the cell cycle regulatory function of C/EBPα is sufficient to initiate AML-like transformation of the granulocytic lineage, but only partially the peripheral pathology of AML.
subtypes. BM transformation was accompanied by expansion of the myeloid progenitor compartment, as well as the c-Kit+ and Lin−Sca-1−c-Kit+ populations, but no detectable increase in long-term repopulating cells. These results provide direct genetic evidence that C/EBPα-mediated E2F repression is important for controlling progenitor proliferation in the myeloid compartment in vivo; that its impairment promotes transformation of the granulocytic compartment of the BM; and therefore, that mutations in a lineage-specific transcription factor may contribute directly to AML.

RESULTS

BM transformation and myeloproliferation in C/EBPα mutant mice

We previously described mouse strains containing point mutations in the basic region of the C/EBPα protein (basic region mutants [BRM] 1, 2, and 5; reference 23). Of these, BRM2 and BRM5 preserve C/EBPα DNA binding and promoter activation, but prevent association of C/EBPα with the E2F complex, and thereby, impair C/EBPα-mediated E2F repression; BRM1 is unaffected in this respect (23, 26). In young adult mice (8 wk) homozygous for the BRM2 (“BRM2 mice”) or BRM5 allele, Mac-1+/Gr-1+ granulo-
cytic cells were few or absent in the BM and peripheral blood. However, examination of older BRM2 mice (36 wk) revealed frequent splenomegaly (Fig. 1 A), accompanied by a strong increase in spleen granulocytes (Fig. 1, B and C). The number of Mac-1+/Gr-1+ BM cells was increased in these mice; in the most severely affected animals the BM consisted almost entirely of granulocytic cells (>85%; Fig. 1, D and F vs. Fig. 1, G and I). Consequently, the number of TER119+ erythroid cells was reduced substantially in the BM of these animals (Fig. 1 E vs. Fig. 1 H). Further analysis confirmed this phenotype and showed that it occurred in a virtually identical manner in BRM5 mice, whereas BRM1 or control knock-in mice were unaffected (unpublished data). At this point we chose the BRM2 line for further analysis.

When compared with littermate control mice, BRM2 mice displayed no detectable excess mortality (unpublished data). Therefore, to evaluate the progression of hematologic disorders we systematically analyzed the granulocytic cells in BM and spleen cells from BRM2 mice of 8, 12, 24, 36, 60, and 75 wk of age. This analysis revealed three distinct phenotypes (data summarized in Fig. 2). The phenotype originally observed in young adult BRM2 mice (i.e., few or no BM granulocytes [<12% Mac-1+/Gr-1+ BM cells] and a relative increase in BM erythroid cells; reference 23)—most likely due to the loss of granulocytic cell types—was found in 6/6 BRM2 mice at 8 wk of age (BRM2-A phenotype; an example is shown in Fig. 3 A); still was observed in a high proportion of mice at 12 wk of age (4/10 mice or 40%); but was more rare in mice of 24 wk or older (5/29 mice or 17%). BRM2-A mice had relatively normal levels of c-Kit+ BM cells (<6%), although overall, a slight elevation relative to the control group was observed (Fig. 2 B). Most likely this is attributable to the loss of the granulocytic compartment which leads to a relative increase in progenitor levels.

Figure 2. Development of myeloproliferative disease. (A) Flow cyto-metric analysis of BM from control (+/+, BRM2+/+) and BRM2 (BRM2/BRM2) mice at 8, 12, 24, 36, 60, and 75 wk of age, performed as in Fig. 1. For each mouse analyzed, a pair of columns shows the percentage of Mac-1+/Gr-1+ and TER119+ cells. Mice in each genotype/age group are organized according to the number of Mac-1+/Gr-1+ cells present (increasing left to right). The cut-offs used to define the BRM2 phenotypes are indicated (BRM2-A: <12% Mac-1+/Gr-1+; BRM2-B: >55% Mac-1+/Gr-1+ BM cells). These cut-off values differ from the mean value (38.2%; n = 30) observed in control mice by more than two standard deviations (SD = 7.1%), and were observed in the control group only once. The level of Ter119+ cells in BRM2-A mice and of Mac-1+/Gr-1+ cells in BRM2-B mice were significantly greater than the levels observed in control mice (P < 10^{-17} and P < 10^{-16}, respectively). (B) Levels of c-Kit positive cells in BM from control mice (n = 11), BRM2-A (n = 4), BRM2-B (n = 4), and BRM2-C (n = 6) mice. *P < 0.005; **P < 0.00005 compared with control mouse. +/+, +/BRM2; A, BRM2-A; B, BRM2-B; C, BRM2-C. (C) Phenotypic progression of BRM2 mice.

Figure 3. Three distinct BRM2 phenotypes. Distinct FACS profiles showing TER119 (a panels) and Mac-1/Gr-1 (b panels) staining and May-Grünwald-Giemsa–stained cytospins (c panels; original magnification, 100) of BM cells from representative mice. The percentage of TER119+ and Mac-1+/Gr-1+ cells are indicated.
At >12 wk of age, most mice contained significant levels of Mac-1⁺/Gr-1⁺ cells. These could be divided into two groups; the BRM2-B group (15/45 mice; 33%) contained highly elevated (>55%) levels of BM Mac-1⁺/Gr-1⁺ cells with the morphology of normally differentiated granulocytes (Fig. 3 B). This group had levels of c-Kit⁺ BM cells that were comparable to WT controls (<6% of nucleated BM cells). The most severely affected BRM2-B animals (>80% Mac-1⁺/Gr-1⁺ BM cells) displayed splenomegaly and elevated levels of spleen granulocytes as described in Fig. 1. The second group (BRM-2C; 10/45 mice; 22%) contained a moderate to high proportion (20–65%) of Mac-1⁺/Gr-1⁺ BM cells that stained weakly for the Gr-1 antigen (Fig. 3 C). Because Gr-1 is a marker for terminal granulocyte differentiation, this suggested a differentiation block along this lineage. Consistent with this, the morphology of BM myeloid cells from BRM2-C mice was immature (elevated numbers of myeloblasts and promyelocytes; Fig. 3 C, panel c). Finally, the BRM2-C mice contained abnormally high (6–20%) c-Kit⁺ positive BM cells (Fig. 2 B). Two mice did not fit this classification, but had normal levels (20–35%) of morphologically normal Mac-1⁺/Gr-1⁺ granulocytes and normal c-Kit⁺ levels; these most likely represent a transition state between the BRM2-A and -B phenotypes. The proportion of BRM2 mice with the three phenotypes progressed from BRM2-A in young mice to >40% BRM2-C in mice >6 mo of age (Fig. 2 C); this suggested a progression from BRM-2A toward BRM2-C, with BRM2-B as a possible intermediate phenotype. No hematopoietic disorder was observed in any age- and litter-matched control WT or heterozygous mice (n = 30).

To determine the differentiation profile of the granulocytic compartment in mice with the three different BRM2 phenotypes, we performed differential counts on BM cytospins (Fig. 4 A). This analysis confirmed that BRM2-A mice had an early block in granulocyte differentiation, few cells progressing beyond the myeloblast stage, and no accumulation of immature myeloid cells (23). In the BRM2-B phenotype, granulocyte differentiation seemed to be morphologically normal, but levels of mature neutrophil granulocytes were abnormally high, consistent with the elevated levels of Mac-1⁺/Gr-1⁺ cells in the BM. In contrast, the BRM2-C phenotype showed loss of granulocyte maturation and accumulation of myeloblasts and early progenitors (mostly promyelocytes) similarly to what is observed in hu
The transformed phenotype of the BM of BRM2-C mice led us to examine whether these mice displayed peripheral phenotypes associated with AML. However, relatively few myeloid blasts (1–6% of nucleated cells) were observed in peripheral blood smears from BRM2-C mice (Fig. 4 A, inset and not depicted). Examination of peripheral hematopoietic organs revealed no increase in immature myeloid cells in the spleens of BRM2-C mice, but 2/10 mice had hematopoietic infiltrations that included immature myeloid cells in the liver (Fig. 4, B–D). Comparison of control and BRM2 mice of >1 yr of age did not reveal thrombocytopenia (Fig. 4 E) or anemia (Fig. 4, F and G) in the BRM2-C group, and BRM2-C white blood cell counts were not significantly different from those of control mice (Fig. 4 H); this was consistent with the low levels of myeloid blasts observed in the peripheral blood of BRM2-C mice. The lack of severe AML–associated peripheral phenotypes most likely explains why no increased mortality of BRM2 mice was detected compared with WT or heterozygous controls.

The defects in granulocyte differentiation may be attributable to deregulation of receptors for myeloid growth fac-

Figure 5. Maintenance and progression of BRM2 phenotypes during BM transplantation. Analysis of recipient BM, from mice transplanted with BM from mice displaying the BRM2-A (A), -B (B), and -C phenotypes (C), as well as control BM (D) (panels d–f). The parallel analysis of the donor BM is shown for comparison (panels a–c). Panels show staining for Mac-1 and Gr-1 (panels a, d, and g), for c-Kit and Mac-1 (panels b, e, and h); and cytopsins stained with May-Grünwald-Giemsa (panels c, f, and i). In the case of BRM2-A and BRM2-B, examples are shown of mice in which phenotypic progression toward the BRM2-C phenotype was observed (panels g–i). In all surviving recipients >90% of hematopoietic cells were of donor origin.

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tors, such as G-, GM- and M-CSF-R, which contain functionally important C/EBPα sites in their promoter (31). Northern blot analysis of BM from WT and BRM2 mice showed that granulocyte colony-stimulating factor receptor (G-CSF-R), macrophage colony-stimulating factor receptor (M-CSF-R), and GM-CSF-Rα levels generally were proportional to the number of granulocytic, and hence, myeloid cells present (Fig. 4, I and J). Therefore, it seems that the BRM2 mutation does not decrease activation of these promoters significantly. Consistent with this activation of a G-CSF-R reporter by C/EBPα BRM2 in transient transfection assays was the same as that seen with WT C/EBPα (unpublished data).

In summary, mice homozygous for the C/EBPα BRM2 mutation, starting at 12 wk of age, progressed with high frequency from a neutropenic state to a myeloproliferative disorder which affected the granulocyte lineage, or to a condition in which the myeloid compartment of the BM was transformed with features of M2 AML, but in which peripheral involvement was relatively weak.

The BRM2 phenotypes are transplantable

The progression observed in BRM2 mice raised the question of how these distinct phenotypes arise. In particular, the possibility existed that the hyperproliferative BRM2-B phenotype was a reactive condition that was induced in response to infection or inflammation. Therefore, BM from BRM2-A, -B, and -C mice, as well as from +/+ controls (all of the CD45.2 allotype), was transplanted into lethally irradiated C57BL/6-CD45.1/2 recipients (results summarized in Table I). In all cases most of the recipients survived and these were engrafted efficiently (>90%). All three BRM2 phenotypes were transplantable, in that most recipients displayed the same BM phenotypes as their respective donors (Fig. 5). In a few cases (3/15), transplantation with BRM2-A or -B BM gave rise to mice that had progressed toward a BRM2-C phenotype (e.g., Fig. 5, A and B, panels g–i). This argued against a reactive or environmental origin of the BRM2 phenotypes, and indicated that they most likely differ as a result of accumulated genetic alterations; in particular, the observed progression of BRM2-A and -B toward BRM2-C—but not the opposite—was consistent with a genetically driven, irreversible process.

Table I. Noncompetitive repopulation

| Donor genotype/phenotype | Recipient phenotype | Recipient survival |
|--------------------------|--------------------|-------------------|
| +/-                      | normal             | 7/7               |
| BRM2-A                   | 4A, 1 progressed towards C | 5/7               |
| BRM2-B                   | 6B, 2 progressed towards C | 8/10              |
| BRM2-C                   | 3C                 | 3/4               |

2 × 10⁶ CD45.2 donor BM cells were injected into the tail vein of lethally irradiated CD45.1/2 C57BL/6 mice. Mice were analyzed 9–12 wk after transplantation. Donor contributions were >90%; all dead recipients died within 3 wk.

C/EBPα mutations increase the proliferative capacity of myeloid progenitors

To determine the cellular mechanisms that underlie the observed transformation of the granulocytic BM compartment, we next investigated the levels and replating efficiency of myeloid progenitors from BRM2 and control mice. BM cells were plated in semi-solid medium to allow outgrowth of all myeloid cell types, and the number of colonies was observed. Colonies were harvested and cells were replated to determine the proliferative ability of myeloid progenitors. Representative data are shown in Fig. 6 A. Progenitors from WT mice had the expected limited proliferative capacity, as illustrated by the rapidly decreasing number of colonies ob-

![Figure 6](image-url)
tained upon successive replatings. BRM2-A and -B mice contained a number of colony-forming progenitors similar to that found in WT BM, but their replicative potential was significantly greater; they continued to proliferate at the fourth replating (and in most cases well beyond; unpublished data). BRM2-C mice consistently contained significantly elevated levels of progenitors (as measured by the initial plating efficiency), also with high self-renewal capacity. The observation that progenitors from mice of all BRM2 phenotypes displayed increased replicative potential in vitro indicates that C/EBPα directly controls the ability of myeloid progenitors to expand. However, this seemed to lead to an in vivo expansion of the progenitor compartment only in mice with BM transformation. To determine the identity of the myeloid progenitors present in the different geno-/phenotypes, colony assays were performed (Fig. 6 B); this showed that in BRM2-A and -B BM, multipotent (CFU-granulocyte/erythrocyte/macrophage/megakaryocyte [GEMM]) and committed (CFU-granulocyte/macrophage [GM], burst-forming units-erythrocyte [BFU-E]) progenitors were present in numbers that were comparable to those found in WT mice. In contrast, BRM2-C mice contained significantly elevated

Figure 7. HSC and progenitor compartments in BRM2 mice. Flow cytometric determination of BM progenitors. Progenitor analysis of BM cells was performed as described previously (52). The percentage of nucleated BM cells with the immunophenotype of HSCs, CMPs, GMPs, and megakaryocyte-erythroid progenitors (MEP) is indicated. The diagram in the (A) panel shows the phenotyping strategy. Typical examples of analyses of WT (B), BRM2-A (C), BRM2-B (D), and BRM2-C (E) mice are shown.
levels of committed and multipotent progenitors; this suggested that an expansion of a very early myeloid progenitor population had occurred in these mice.

**Early myeloid progenitors and Lin^-Sca-1^-c-Kit^+ cells are expanded in BM from transformed BRM2 mice**

The above results suggested that mutation of C/EBPα, in addition to its well-characterized effects on granulocyte differentiation, affected an immature, proliferative compartment in the BM. To substantiate this finding we used the immunophenotyping protocol developed by Weissman and colleagues (32, 33). Typical examples of such analyses, performed on control and BRM2 mice, are shown in Fig. 7; results are summarized in Fig. 8A. These experiments revealed that bipotent granulocyte/macrophage progenitors (GMP), multipotent common myeloid progenitors (CMPs), and phenotypically defined (Lin^-Sca-1^-c-Kit^+) hematopoietic stem cells (HSCs) were increased significantly in BRM2-C mice compared with nontransformed BRM2-A/B mice. In particular, the HSC population was increased by an average of 10-fold compared with nontransformed BRM2 mice. Finally, we observed an increase in the proportion of c-Kit^+ cells in the Lin^-Sca-1^-IL-7R^- progenitor population in all BRM2 mice, regardless of their BM phenotype (Fig. 7, B–E, d panel; and not depicted).

The HSC population as defined above are not only repopulating stem cells, but are a mixture of repopulating stem cells and short-term pluripotent myeloid/lymphoid progenitors (sometimes referred to as “short-term” HSCs). To address the nature of the expanded Lin^-Sca-1^-c-Kit^+ compartment in BRM2-C mice, we performed competitive repopulation assays in which WT CD45.1 competitor BM cells were cotransplanted with BRM2-B, BRM2-C, or WT control BM cells into irradiated CD45.1/2 recipients at a 10:1 ratio. These assays showed a decreased hematopoietic contribution of BRM2 BM compared with WT BM, with no difference seen between the BRM2-B and -C phenotypes (Fig. 8B). This indicated that repopulating activity was not greater in the transformed BRM2-C BM, and that the increased proportion of Lin^-Sca-1^-c-Kit^+ cells most likely reflects an increase in multi-/pluripotent progenitors, rather than repopulating cells.

**DISCUSSION**

The results presented here allow us to reach three main conclusions. First, mutations in C/EBPα that impair C/EBPα–E2F association have the capacity to induce transformation of the myeloid compartment of the BM. This phenotype was seen only in mice homozygous for the mutations—indicating their recessive nature—and was consistent with loss-of-function as their main effect. Second, our data indicate that the cellular mechanism involves an increased replating efficiency of myeloid progenitors, correlating with c-Kit up-regulation in the progenitor-containing Lin^-Sca-1^-IL-7R^- BM population. This indicates a role for C/EBPα-mediated E2F repression in controlling the proliferative capacity of early myeloid progenitors. Finally, although BM transformation did not lead to an increase in long-term repopulating cells, it was associated with expansion of the c-Kit^+ and Lin^-Sca-1^-c-Kit^+ populations as well as CMP/CFU-GEMM and GMP/CFU-GM progenitors; this indicated that the target for BM transformation is an early multipotent progenitor/stem cell population. These results provide genetic evidence that targeting a myeloid lineage–specific transcription factor, essential for normal granulocyte differentiation.
tion, disrupts the normal regulation of progenitor cell proliferation/differentiation and directly contributes to transformation of the myeloid compartment of the BM, one of the key features of AML.

**C/EBPα mutation is sufficient to initiate BM transformation**

Definitive evidence that gain or loss of function of a particular protein is important for tumor development only can be obtained by direct in vivo mutagenesis. The present data, showing that C/EBPα mutation by itself is sufficient to initiate the development of granulocytic myeloproliferative disease and an M2 AML-like BM transformation, provide genetic evidence to support the role of C/EBPα as a myeloid-specific tumor suppressor. Thus, our observations strongly support the etiological relevance of *c/ebp* mutations in patients who have AML, and inhibition of C/EBPα function by translocation products found in myeloid leukemias. Previously, induction of AML-like disease in vivo by expression of PML-RARα or BCR-ABL transgenes (34–36), by ectopic Bcl-2 expression combined with the absence of Fas signaling (37), and by ectopic AML-ETO expression in conjunction with N-ethyl-N’-nitrosourea mutagenesis (38, 39) has been observed. However, the precise mechanisms of action of translocation-derived oncproteins remain unclear, not least because each transgenically expressed oncprotein generally affects several cellular targets, in the case of AML-ETO and BCR-ABL including C/EBPα. In the present study the C/EBPα BRM2 specifically targets the ability of C/EBPα to repress E2F activity and identifies a specific cellular pathway, the deregulation of which leads to BM transformation. Because a common *c/ebp* mutation in AML involves loss of expression of C/EBPα p42 and overexpression of the short p30 form of C/EBPα (4) which is incapable of repressing E2F activity (24), this indicates strongly that E2F repression is a critical feature of C/EBPα tumor suppressor function. Although the BRM2 knock-in allele produces the p42 and p30 isoforms at their normal ratio (23), we recently observed that mice in which C/EBPα p42 (but not p30) translation has been ablated and leads to a molecularly distinct but functionally similar loss of E2F repression, undergo BM transformation in a manner similar to that described here for BRM2 mice (M. Schuster, B. Porse, D. Tenen, and C. Nerlov, unpublished data); this is consistent with the above notion.

**Control of myeloid progenitor proliferation by C/EBPα**

We find that homozygosity for the C/EBPα BRM2 allele increases the proliferative capacity of BM myeloid progenitor cells in vitro. Although C/EBPα mRNA expression has been observed in CMPs and GMPs (32), the function of C/EBPα in these cell types has remained unclear. Our results indicate that in addition to its role in promoting neutrophil and eosinophil granulopoiesis (2, 40, 41), C/EBPα plays a direct role in the control of early myeloid progenitor (CMP/GMP) proliferation versus differentiation, and correlates with up-regulation of c-Kit in the CMP/GMP-containing Lin^-Sca-1^-IL-7R^- compartment. Our results further suggest that loss of this control mechanism contributes to early progenitor expansion during BM transformation. Two recent reports have shown that the CMP to GMP transition is impaired severely in this complete absence of C/EBPα, and this was associated with increased proliferative capacity of myeloid progenitors (42, 43). In neither study was overt granulocytic AML observed to develop from C/EBPα-deficient cells, most likely because C/EBPα is required for granulocyte lineage commitment, without which granulocyte-type AML cannot occur (for discussion see reference 29). Instead, accumulation of blasts in the BM was observed with virtually no granulocytic maturation (42). We also see increased progenitor proliferation and accumulation of immature myeloid BM cells without development of overt AML in BRM2-C mice. However, formation of GMPs is not impaired in BRM2 mice, and some maturation along the granulocytic lineage is seen in transformed BRM2-C mice. Therefore, the BM transformation that is caused by the BRM2 is likely to have a molecular basis distinct from the AML-like BM phenotype that was observed by Zhang and co-workers (42), which seems to be due to an early block in myeloid lineage commitment. This also is supported by the delayed development of BM transformation in BRM2 mice compared with the acute differentiation block seen by complete deletion of C/EBPα, which suggests the requirement of additional mutations only in the case of BRM2 mice.

Although BRM2 impairs the ability of C/EBPα to repress E2F activity (23, 26) it was proposed instead that this mutation reduces C/EBPα DNA binding capacity which contributes to its inability to arrest cell proliferation (44). However, this proposal is not consistent with the inability of other investigators, as well as ourselves, to detect any loss of C/EBPα transactivation of or binding to the G-CSF-R promoter, either in vivo or in tissue culture experiments upon introduction of this mutation (23, 25), nor does it explain the observation that C/EBPα alleles with complete loss of DNA binding are less defective in arresting myeloid cell cycle progression than the C/EBPα BRM2 allele (25). Granulocytes from BRM2 mice express the G-CSF-R mRNA—a genetically defined C/EBPα target gene (2)—at levels similar to those seen in WT granulocytes. Therefore, although we cannot rule out formally that interactions with factors other than E2F are impaired by BRM2, the available evidence indicates loss of E2F interaction as the most likely basis for the phenotypes observed. This is supported by studies in the K562 cell line: here C/EBPα BRM2 is incapable of down-regulating *c-myc* transcription via an E2F binding site in the *c-myc* promoter (24, 26), showing that the BRM2 deregulates E2F in differentiating granulocytes in vitro. This deregulation of the E2F-Myc axis is the likely cause of the initial loss of granulocytic cells (for discussion see reference 23). Current efforts are directed at the identification of promoters that are deregulated by C/EBPα BRM2 in vivo. Although the increased
self-renewal capacity correlated with an increase in the proportion of Lin–Sca-1–IL-7R– cells that were c-Kit positive, it is not clear whether deregulation of c-Kit expression contributes to, or merely reflects, this phenomenon. Although a role for c-Kit in M2 AML is supported by the occurrence of activating mutations in the c-kit gene in this leukemic subtype (45) and c-Kit overexpression in BM cells of patients who have AML (46), further analysis of the regulation of c-Kit expression by C/EBPα controlled pathways will be required to determine the functional relevance of elevated levels of c-Kit–expressing cells in the BRM2 mice.

Involvement of the stem cell compartment in BM transformation

BM transformation was observed in ~22% of the BRM2 mice of 12 wk of age or older. The incomplete penetrance and delayed occurrence indicates that BM transformation requires additional genetic events. Although the nature of such events is not clear, the consistent increase of bipotent (GM/CFU-GM), multipotent (CMP/CFU-GEMM), and pluripotent (Lin–Sca-1–c-Kit+) cells in transformed BM indicates that the target cell resides very early in the hematopoietic differentiation hierarchy. Although the AML-like BRM2-C phenotype is transplantable, we do not find that the long-term repopulating activity is increased in transformed BM relative to the preleukemic state. Therefore, the most likely explanation seems to be that the number of stem cells is not increased in the leukemic mice, but that a subset of these, or of early Lin–Sca-1–c-Kit+ progenitor cells, has undergone secondary mutations that generate transplantable leukemic stem cells. This results in production of elevated levels of multipotent and myeloid-restricted progenitors—which fail to mature along the granulocytic lineage—and in the transformed BM phenotype. This is in line with earlier transplantation studies that identified the “AML stem cell” from all tested human acute leukemic subtypes as having a phenotype similar to the HSC (CD34+/CD38–), and thereby, identified AML as a disease residing in the stem cell compartment (47–49). Thus, BRM-2 mutant mice may prove to be a valuable system for the identification of transforming stem cell mutations in AML.

The observation that BRM2 HSCs have decreased competitive repopulating activity compared with WT HSCs contrasts with the observation that HSCs in which C/EBPα has been deleted display increased competitive repopulating activity (42). This indicates that the effect of the BRM2 mutation in HSCs is distinct from complete loss of function, and that the positive effect of C/EBPα deletion on HSC function may not be related to C/EBPα cell cycle control, but rather to effects on gene expression. This is consistent with the increased Bmi-1 expression that is seen in HSCs that lack C/EBPα (42).

The BRM2 mice undergo phenotypic progression, from the BRM2-A phenotype (in mice <2 mo) toward BRM2-C (which is the most prevalent phenotype in mice >6 mo). These phenotypes are stable during transplantation which suggests that the progression is due to accumulation of secondary mutations, rather than proliferative syndromes that are induced by stromal defects, infection, or inflammation. The observation that a proportion of mice that was transplanted with BRM2-A or –B BM progressed toward a BRM2-C phenotype is consistent with this notion, and indicates that the myeloproliferative state can serve as an intermediate step in BM transformation. A preleukemic phase with elevated BM neutrophil levels is seen in mice homozygous for a PU.1 knockout allele before progression to overt AML (18). PU.1 and C/EBPα antagonize each other during the granulocyte-macrophage lineage decision, possibly by displacement of c-Jun—a PU.1 coactivator—by C/EBPα (50, 51). However, the above observations suggest that PU.1 and C/EBPα contribute to control of neutrophil proliferation in vivo. The observed deregulation could involve c-Myc overexpression, because c-Myc is up-regulated in a significant proportion of PU.1 knockdown mice, possibly through chromosomal amplification (18), and is repressed by C/EBPα via E2F sites in the c-myc promoter (24). However, it also is possible that if PU.1 levels are low it may fail to sequester c-Jun, which could inhibit C/EBPα (52). The individual contributions of these pathways to granulocyte hyperproliferation remain to be determined.

In summary, we have found that loss of C/EBPα-mediated E2F repression results in deregulation of progenitor proliferation in the myeloid compartment, and predisposes to the development of transplantable myeloproliferative disorders of the granulocytic lineage, including BM transformation with features of M2 AML and in vivo expansion of multi- and bipotent myeloid progenitors, and the Lin–Sca-1–c-Kit+ compartment. Thus, our results provide a molecular link between the clinically observed mutations in the cebpa gene in patients who have AML and the finding that transplantation of AML requires the early progenitor/stem cell compartment; this indicates that C/EBPα mutant mice will prove to be a valuable tool in studying the role of the stem cell compartment in AML development. More generally, our results support the idea that mutations in lineage-specific transcription factors play a direct role in myeloid leukemogenesis. This notion is consistent with the recent observation that reduced expression of PU.1 leads to AML (18); it will be relevant to examine mice containing appropriate PU.1 and GATA-1 knock-in mutations.

MATERIALS AND METHODS

Mouse strains. BRM2 mice were maintained on a mixed C57BL/6-129/Ola background and genotyped as described previously (23). Mice with the desired genotypes were maintained under specific pathogen-free conditions for 8–75 wk before analysis. C57BL/6-CD45.1 or C57BL/6-CD45.2 mice were bred at B & S. Animal care was in accordance with the institutional guidelines of the Universities of Lund and Copenhagen, as well as Danish and Swedish National guidelines.

Transplantation assays. BM transplants were performed essentially as described (53). For simple transplantation (noncompetitive repopulation), lethally irradiated C57BL/6-CD45.1/2 recipients were injected into the tail vein with $2 \times 10^6$ BM cells from WT donors or donors displaying each of the three BRM2 phenotypes. The BM phenotype of the recipients...
was analyzed 9–12 wk after transplantation. For competitive repopulation assays, CD45.1/2 recipients received 2 × 10^6 C57BL/6–CD45.1 competitor cells and 2 × 10^6 CD45.2 test BM cells from B6.Ms or their WT controls. Relative engraftment of the CD45.2 cells was measured by flow cytometry of peripheral blood leukocytes 8 wk after transplant. Although donor cells were of a mixed C57BL/6–129/Ola background, this did not affect detectably their ability to function in competitive repopulation against C57BL/6 cells in C57BL/6 recipients, as observed previously (54).

**Histology and peripheral blood analysis.** BM cells were harvested as described previously (23). Spleen cells were dissociated through a fine-gauge needle in medium containing DMEM + 10% FCS + 20 mM Hepes-KOH. Peripheral blood was used directly for blood smears. Methanol-fixed cytopsins and smears were stained with May-Grünwald-Giemsa. For differential counts, >300 cells from BM cytopsins were counted for each sample. Blood samples were examined on a TXK21 automated hematologist analyzer (TOA Medical Electronics Co.). For histologic analysis, formalin-fixed (4% paraformaldehyde) tissues were paraaffin embedded, sectioned at 4 μm, and stained with hematoxylin-eosin.

**Flow-cytometry and progenitor phenotyping.** Flow cytometry was performed by staining with anti–Mac-1–FITC, anti–TER119–PE, anti–Gr-1–APC, anti–c–Kit–APC, or the corresponding isotype control antibodies (BD Biociences) and analysis on a FACScalibur flow cytometer (Becton Dickinson). Progenitor analysis of BM cells was performed using antibodies against lineage markers (B220, CD3, Mac-1, Gr-1, TER119, CD4, CD5, CD8), c-Kit, Sca-1, IL-7R, FcγRII/III, and CD34 to define common myeloid progenitor cells (CMP; Lin–Sca-1–IL7R–c–Kit–CD34+/−FcyRII/III+), GMP cells (Lin–Sca-1–IL7R–c–Kit–CD34+/−FcyRII/III+), and megakaryocyte-erythroid progenitors (Lin–Sca-1–IL7R–c–Kit–CD34+/−FcyRII/III+) and with antibodies against lineage markers, c-Kit, Sca-1, and IL-7R to define HSCs (Lin–Sca-1–c–Kit–) and common lymphoid progenitor cells (Lin–IL7R–Sca-1–c–Kit–) as described previously (32, 33, 35). For analysis of BM transplanted mice, peripheral blood leukocytes were stained with anti–CD45.2–FITC and anti–CD45.1–PE to identify donor-derived cells. Staining with anti–CD4, anti–CD8, anti–B220 (all PE-Cy5 conjugated), anti–B220, and anti–Mac-1 (both allophycocyanin conjugated) was used to distinguish T lymphocytes, B lymphocytes, and myeloid cells.

**Colony assays and serial replating experiments.** For the analysis of colony-forming potential, BM cells (5,000–20,000 cells/35-mm dish) were seeded in methylcellulose-based medium (M3434, StemCell Technologies Inc.) supplied with erythropoietin, IL-3, IL-6, and stem cell factor. After 10–12 d in culture, the colonies were scored as CFU-GM, BFU-E, or CFU-GEMM. In the serial replating experiments, a similar number of BM cells was seeded in M3434 medium, cultured for 7 d, and the number of colonies was counted. The cells were harvested, washed with PBS, diluted, and replated in fresh M3434 medium and cultured for an additional 7 d. This procedure was repeated for 5 wk.

**Northern blotting.** Total RNA was isolated from BM cells and Northern blotting was performed as described (23) using cDNA probes for the G-CSF–R, M-CSF–R, and GM-CSF–Rα (18). An 18S rRNA probe (5′-GTGGGTACTTACGACATGCATG-3′) was used as a loading control.

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