ORIGINAL ARTICLE

Granulocyte–Macrophage Progenitors as Candidate Leukemic Stem Cells in Blast-Crisis CML

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ABSTRACT

BACKGROUND

The progression of chronic myelogenous leukemia (CML) to blast crisis is supported by self-renewing leukemic stem cells. In normal mouse hematopoietic stem cells, the process of self-renewal involves the β-catenin–signaling pathway. We investigated whether leukemic stem cells in CML also use the β-catenin pathway for self-renewal.

METHODS

We used fluorescence-activated cell sorting to isolate hematopoietic stem cells, common myeloid progenitors, granulocyte–macrophage progenitors, and megakaryocyte–erythroid progenitors from marrow during several phases of CML and from normal marrow. *BCR-ABL*, *β-catenin*, and *LEF-1* transcripts were compared by means of a quantitative reverse-transcriptase–polymerase-chain-reaction assay in normal and CML hematopoietic stem cells and granulocyte–macrophage progenitors. Confocal fluorescence microscopy and a lymphoid enhancer factor/T-cell factor reporter assay were used to detect nuclear β-catenin in these cells. In vitro replating assays were used to identify self-renewing cells as candidate leukemic stem cells, and the dependence of self-renewal on β-catenin activation was tested by lentiviral transduction of hematopoietic progenitors with axin, an inhibitor of the β-catenin pathway.

RESULTS

The granulocyte–macrophage progenitor pool from patients with CML in blast crisis and imatinib-resistant CML was expanded, expressed *BCR-ABL*, and had elevated levels of nuclear β-catenin as compared with the levels in progenitors from normal marrow. Unlike normal granulocyte–macrophage progenitors, CML granulocyte–macrophage progenitors formed self-renewing, replatable myeloid colonies, and in vitro self-renewal capacity was reduced by enforced expression of axin.

CONCLUSIONS

Activation of β-catenin in CML granulocyte–macrophage progenitors appears to enhance the self-renewal activity and leukemic potential of these cells.

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N Engl J Med 2004;351:657-67.

THE PHILADELPHIA CHROMOSOME IN chronic myelogenous leukemia (CML) gives rise to the *BCR-ABL* proto-oncogene and its constitutively active protein tyrosine kinase product $p210^{BCR\text{-}ABL}$.[1-16] *BCR-ABL* is important because in patients with CML, there is clonal expansion of hematopoietic cells that express this fusion gene. Moreover, continued expression of *BCR-ABL* is required for sustained proliferation of leukemic cells in mouse models of CML.[6-9]

Imatinib, a potent inhibitor of $p210^{BCR\text{-}ABL}$, can induce remission in patients with chronic-phase CML, but despite this event, cells in the marrow may continue to produce *BCR-ABL* transcripts.[17,18] In some patients, resistance to imatinib develops as a result of *BCR-ABL* amplification or mutations in the binding site of $p210^{BCR\text{-}ABL}$ for imatinib.[19,20] In another myeloid leukemia, t(8;21) acute myelogenous leukemia, marrow from patients in complete remission contains apparently normal hematopoietic stem cells that produce *AML1-ETO* transcripts. The presence of these stem cells during remission suggests that they are preleukemic rather than leukemic cells. (These transcripts participate in the development of acute myeloid leukemia; *AML1-ETO* is formed by the fusion of part of the *AML1* gene on chromosome 8 with part of the *ETO* gene on chromosome 21.[21]) Similarly, genomic *BCR-ABL* persists in the marrow of some patients with CML who are in a sustained complete cytogenetic remission,[22] and it has been detected at very low levels in leukocytes from healthy persons,[23] which suggests that preleukemic hematopoietic stem cells or more differentiated progenitor cells need additional mutations for progression to overt leukemia to occur.[15,16,24-26]

In mice, self-renewal of hematopoietic stem cells entails activation of the β-catenin pathway,[27,28] which results in the translocation of β-catenin to the nucleus, where it interacts with lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors and regulates the transcription of genes such as *c-myc* and cyclin D1.[29-32] Activating β-catenin mutations occur in many epithelial cancers.[33-37] The ability to isolate purified populations of hematopoietic stem cells and myeloid progenitors[38-41] has made it possible to identify genes involved in the self-renewal of hematopoietic stem cells (the ability to make more daughter cells at the same stage of differentiation).[27,28,42-45] Deregulation of self-renewal pathways, which are normally tightly regulated in hematopoietic stem cells,[27,28,39-45] has recently been recognized as an important step in leukemic progression.[46,47] We conducted a study to identify candidate leukemic stem cells[13,21,24-26] that are responsible for disease progression and resistance to imatinib in patients with CML and to determine whether these stem cells acquire the potential for self-renewal by activating β-catenin.[29-32]

METHODS

BONE MARROW AND PERIPHERAL-BLOOD SAMPLES

Samples of normal bone marrow (All Cells) or peripheral blood mobilized by granulocyte colony-stimulating factor were obtained from 11 healthy volunteers as previously described.[41] Samples were obtained from 20 patients with CML in chronic phase, 26 patients with accelerated-phase CML, and 13 patients with CML in blast crisis. All subjects provided written informed consent, and the study was conducted according to the regulations of the institutional review boards of Stanford University and the University of California, Los Angeles.[41] Cells were obtained from patients before they began treatment with imatinib, from patients who had received imatinib for 6 to 15 months (22 with chronic-phase CML, 11 with accelerated-phase CML, and 2 with CML in blast crisis), and from patients with imatinib-resistant CML (5 with accelerated-phase CML and 1 with CML in blast crisis). Patients with chronic-phase CML received interferon alfa, whereas patients with advanced disease were often treated with cytoreductive agents before receiving imatinib (described in Table 1 of the Supplementary Appendix, available with the full text of this article at www.nejm.org).

ISOLATION OF HEMATOPOIETIC STEM CELLS AND MYELOID PROGENITORS

Hematopoietic stem cells ($CD34^+CD38^-CD90^+$ [$Thy1^+$]Lin^- cells) and myeloid progenitors, including common myeloid progenitors ($CD34^+CD38^+$ interleukin-3 receptor $\alpha^+CD45RA^-$), granulocyte–macrophage progenitors ($CD34^+CD38^+$interleukin-3 receptor $\alpha^+CD45RA^+$), and megakaryocyte–erythroid progenitors ($CD34^+CD38^+$interleukin-3 receptor $\alpha^-CD45RA^-$), were isolated from normal and CML mononuclear cells by fluorescence-activated cell sorting (FACS), as described previously.[41]

COLONY-FORMING CELL ASSAYS

Colony-forming cell assays were performed as described previously.[41] In replating experiments, indi-

vidual colonies were obtained on day 14, replated in 96-well plates, and analyzed 14 days later. In some experiments, lentiviral constructs containing an LEF/TCF–green fluorescent protein (GFP) reporter, a phosphoglycerate kinase promoter–β-catenin–internal ribosome entry site (IRES)–GFP cassette, or a phosphoglycerate kinase–axin–IRES–GFP cassette were added to colony-forming cell assays (described in detail in Method 1 of the Supplementary Appendix).[27]

ASSAY FOR *BCR-ABL*

RNA was isolated from 40 to 300 hematopoietic stem cells, common myeloid progenitors, granulocyte–macrophage progenitors, or megakaryocyte–erythroid progenitors from five control subjects, four patients with chronic-phase CML, seven with accelerated-phase CML, three with CML in blast crisis, four after imatinib therapy, and five with imatinib-resistant CML. Quantitative reverse-transcriptase–polymerase-chain-reaction (RT-PCR) analysis of the expression of *BCR-ABL*, β-catenin, *LEF-1*, and *HPRT* was performed (described in Method 2 of the Supplementary Appendix).[48,49]

β-CATENIN FACS ANALYSIS

Normal or CML mononuclear cells were stained with phycoerythrin-conjugated antibody against human CD90 or interleukin-3 receptor α, in addition to allophycocyanin-conjugated antibody against human CD34 and biotinylated antibody against human CD38 followed by staining with an alexa 594–conjugated antibody against human streptavidin. Cells were then fixed with 0.8 percent paraformaldehyde and made permeable by exposure to 0.3 percent saponin.[41] Cells were stained overnight with a fluorescein isothiocyanate–conjugated antibody against β-catenin (Transduction Laboratories) or a control fluorescein isothiocyanate–conjugated antibody against IgG1 isotype, washed, and analyzed by means of FACS.

CONFOCAL FLUORESCENCE MICROSCOPY

Hematopoietic stem cells or granulocyte–macrophage progenitors from six controls and nine patients with CML were sorted onto glass slides by means of FACS, stained with a fluoroscein isothiocyanate–conjugated antibody against human CD45, fixed in 4 percent paraformaldehyde, and stained with a mouse monoclonal antibody against activated β-catenin (clone 8E4, Upstate Biotechnology) at a dilution of 1:200 (2.5 μg per milliliter), as described previously.[50] Slides were then stained with an alexa 594–conjugated goat antimouse antibody and a nuclear stain (Hoechst 33342, Molecular Probes). Confocal images were obtained with the use of a dual-photon confocal fluorescence microscope (model LSM510, Zeiss) at a magnification of 100×, and three-dimensional images were rendered with the aid of Volocity software (described in Method 3 of the Supplementary Appendix).

LEF/TCF REPORTER ASSAY

A lentiviral LEF/TCF reporter (Fig. 1 of the Supplementary Appendix) was used to assess the capacity of β-catenin to activate transcription by means of LEF/TCF in sorted populations of normal and CML hematopoietic stem cells and myeloid progenitors, essentially as previously described, with the substitution of human cytokines interleukin-6 (10 ng per milliliter), Flt3 ligand (50 ng per milliliter), steel factor (50 ng per milliliter), and thrombopoietin (10 ng per milliliter).[27,51]

RESULTS

HEMATOPOIETIC STEM CELLS AND PROGENITOR CELLS IN CML

FACS analysis revealed higher levels of the progenitor pool ($CD34^+Lin^-$ cells) in bone marrow from patients with CML in the accelerated or blast phase than in normal bone marrow (Fig. 1A in the Supplementary Appendix). Within the progenitor pool, the population of hematopoietic stem cells ($CD34^+CD38^-CD90^+Lin^-$) did not expand significantly with disease progression (Fig. 1A). However, an evaluation of individual populations of myeloid progenitors ($CD34^+CD38^+Lin^-$) revealed that in comparison with normal bone marrow, increased numbers of megakaryocyte–erythroid progenitors were present in marrow from patients with chronic-phase CML ($P<0.001$), levels of common myeloid progenitors were increased in patients with accelerated-phase CML ($P=0.004$), and levels of granulocyte–macrophage progenitors were increased in marrow from patients with CML in blast crisis ($P=0.02$) (Fig. 1B). Among cells from patients with a response to imatinib, there was a significant decrease in the number of $CD34^+Lin^-$ cells, as compared with their normal bone marrow counterparts ($P=0.03$), and the proportion of individual myeloid progenitors reverted to normal, whereas samples from patients with imatinib-resistant CML had increased numbers of granulocyte–macrophage pro-

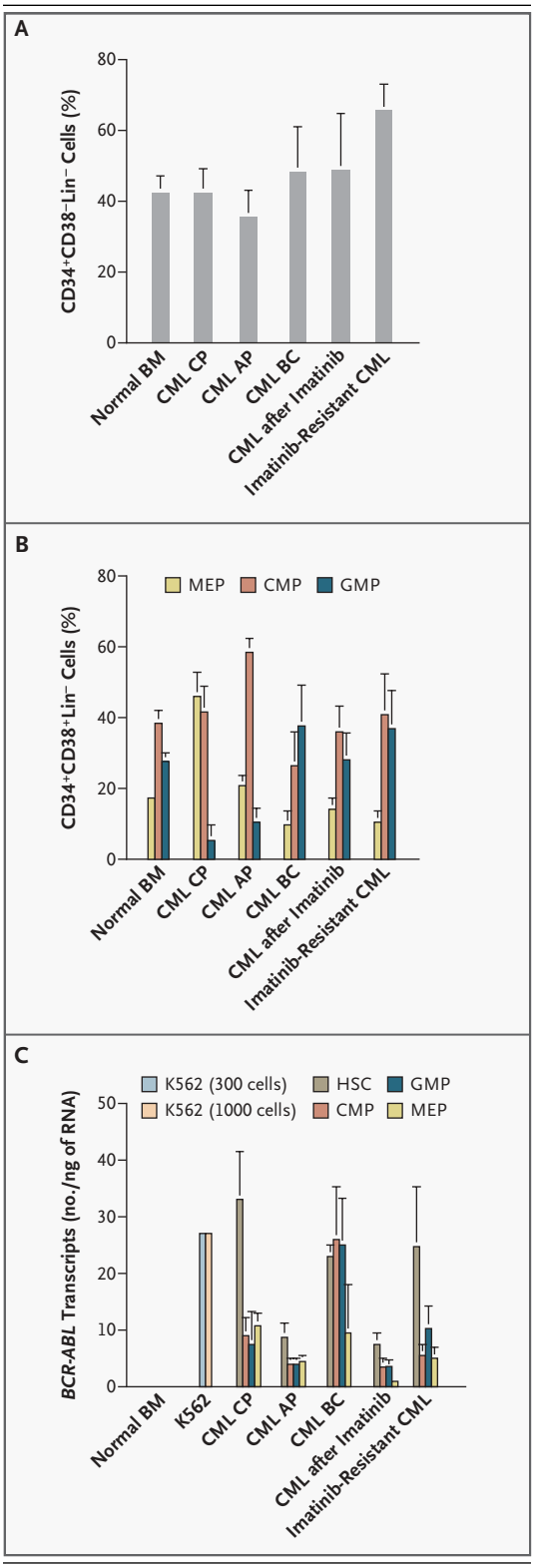


Figure 1. Mean (+SE) Proportions of CD90+ Hematopoietic Stem Cells (Panel A) and Myeloid Progenitor Populations (Panel B) and Levels of *BCR-ABL* Transcripts (Panel C).

Panel A shows the proportion of hematopoietic stem cells (HSC) within the $CD34^+CD38^-Lin^-$ population of cells in bone marrow (BM) from 11 control subjects, 5 patients with CML in chronic phase (CP), 6 patients with CML in accelerated phase (AP), 4 patients with CML in blast crisis (BC), 6 patients after treatment with imatinib, and 4 patients with imatinib-resistant CML. Panel B shows the percentage of individual populations of myeloid progenitors, including common myeloid progenitors (CMP), granulocyte–macrophage progenitors (GMP), and megakaryocyte–erythroid progenitors (MEP), expressed as the percentage of the $CD34^+CD38^+Lin^-$ fraction of the same samples. Statistical analyses were performed with the use of Excel software and Student's two-tailed unpaired t-test. Panel C shows the average number of *BCR-ABL* transcripts per nanogram of RNA derived from K562 (a Philadelphia chromosome–positive cell line) or bone marrow from 5 controls, 14 patients with CML (in chronic phase, accelerated phase, or blast crisis) before the initiation of imatinib therapy, 4 patients after treatment with imatinib, and 6 patients with imatinib-resistant CML, as well as from hematopoietic stem cells, common myeloid progenitors, granulocyte–macrophage progenitors, and megakaryocyte–erythroid progenitors.

genitors (Fig. 1A and 1C in the Supplementary Appendix).

Quantitative RT-PCR analysis demonstrated that in samples from patients with chronic-phase CML, *BCR-ABL* transcripts were more abundant in hematopoietic stem cells than in myeloid progenitors. Conversely, blast crisis was associated with increased numbers of *BCR-ABL* transcripts in myeloid progenitors, particularly common myeloid progenitors and granulocyte–macrophage progenitors (Fig. 1C). Because of the small numbers of progenitors we were able to obtain from banked samples, we performed RT-PCR analysis for *BCR-ABL* rather than fluorescence in situ hybridization. For this reason, we cannot exclude the possibility of a mixture of Philadelphia chromosome–positive and Philadelphia chromosome–negative cells within the individual progenitor subgroups.

β-CATENIN ACTIVATION

FACS analysis revealed that total intracellular β-catenin levels in hematopoietic stem cells did not

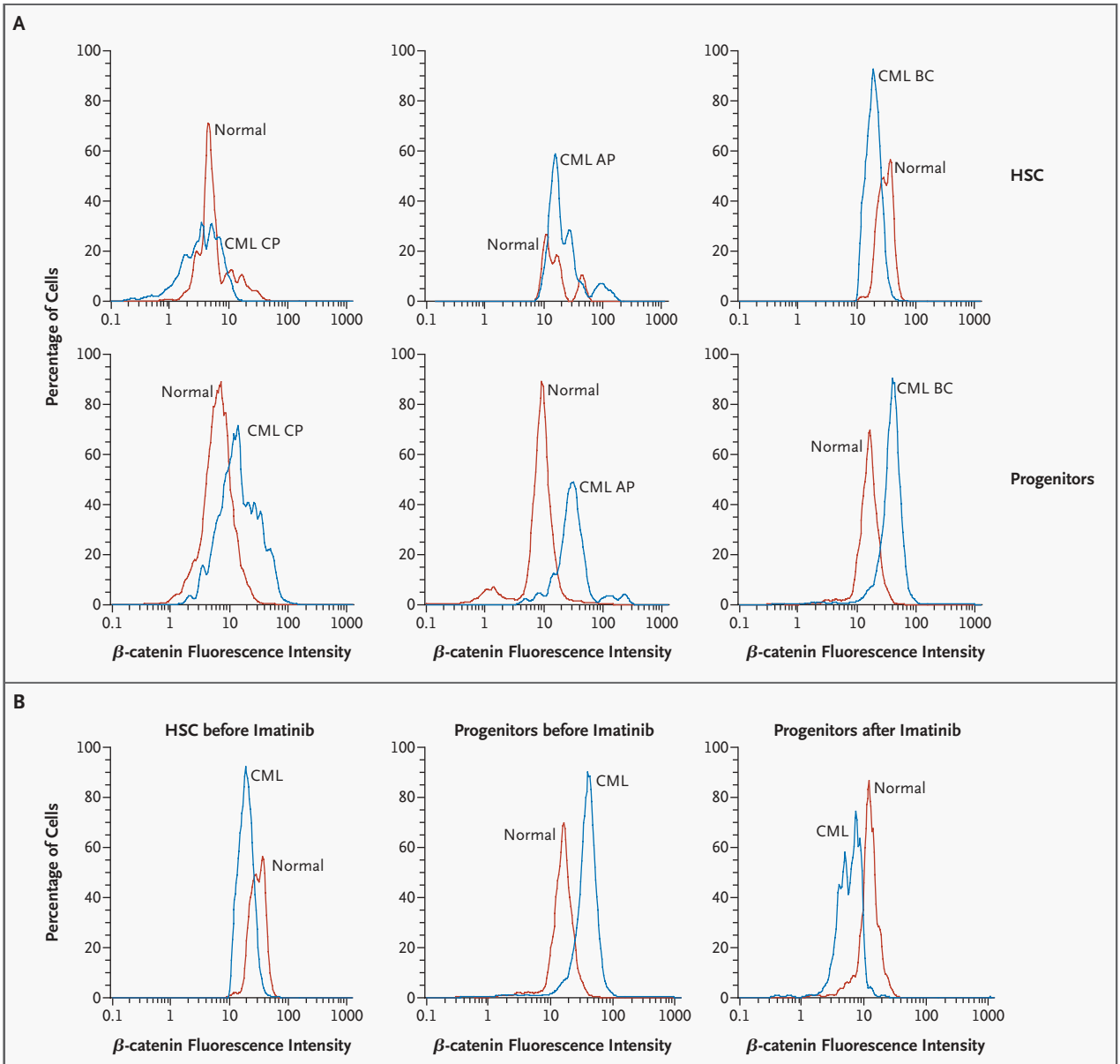


Figure 2. Results of Fluorescence-Activated Cell-Sorting Analysis of β-Catenin Expression.

Panel A shows the fluorescence intensity of fluorescein isothiocyanate (FITC)–conjugated antibody against β-catenin in hematopoietic stem cells (HSC; upper three histograms) from six controls, as compared with five patients with CML in chronic phase (CP; P=0.36 by Student's two-tailed unpaired t-test), five with CML in accelerated phase (AP; P=0.30), and four with CML in blast crisis (BC; P=0.33), and in myeloid progenitors (lower three histograms) from controls, as compared with patients with CML in chronic phase (P=0.96), accelerated phase (P=0.009), and blast crisis (P=0.04). Panel B shows the representative fluorescence intensity of FITC-conjugated antibody against β-catenin in hematopoietic stem cells or progenitor cells from six controls, as compared with three patients with CML in blast crisis before imatinib therapy and after imatinib therapy. Hematopoietic stem cells are $CD34^+CD38^-CD90^+Lin^-$[38,39]; progenitors are $CD34^+CD38^+IL3R\alpha^+Lin^-$.[41] There was a significant difference in the mean fluorescence intensity of progenitor cells before and after imatinib therapy from patients with CML in accelerated phase (not shown) (P=0.03) and patients with CML in blast crisis (P=0.03).

differ significantly between controls and patients with CML at any stage of the disease. In contrast, myeloid progenitors from patients with CML in an accelerated phase or blast crisis had increased β-catenin levels, as compared with levels in controls (Fig. 2A). These levels normalized in patients who had received imatinib (Fig. 2B). In the absence of phosphorylation, activated β-catenin translocates to the nucleus (Fig. 2 in the Supplementary Appendix). Confocal fluorescence microscopy with monoclonal antibodies against unphosphorylated β-catenin showed that the staining intensity of nuclear β-catenin was similar in hematopoietic stem cells from controls, patients with accelerated-phase CML, and patients with CML in blast crisis. However, there was a striking increase in activated β-catenin in granulocyte–macrophage progenitors from patients in blast crisis (Fig. 3B) or those who had imatinib-resistant CML, as compared with such progenitors from normal marrow (Fig. 3C). Concordantly, an LEF/TCF–GFP reporter assay of β-catenin–mediated transcriptional activation revealed similar levels of GFP in hematopoietic stem cells from controls, patients with chronic-phase CML (not shown), and patients with CML in blast crisis, but an increase in GFP — and thus in nuclear β-catenin — in granulocyte–macrophage progenitors from patients in blast crisis (Fig. 3D). Moreover, colonies derived from blast crisis CD34+Lin– cells that were transduced with the LEF/TCF–GFP reporter had higher levels of GFP than did their normal counterparts (Fig. 3 in the Supplementary Appendix). Whereas normal granulocyte–macrophage progenitors had reduced expression of β-catenin or its transcriptional coactivator, LEF-1, as compared with normal hematopoietic stem cells, CML granulocyte–macrophage progenitors from patients in blast crisis expressed increased levels of both transcripts (Fig. 4 in the Supplementary Appendix), which most likely contributed to the nuclear accumulation of β-catenin.

Figure 3 (facing page). Confocal Fluorescence Microscopical Images of β-Catenin Nuclear Localization (Panels A, B, and C) and the Results of Fluorescence-Activated Cell-Sorting Analysis of LEF/TCF Reporter Activity.

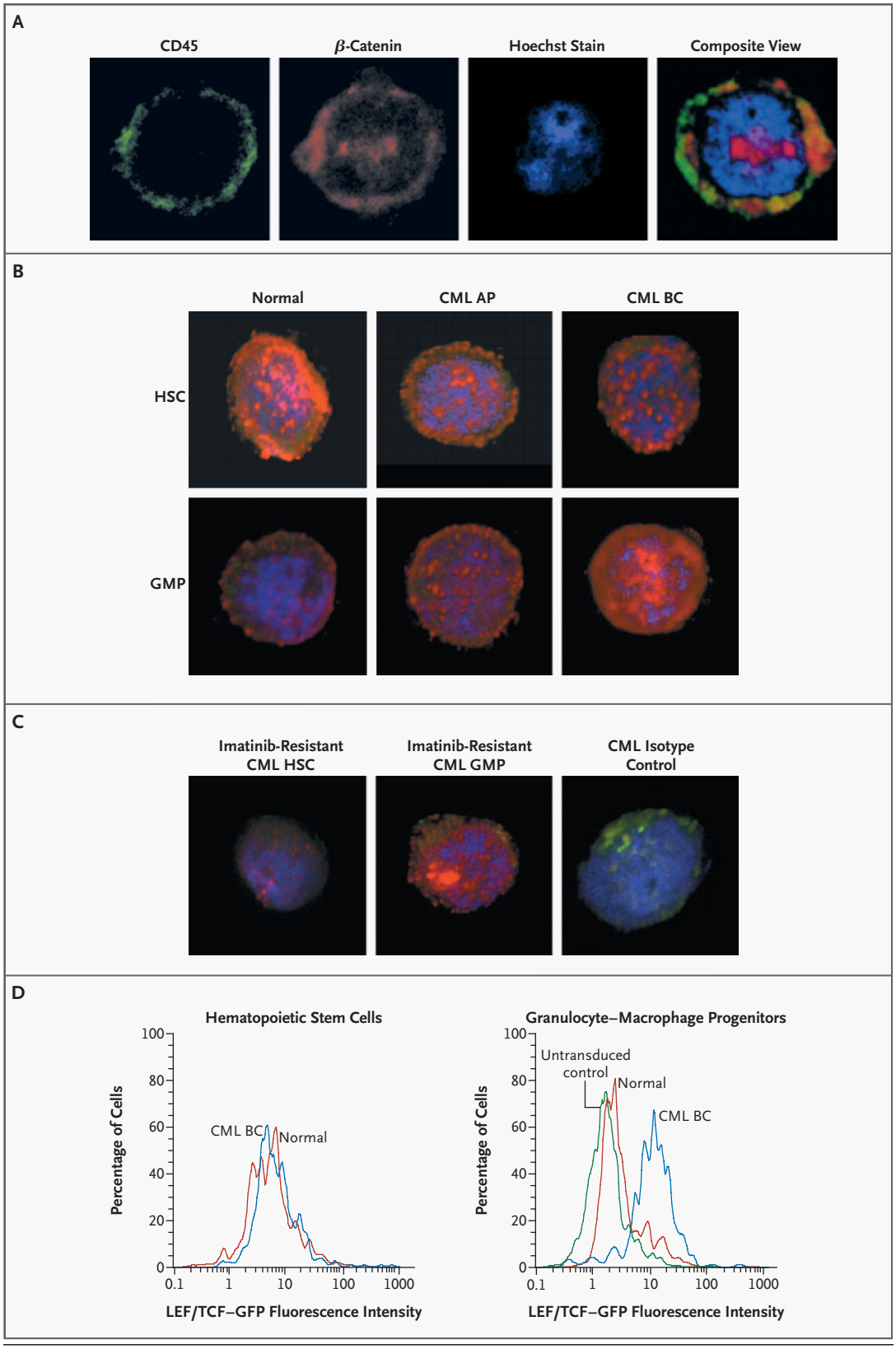
Panel A shows confocal fluorescence microscopical images of $CD34^{+}Lin^{-}$ cells from a patient with accelerated-phase CML stained with fluorescein isothiocyanate–conjugated antibody against CD45 (green); an antibody specific for activated β-catenin (red); and Hoechst, a blue nuclear stain. Panel B shows the localization of β-catenin in hematopoietic stem cells (HSC; top three images) and granulocyte–macrophage progenitors (GMP; bottom three images) representative of five controls, three patients with CML in the accelerated phase (AP), and four patients with CML in blast crisis (BC). Panel C shows the localization of β-catenin in hematopoietic stem cells representative of imatinib-resistant CML, granulocyte–macrophage progenitors representative of imatinib-resistant CML, and an isotype control. Histograms in Panel D show the relative percentage of cells with the expression of LEF/TCF–green fluorescent protein (GFP) reporter in hematopoietic stem cells and granulocyte–macrophage progenitors from controls and patients with CML in blast crisis. In the histogram on the right, untransduced granulocyte–macrophage progenitors from a patient with CML in blast crisis was used as a control (green).

SELF-RENEWAL OF CML GRANULOCYTE–MACROPHAGE PROGENITORS

We assessed whether granulocyte–macrophage progenitors in CML gained the high proliferative potential and self-renewal capacity of hematopoietic stem cells as a result of β-catenin activation.[27-32] $CD34^{+}Lin^{-}$ cells from patients with CML in the accelerated phase that were transduced with lentiviral β-catenin formed colonies that were larger than normal, whereas transduction of the cells with axin decreased the size of the colonies (Fig. 4A; and Fig. 5 in the Supplementary Appendix). In addition, enforced expression of β-catenin provided normal granulocyte–macrophage progenitors with self-renewal capacity in a replating assay (Fig. 4B). CML granulocyte–macrophage progenitors, but not normal granulocyte–macrophage progenitors, had a high replating capacity and retained the capacity to form myeloid colonies in colony-forming cell assays (Fig. 4C). Finally, transduction of CML granulocyte–macrophage progenitors with axin, an inhibitor of β-catenin signaling, reduced the replating capacity of leukemic cells (Fig. 4C).

DISCUSSION

CML is believed to arise as a consequence of the clonal expansion of hematopoietic stem cells that express the *BCR-ABL* fusion gene.[13-15,18-24] However, recent work with transgenic mouse models of CML showed that although *BCR-ABL* is necessary for the development of a myeloproliferative syndrome resembling CML, hematopoietic stem cells need not be involved.[8] Moreover, additional genetic or epigenetic events are required for progression



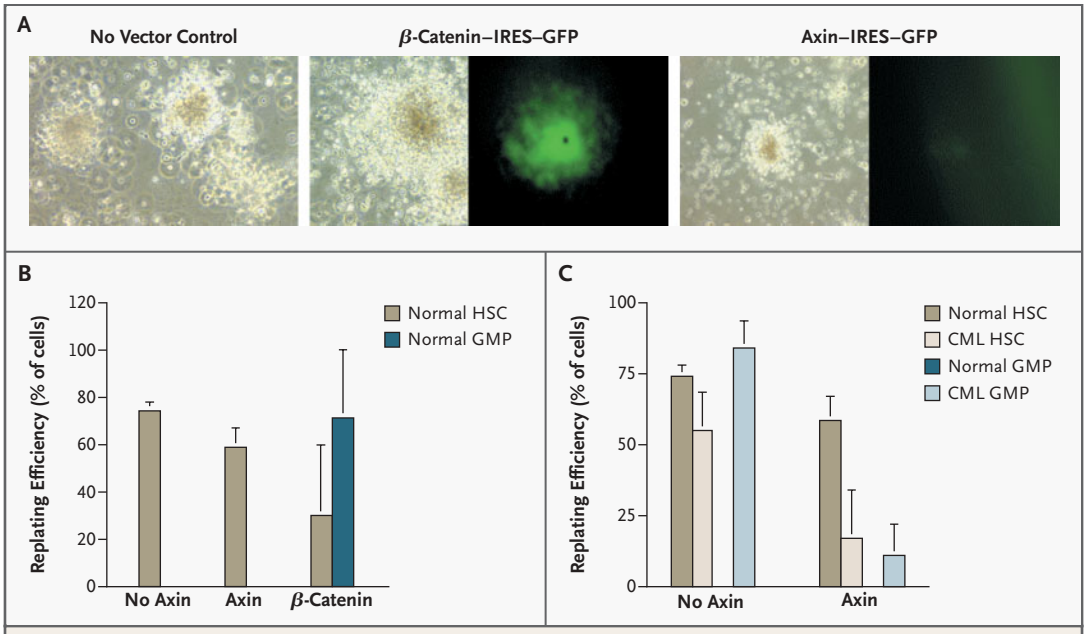


Figure 4. Phase-Contrast Photomicrographs (Panel A) and Replating Efficiency of Cells (Panels B and C).

Panel A shows phase-contrast photomicrographs of colonies of untransduced accelerated-phase CML, colonies derived from cells transduced with β-catenin–internal ribosome entry site in viral RNA (IRES)–green fluorescent protein (GFP), and colonies derived from cells transduced with axin–IRES–GFP. Panel B shows the replating efficiency of individual colonies derived from hematopoietic stem cells (HSC) and granulocyte–macrophage progenitors (GMP) from three control subjects in the presence or absence of lentivirally enforced expression of axin and β-catenin. Panel C shows the replating efficiency of single colonies derived from hematopoietic stem cells and granulocyte–macrophage progenitors from three controls and three patients with CML before and after transduction with a lentiviral axin–GFP vector.[1]

to blast crisis.[8-10,15,16] In humans and mice, hematopoietic stem cells are the only normal progenitors that renew themselves,[38-45] and therefore, they are widely considered to be the only cells in the marrow in which preleukemic changes can accumulate, whether by genetic or epigenetic means. However, it is also possible that a downstream progenitor can acquire self-renewal capacity.[24-27,41] It is therefore important to identify the population that contains leukemic stem cells in CML and other myeloid leukemias and to identify events leading to the progression of leukemia, the outcomes of these events, and the order of their appearance in leukemic stem cells and their precursors.[24-26]

Several of our findings in CML cells were unanticipated. First, progression to blast crisis was associated with expansion of the myeloid progenitor fraction, which consists mainly of granulocyte–macrophage progenitors, rather than expansion of the pool of hematopoietic stem cells. Second, *BCR-ABL* amplification occurred in granulocyte–macrophage progenitors, whereas the *BCR-ABL* transcript levels in hematopoietic stem cells remained relatively constant during progression of the disease. Third, the β-catenin pathway was activated in granulocyte–macrophage progenitors from patients with CML in accelerated phase and from those with CML in blast crisis, as well as in patients with imatinib-resistant CML. Fourth, granulocyte–macrophage progenitors in CML have self-renewal capacity, at least in vitro, although our data do not exclude the possibility that this capacity may be more limited than that of normal hematopoietic stem cells in vivo. Fifth, β-catenin–mediated renewal of granulocyte–macrophage progenitors in CML may be inhibited by enforced expression of axin — a potent and highly specific β-catenin antagonist.

We propose that in chronic-phase CML, there is an expansion of the progenitor pool and the downstream progeny of these progenitors that is due in part to the expression of the *BCR-ABL* fusion gene. This expansion causes the myeloproliferative syndrome (chronic-phase CML), but apoptosis and differentiation pathways remain intact. Progression of

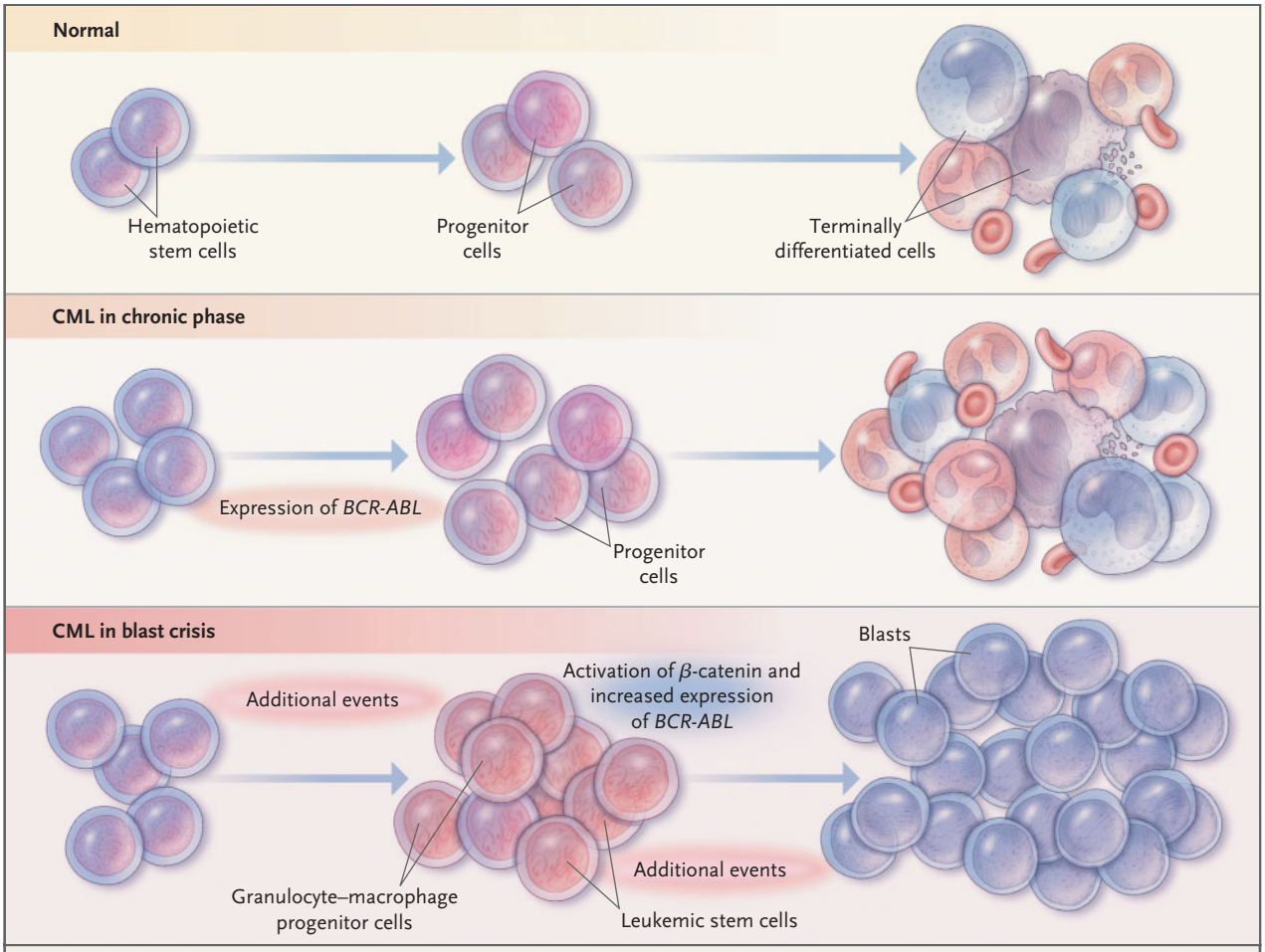


Figure 5. Model of the Role of Activated β-Catenin in the Progression of CML.

Unlike the progenitor pool in healthy subjects, in patients with chronic-phase CML, cells within the progenitor pool have an increased proliferative capacity owing to elevated expression of *BCR-ABL*. This increased proliferative capacity leads to a myeloproliferative syndrome but does not affect the pathways of cell death and differentiation. Progression to blast crisis results from additional events, including the activation of β-catenin in the granulocyte–macrophage progenitor population, increasing their proliferative and self-renewal capacity and possibly allowing them to become leukemic stem cells. Avoidance of cell death, evasion of innate and adaptive immune responses, and a block in differentiation must also occur for CML to progress.

CML to blast crisis probably involves several events in primitive progenitors, including *BCR-ABL* amplification, acquisition of resistance to apoptosis, genomic instability, escape from innate and adaptive immune responses, and activation of β-catenin in granulocyte–macrophage progenitors, resulting in the acquisition of self-renewal capacity (Fig. 5).[15,16,24-26,52] Our in vitro replating data indicate that the activation of the self-renewal process by means of the β-catenin pathway in granulocyte–macrophage progenitors, which normally have no capacity for self-renewal, may not only lead to expansion of the granulocyte–macrophage progenitor pool but also play a role in the subsequent production of blasts that occurs in advanced phases of CML, although such blasts may be nonmalignant progeny of leukemic stem cells, rather than leukemic stem cells themselves.

The cause of activation of the β-catenin pathway in CML is unknown, and whether β-catenin or other components of the signaling pathway and $p210^{BCR-ABL}$ interact directly has yet to be determined. Transcriptional targets of β-catenin/LEF-1 signaling, including the cyclin D1 and *c-myc* genes, also play a critical role in transformation mediated by Abl, a member of the Src kinase family of proto-

oncogenes, and by BCR-ABL; thus, these genes may provide a final common pathway between *BCR-ABL* and β-catenin leading to the progression of CML.[53,54] Additional members of the Src family of kinases have also been shown to augment the activation of β-catenin.[55,56] Other proto-oncogenes and tumor suppressors, such as *bmi-1*, *HOXB4*, and *notch*, also play a role in the self-renewal of hematopoietic stem cells and may interact with β-catenin during leukemogenesis.[27,46,47,57,58] Detection of activated β-catenin in highly purified CML progenitors might be used to predict progression, relapse, or the development of imatinib resistance. Moreover, components of the β-catenin–signaling pathway, especially ones that are mutated, may provide new targets for the development of molecular and immune therapies for CML.

Supported by grants (CA55209 and CA86017, to Dr. Weissman; 2PO1CA49605, to Ms. Jones and Dr. Zehnder; and K23 HL04409, to Dr. Gotlib) from the National Institutes of Health, a de Villiers grant from the Leukemia Society (to Dr. Weissman), a Stanford Center for Clinical Immunology Yu–Bechmann fellowship for Genomics and Oncology (to Dr. Jamieson), the Walter and Beth Weissman Fund, the Smith Family Fund, a Leukemia/Lymphoma Society Special Fellowship (to Dr. Ailles), and an American Cancer Society Edward Albert Bielfelt Postdoctoral Fellowship (to Dr. Dylla). Dr. Dylla is the recipient of a Stanford Immunology Fellowship (5T32AI07290) funded by the National Institutes of Health. Dr. Sawyers is an Investigator of the Howard Hughes Medical Institute and a Doris Duke Distinguished Clinical Scientist.

Drs. Jamieson, Ailles, and Weissman have applied for a U.S. patent entitled "Use of Beta-Catenin in the Diagnosis and Treatment of Leukemia and Lymphomas" through the Stanford University Office of Technology and Licensing. Dr. Weissman receives consulting fees from and has equity ownership or stock options in Cellerant.

We are indebted to Drs. S. Schrier, L. Leung, L. Boxer, R. Negrin, J. Shizuru, G. Beilhack, R. Nusse, F. Staal, and W.J. Nelson for indispensable advice; to R. Falkow and Dr. P. Lee at Stanford and Dr. N. Shah at UCLA for providing samples from patients with CML; to L. Jerabek for excellent laboratory management; to the Stanford Cell Sciences Imaging Facility and the Stanford FACS facility for expert assistance; to members of the Center for Clinical Immunology at Stanford as well as the Division of Bone Marrow Transplantation at Stanford for their support; and to the patients who made this research possible.

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