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A murine model of CML blast crisis induced by cooperation between BCR/ABL and NUP98/HOXA9

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Constitutive activation of tyrosine kinases, such as the BCR/ABL fusion associated with t(9;22)(q34;q22), is a hallmark of chronic myeloid leukemia (CML) syndromes in humans. Expression of BCR/ABL is both necessary and sufficient to cause a chronic myeloproliferative syndrome in murine bone marrow transplantation models, and absolutely depends on kinase activity. Progression of CML to acute leukemia (blast crisis) in humans has been associated with acquisition of secondary chromosomal translocations, including the t(7;11)(p15;p15) resulting in the NUP98/HOXA9 fusion protein. We demonstrate that BCR/ABL cooperates with NUP98/HOXA9 to cause blast crisis in a murine model. The phenotype depends both on expression of BCR/ABL and NUP98/HOXA9, but tumors retain sensitivity to the ABL inhibitor STI571 *in vitro* and *in vivo*. This paradigm is applicable to other constitutively activated tyrosine kinases such as TEL/PDGFβR. These experiments document cooperative effects between constitutively activated tyrosine kinases, which confer proliferative and survival properties to hematopoietic cells, with mutations that impair differentiation, such as the NUP98/HOXA9, giving rise to the acute myeloid leukemia (AML) phenotype. Furthermore, these data indicate that despite acquisition of additional mutations, CML blast crisis cells retain their dependence on BCR/ABL for proliferation and survival.

Chronic myeloid leukemia (CML) syndromes in humans are phenotypically characterized by leukocytosis with normal maturation and differentiation of myeloid lineage cells. Cloning of recurring chromosomal translocations associated with the CML phenotype has invariably identified constitutively activated tyrosine kinases that are expressed as a consequence of the translocation. The most common of these is the t(9;22)(q34;q22) associated with the *BCR/ABL* fusion transcript (1). Other examples include the t(5;12)(q33;p13), t(9;12)(q34;p13), t(9;12)(p24;p13), and t(5;10)(q33;q11.2) translocations associated with the *TEL/PDGFβR* (2), *TEL/ABL* (3), *TEL/JAK2* (4), and *H4/PDGFβR* (5, 6) fusions, respectively. Expression of these constitutively activated tyrosine kinases in a murine bone marrow transplantation assay system is both necessary and sufficient to cause a myeloproliferative disease in animals that has many of the features of CML in humans (7–9). The phenotype includes leukocytosis, splenomegaly, and extramedullary hematopoiesis with normal maturation and differentiation of myeloid lineage cells. Transformation is dependent on kinase activity, as demonstrated by point mutations that abrogate kinase activity, as well as by ABL and PDGFβR kinase-specific inhibitors *in vitro* and *in vivo* (8, 10–15).

In contrast to chronic myeloid leukemias, cloning of recurrent chromosomal translocation breakpoints in acute leukemias has identified fusion genes that encode transcription factors or transcription regulatory proteins such as *CBFB/MYH11* (16), *PML/RAR* (17), *MLL/CBP* (18), *MLL/ENL* (19) or *NUP98/HOXA9* (20, 21). Expression of certain of these transcription factor fusions in murine bone marrow transplantation assays or transgenic systems may result in acute myeloid leukemia after latency of 6–12 months, indicating a requirement for additional cooperating mutations. Although the functional contribution of these fusion proteins to the leukemic phenotype is not completely understood, many impair normal hematopoietic development (22–27). In addition, none of the aforementioned fusion genes is sufficient to cause acute leukemia in murine models. We hypothesize that one class of mutations, exemplified by constitutively activated tyrosine kinases in CML, confer a proliferative and/or survival advantage but do not affect differentiation. A second class of mutations, involving mutations in hematopoietic transcription factors, impairs hematopoietic differentiation.

Disease progression from CML to AML (or CML "blast crisis") in patients provides indirect support for this hypothesis. *BCR/ABL*-positive CML may progress to AML with the acquisition of either t(7;11)(p15;p15) or t(3;21)(q26;q22) associated with expression of the NUP98/HOXA9 fusion (20, 21, 28–30) or the AML1/EVI1 fusion (31–33), respectively. In other examples, progression of t(5;12) CML with the *TEL/PDGFR* fusion to AML may be associated with the acquisition of the t(8;21)(q22;q22) translocation generating the AML1/ETO fusion protein (2).

Here we report a murine model of CML blast crisis mediated by a cooperation between *BCR/ABL*, a constitutively activated tyrosine kinase, and the *NUP98/HOXA9* fusion. The tumors retain sensitivity to the ABL inhibitor STI571. This model demonstrates that AML although genetically complex, may retain dependence on an initial oncogenic event. This has important implications for the use of tyrosine kinase inhibitors in the therapy of *de novo* AML.

Materials and Methods

Constructs and Cloning. An *Eco*RI fragment from *pBSNUP98/HOXA9* (20) containing the entire coding sequence of the *NUP98/HOXA9* fusion gene was cloned into the *Eco*RI site of the retroviral bicistronic expression vector *MSCV-IRES-EGFP* (a kind gift from W. Pear, University of Pennsylvania, Philadelphia). *MSCV-IRES-EGFP* without insert was used as a vector control. *p210 BCR/ABL*, *TEL/PDGFβR* (8), and *mutSTAT5a* (9) were all cloned into the retroviral vector *MSCVneo* (kindly provided by R. Hawley, Red Cross, Rockville, MD).

Viral Supernatants and Bone Marrow Reconstitution Assay. Retroviral supernatants were generated and bone marrow transplants were performed as described (9), except that retroviral transduction of bone marrow cells was done by spinoculation (34). Viral titers of the *MSCV-NUP98/HOXA9* or the *MSCV-IRES-EGFP* viral stocks were determined by assaying for EGFP expression 48 h post transduction by flow cytometry. Equal titers of the *MSCV-NUP98/*

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Abbreviations: CML, chronic myeloid leukemia; AML, acute myeloid leukemia; BA/NH, BCR/ABL-NUP98/HOXA9.

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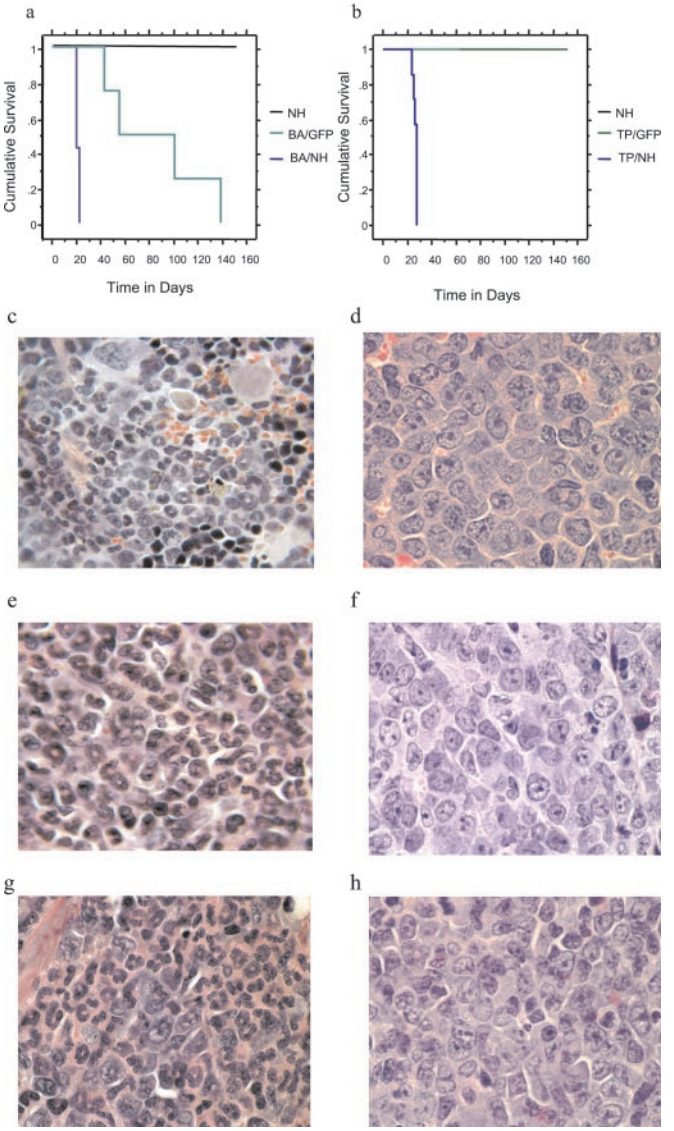

Fig. 1. Activated tyrosine kinase fusions cooperate with NUP98/HOXA9 (NH) to induce an acute myeloid leukemia with features of CML blast crisis. (*a* and *b*) Kaplan–Meier survival analysis of mice that have undergone bone marrow transplantation. Mice transplanted with bone marrow that was transduced with *NH* alone did not develop disease with >5 months of follow up (4 mice in cohort). In contrast, bone marrow cotransduced with *BCR/ABL* (*BA*) and *NH* induced an acute myeloid leukemia with a latency of 21 days (12 mice in cohort). Under these experimental conditions, BA alone induced a myeloproliferative disease with a latency of 5–17 weeks (6 mice in cohort). Viral supernatants were titered to ensure transduction of 10% and 1% of cells by *NUP98/HOXA9* and *BCR/ABL*, respectively. Thus, under these conditions only 0.1% of cells would be doubly transduced with both retroviruses. The amount of the *MSCVneo-BCR/ABL* supernatant used in the control experiments, as well as the *BA/NH* synergy experiments, was 10% of the amount that reproducibly caused a short latency rapid myeloproliferative disease in control mice (data not shown). (*b*) Cotransduction with *TEL/PDGFβR* (*TP*) and *NH* induced an acute myeloid leukemia with a latency of 25 days, whereas under these experimental conditions *TP* alone gave no disease with >8 weeks of follow-up. The control *NH* mice indicated in *b* are the same as those represented in *a*. (*c–h*) Histopathological analysis of spleens derived from transplant recipients. (*c*) Spleens from animals reconstituted with marrow transduced with *NH* alone killed at 21 days posttransplant as a control, and (*d*) at 7 months post transplantation, with onset of severe clinical disease. (*e*) *BA* + *MSCV-IRES-GFP* vector control killed at 21 days (before onset of full-fledged disease) to serve as a control for (*f*) *BA+NH* animal killed at 21 days, with onset of severe clinical disease. (*g*) *TP* + *MSCV-IRES-GFP* vector control killed at 24 days (before onset of full-fledged disease) as a control for (*h*) *TP+NH* animal killed at 24 days at onset of severe clinical disease. Spleen sections were stained with hematoxylin/eosin.

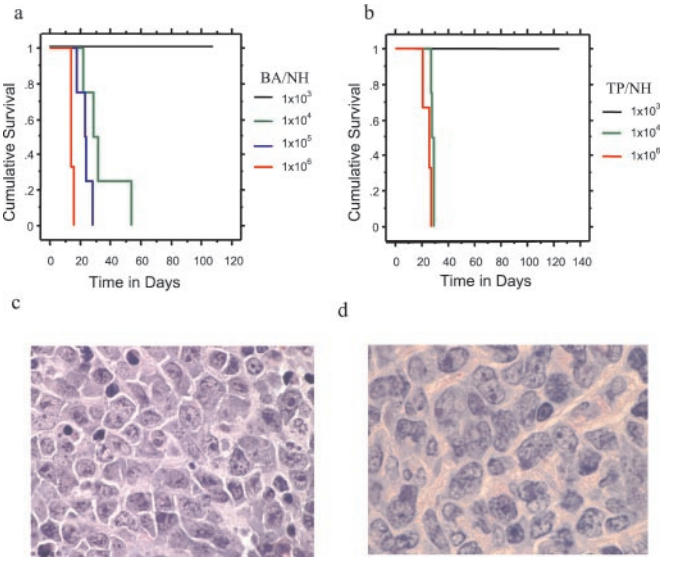

Fig. 2. Limiting dilution experiments demonstrate transplantability of BA+NH- and TP+NH-induced disease. (*a* and *b*) Kaplan–Meier survival analysis of secondary recipient mice. Secondary mice received 10^6, 10^5, 10^4, or 10^3 cells from the primary donor mouse. Eight recipient mice were used for dilutions 10^6–10^4, whereas four mice received 10^3 cells. As few as 10^4 spleen cells from a mouse with either (*a*) BA/NH or (*b*) TP/NH disease, when transplanted into secondary, sublethally irradiated recipient mice, was sufficient to recapitulate the primary disease. The histopathologic analysis of spleens from the secondary recipients was similar to that seen in primary transplant recipients [BA/NH (*c*), TP/NH (*d*)], consisting of a diffuse proliferation of myeloid blast forms with a myeloid maturation arrest.

HOXA9 or the *MSCV-IRES-EGFP* viral stocks were used in all of the experiments. The amount of the *MSCVneo-BCR/ABL* supernatant used in these experiments was 10% (i.e., 100 μl) of the amount (i.e., 1,000 μl) that reproducibly caused a short latency rapid myeloproliferative disease in control experiments (data not shown). For the secondary bone marrow transplants, frozen spleen cells from the donor mice were thawed, then specified numbers of viable cells were injected into sublethally irradiated [1 × 450 centigray (cGy)] BALB/c mice. At least two independent transduction/transplantation experiments were carried out for each of the combinations of genotypes with similar results, and the data combined to generate the survival curves. Kaplan–Meier survival analyses were performed using the program STATVIEW (SAS Institute, Cary, NC).

DNA Isolation and Southern Analysis. Genomic DNA was isolated from various tissues by using a PUREGENE DNA isolation kit (Gentra Systems, Minneapolis). For analysis of proviral integrations, genomic DNA was digested with *Xba*I. For assay of clonality,

Animals transduced with *NH* alone showed splenic morphology typical of posttransplant control mice with a mild degree of extramedullary hematopoiesis at 21 days (*c*). At 7 months, however (*d*), myeloid maturation was not seen and a proliferation of myeloid blast forms was present consistent with the development of myeloid leukemia [as has been reported (27)]. Animals transplanted with marrow transduced with *BA* (*e*) or *TP* (*g*) alone developed a myeloproliferative disease with varying degrees of myeloid maturation and extensive extramedullary hematopoiesis, particularly in the case of *BA*. In sharp contrast both *BA* and *TP*, when cotransduced with *NH* (*f* and *h*, respectively), induced a morphologically identical short latency disease demonstrating a myeloid maturation arrest and a sheet-like proliferation of myeloid blast forms, with a diminished degree of extramedullary hematopoiesis. Similarly, bone marrow and liver demonstrated extensive replacement by myeloid blast forms (data not shown).

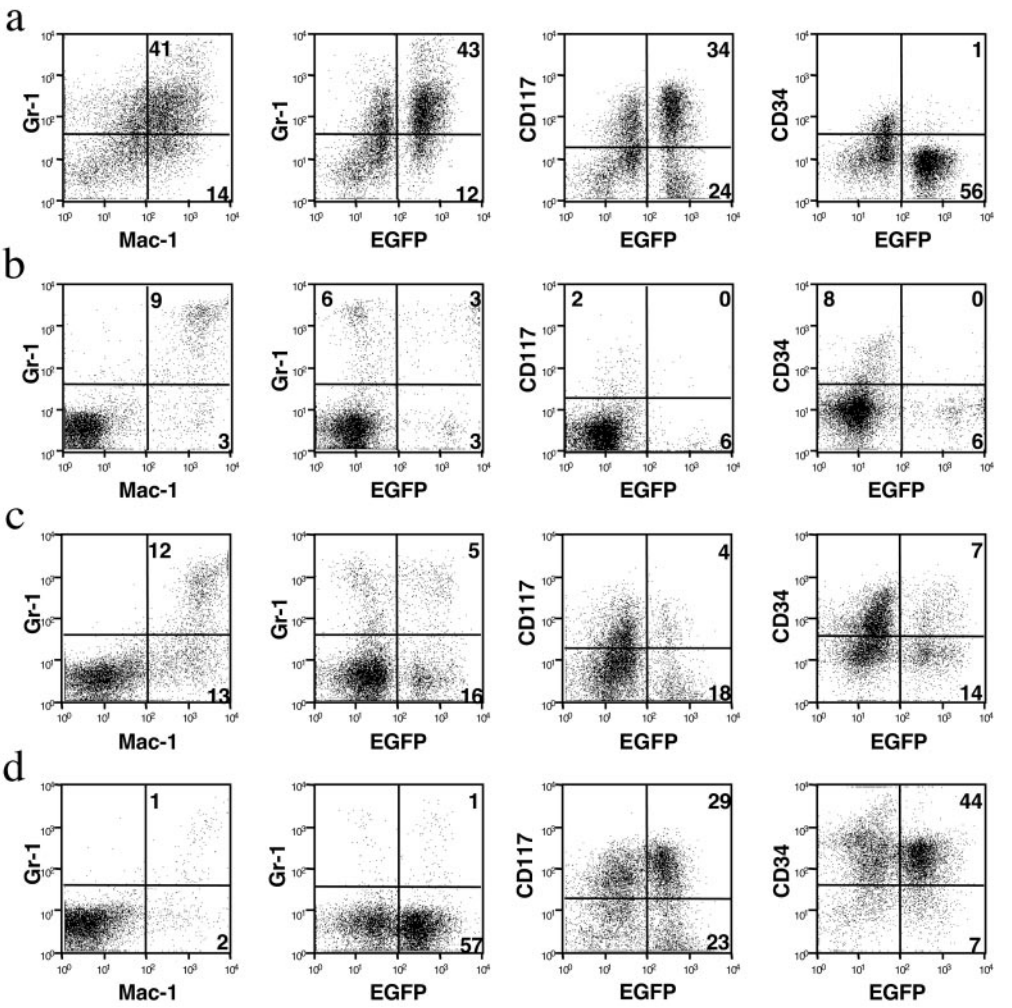

Fig. 3. Mice receiving transplants of bone marrow cells transduced with both *BA* and *NH* fusion genes develop an acute myeloid leukemia that includes a population of immature cells displaying stem cell markers. Spleen cells from mice that received primary or secondary bone marrow transplants were stained with APC-conjugated anti-Gr-1 and PE-conjugated anti-Mac-1, PE-conjugated anti-CD117, or biotin-conjugated anti-CD34 followed by APC-conjugated streptavidin; results presented as dot plots. (*a*) *NH* primary transplant; (*b*) *BA* + *MSCV-IRES-GFP* vector control transplant; (*c*) *BA*/*NH* primary transplant; (*d*) *BA*/*NH* secondary transplant receiving 1×10^6 cells. The numbers in the upper right and lower right quadrants represent the percentages of the total number of gated cells present in those quadrants. A schematic representation of the viral constructs used in the cotransductions is given in Fig. 4. The NH primary transplant animal was killed 7 months post-transplant after the onset of clinical disease. The *BA*/*NH* primary transplant animal (*c*) was killed at 21 days after bone marrow transplant after onset of disease, at which time the *BA* + *MSCV-IRES-GFP* vector control animal (*b*) was also killed as a control.

genomic DNA was digested with *Eco*RI. Hybridization probes were the cDNAs for either Neo^R or *EGFP* genes.

Flow Cytometric Immunophenotyping. Single-cell suspensions from spleen, blood, and bone marrow were prepared as described (9). Aliquots of 0.5 to 2.0×10^6 cells were stained for 20 min on ice with APC-conjugated anti-Gr-1, PE-conjugated anti-Mac-1, PE-conjugated anti-CD117, biotin-conjugated anti-CD19, biotin-conjugated anti-CD34 (PharMingen), APC-conjugated anti-CD4, or APC-conjugated anti-CD8 (Caltag, South San Francisco, CA). APC-conjugated streptavidin (Caltag) was used as a secondary reagent to detect the binding of the biotinylated primary antibody. Multicolor flow cytometric analysis was done with a FACSort (Becton-Dickinson). A minimum of 10,000 events was acquired and the data were analyzed using CELLQUEST software (Version 3.1, Becton-Dickinson).

Histopathology. Murine tissues were fixed for at least 72 h in 10% neutral buffered formalin (Sigma), dehydrated in alcohol, cleared in xylene, and infiltrated with paraffin. Four-micrometer-thick tissue sections from paraffin embedded tissue blocks were placed on charged slides, deparaffinized in xylene, rehydrated through graded alcohol solutions, and stained with hematoxylin/eosin.

Primary Cell Cultures and Clonal Cell Lines. Spleen cells were initially grown in IMDM medium containing 10% FBS, 10 ng/ml, respectively, of recombinant murine Interleukin-3 (R & D Systems), recombinant human interleukin-6 (PeproTech, Rocky Hill, NJ), and recombinant murine granulocyte–macrophage colony-stimulating factor (GM-CSF, R & D Systems), and 100 ng/ml recombinant murine stem cell factor (SCF, R & D Systems). Growth factors were sequentially deleted from the growth medium until the cells were growing independent of growth factors. Clonal cell lines were established by limiting dilution.

Methylcellulose Assay and STI571 Treatment. Spleen cells (2.5×10^5) and serial dilutions of STI-571 were mixed with MethoCult M3334 (StemCell Technologies, Vancouver). Assays were plated in 35-mm dishes in triplicate and the colonies were scored at day 7. To test the ability of STI571 treated cells to cause disease when transplanted into recipient mice, 10^4 cells from the NH/BA cell culture were incubated for 48 h with 0.3 μM STI571, 1 μM STI571, or DMSO alone. The cells were then harvested and injected into sublethally irradiated (1×450 cGy) BALB/c mice. To test the ability of STI571 to abrogate disease *in vivo*, 1×10^5 cells from the BA/NH cell culture were transplanted into recipient mice that were then treated with STI571 by gavage every 12 h for 29 days (50 mg/kg in PBS). Treatment was started 5 days posttransplant. The placebo group received PBS alone.

Results

BCR/ABL and NUP98/HOXA9 Cooperate to Induce an Acute Myeloid Leukemia. To determine whether NUP98/HOXA9 cooperated with BCR/ABL to cause an acute myeloid leukemia, we cotransduced murine bone marrow cells with murine ecotropic retrovi-

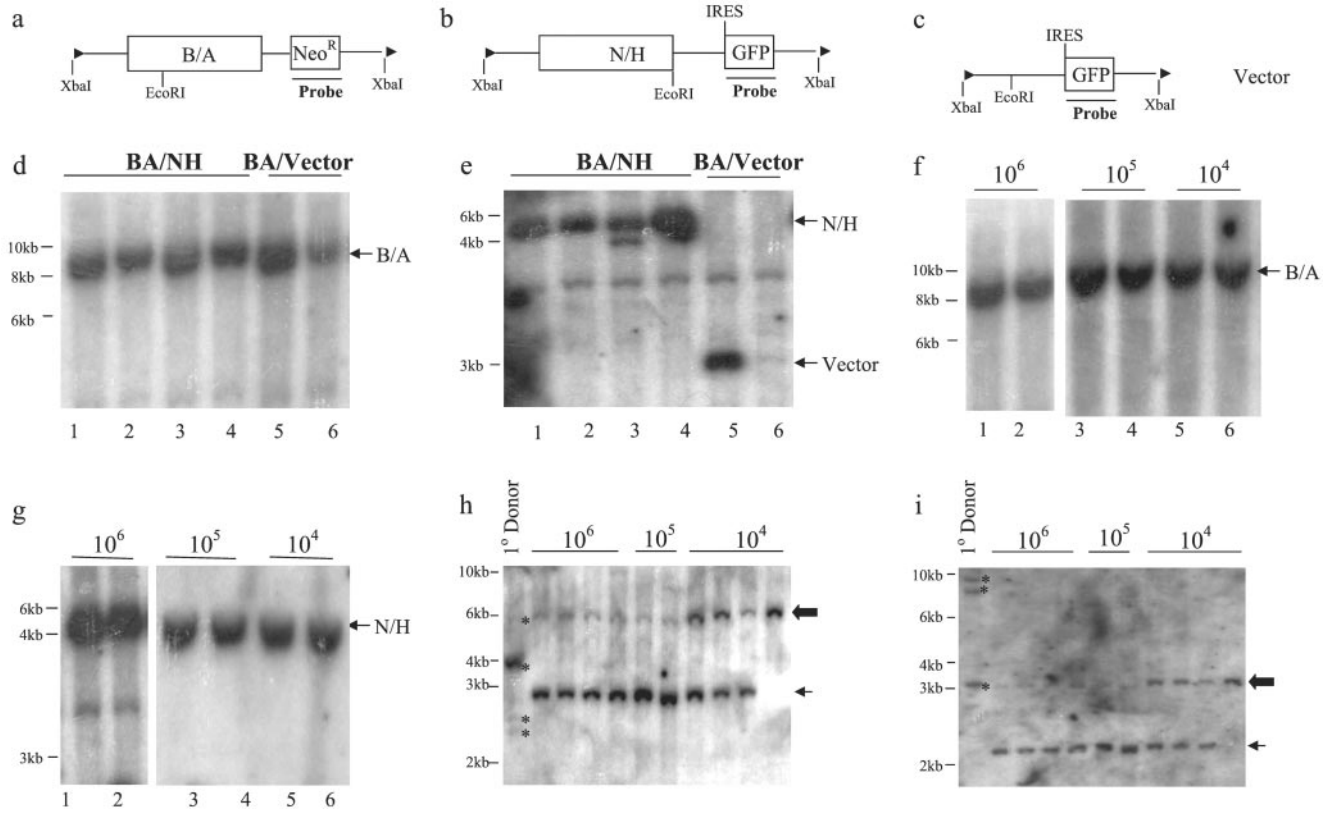

Fig. 4. Both BCR/ABL (BA) and NUP98/HOXA9 (NH) are required to generate the acute myeloid leukemia phenotype. (*a–c*) Schematic representation of the retroviral constructs used in the bone marrow transplant assays. *MSCVneoBCR/ABL* (*a*; ref. 45), *MSCV-IRES-GFP NUP98/HOXA9* (*b*), and *MSCV-IRES*-GFP (37) control vector (*c*). The restriction endonuclease sites used in the Southern blot analysis of integration and clonality are indicated. For Southern blot analysis of proviral integration (*d–g*), genomic DNA from the primary BA+NH mice (*d* and *e*) and secondary BA+NH (*f* and *g*) was cleaved with *Xba*I and probed for *BA* integration with either *Neo*^{R} (*d* and *f*) or for *NH* integration with *EGFP* (*e* and *g*) probes. Each lane represents an individual animal. Secondary mice received 10^6, 10^5, or 10^4 cells from the primary donor mouse. These data demonstrate the presence of both *BA* and *NH* provirus in leukemic cells. For Southern blots assaying clonality (*h* and *i*), genomic DNA from primary and secondary leukemic mice was cleaved with *Eco*RI and probed for *BA* clonality with *Neo*^{R} (*h*) or *NH* clonality with *EGFP* (*i*). Each lane represents an individual animal. In limiting dilution experiments, secondary mice received 10^6, 10^5, or 10^4 cells from primary donors. Primary disease was oligoclonal in nature, with several independent clones indicated by asterisks, whereas the secondary recipients had either a biclonal or a monoclonal disease. The presence of a single clonal integrant for *BA* and *NH*, respectively, at the limit dilution of 10^4 cells demonstrates the requirement for integration of both *BA* and *NH* provirus for the acute leukemia phenotype. The large arrow points to the predominant leukemic clone selected for by limit dilution, whereas the small arrow points to additional leukemic clones seen in most of the secondary transplants.

ruses encoding the *BCR/ABL* or *NUP98/HOXA9* fusion genes, respectively. Transduced bone marrow cells were then introduced into syngeneic, lethally irradiated, recipient mice, as described (9). Bone marrow cells cotransduced with *BCR/ABL* and *NUP98/HOXA9* retrovirus induced an acute myeloid leukemia with a latency of 21 days and with features of myeloid blast crisis of CML (Fig. 1 *a* and *f*). The histopathologic features of the leukemia included a myeloid maturation arrest with a concomitant proliferation of myeloid blast cells in the spleen (Fig. 1*f*), liver, and bone marrow (data not shown). We identified these cells as immature myeloid cells displaying the stem cell markers CD34 and CD117 (Fig. 3*c*). These cells stained negative for lymphoid markers CD4, CD8, and CD19, confirming their myeloid nature (data not shown). Ten thousand cells obtained from primary recipient mice were transplantable into secondary recipients, and caused a fatal acute myeloid leukemia with a latency of 2 weeks (Fig. 2*a*). AML in secondary recipients had a shift toward a more immature phenotype, as shown histopathologically (Fig. 2*c*) and by flow cytometry as evidenced by increased numbers of $CD34^+$ and $CD117^+$ cells (Fig. 3*d*). In control experiments, bone marrow cells were also transduced with retroviruses encoding either fusion gene alone. Under the conditions used for this experiment, BCR/ABL induced a myeloproliferative disease with a latency of 5–17 weeks and normal myeloid maturation, and comprised predominantly of neutrophils as described (ref. 7; see Fig. 1 *a* and *e*). *NUP98/HOXA9* alone gave an AML phenotype, but with markedly prolonged latencies (Fig. 1 *a* and *d*). This observation is consistent with previous reports that *NUP98/HOXA9* requires additional mutations to cause AML (27).

Limiting Dilution Experiments Demonstrate the Requirement for Both *BCR/ABL* and *NUP98/HOXA9* Provirus. Cooperation between the two fusion genes in the generation of AML would predict a selective growth advantage for rare double transductants versus cells transduced with a single retrovirus. Therefore, the cotransduction experiments were designed using low titer retroviral supernatants such that a fraction of bone marrow cells would be transduced with either the *BCR/ABL* or the *NUP98/HOXA9* retrovirus, whereas only rare cells would be transduced with both retroviruses. The amount of the *MSCVneo-BCR/ABL* supernatant used in these experiments was 10% of the amount that reproducibly caused a short latency rapid myeloproliferative disease in control mice (see Figs. 6–8, which are published as supporting information on the PNAS web site, www.pnas.org). There was purposefully no selection for doubly transduced cells using either flow cytometry or antibiotic selection. Thus, the observation that cotransduction resulted in a short latency acute myeloid leukemia phenotype that was readily distinguishable from the phenotype observed with

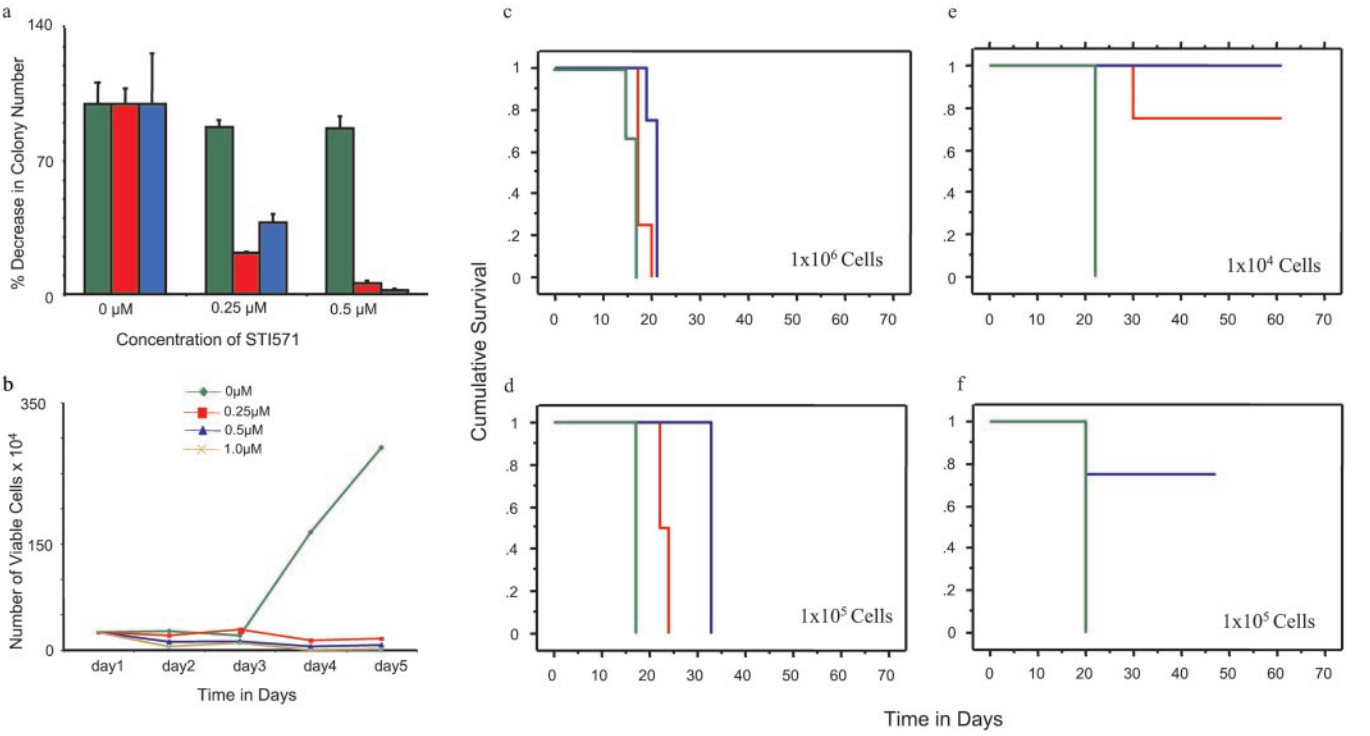

Fig. 5. STI571 inhibits growth of BA/NH cells in culture and abrogates their leukemogenic potential *in vivo*. (*a*) Cells (1×10^4) from the mutSTAT5a/NH control (green bars), primary BA/NH (red bars), or the secondary BA/NH (blue bars) splenic cell cultures were seeded in methylcellulose medium containing 0 μM, 0.25 μM, or 0.5 μM STI571. MutStat5a (37) is a constitutively activated mutant of Stat5a that is insensitive to the effects of STI571 and serves as a control for nonspecific toxicity (35). Colonies were counted after 7 days. Two independent experiments were performed in triplicate. (*b*) The ability of the BANH cells to grow in liquid culture in the presence of STI571 was tested. BA/NH cells (1×10^5) were seeded in RPMI medium containing 0 μM (green line), 0.25 μM (red line), 0.5 μM (blue line), and 1.0 μM (yellow line) STI571. The cell numbers plotted represent the viable cells in each population over a period of 5 days. (*c–e*) Kaplan–Meier survival curves for mice transplanted with 10^4, 10^5, and 10^6 cells from the BA/NH cell cultures. The transplanted cells were incubated for 48 h in the presence of 0.3 μM STI571 (IC_{50}, red line), 1 μM STI571 (IC_{90}, blue line), or DMSO alone (green line) before being transplanted into the secondary recipients. (*f*) Kaplan–Meier survival curves of secondary recipient mice that initially received 1×10^5 BA/NH cells and were then treated with STI571 by gavage (blue line) or a vehicle (green line).

transduction of either fusion gene alone provided strong support for cooperativity between *BCR/ABL* and *NUP98/HOXA9*. To confirm that cooperativity between *BCR/ABL* and *NUP98/HOXA9* was essential for the disease phenotype, secondary transplants were performed using 10^6, 10^5, 10^4, or 10^3 primary leukemic cells transplanted into sublethally irradiated secondary recipient mice. Limiting dilution experiments showed that as few as 10^4 cells could recapitulate the blast crisis phenotype in secondary recipient animals, whereas no disease was observed when 10^3 or fewer cells were transplanted (Fig. 2*a*). These data indicated that 10^4 cells from primary BMT recipients contained ≈1 leukemia repopulating cell. Southern blot analysis was performed with Neo^R and *EGFP* c-DNA probes, respectively, on spleen cells derived from diseased secondary recipient animals. These blots (Fig. 4 *d–g*) confirmed that leukemic cells transplanted at limit dilution contained both the *BCR/ABL* and *NUP98/HOXA9* proviruses. Furthermore, analysis using restriction enzymes that cleave once within the proviral insert demonstrated monoclonality of these leukemic cells, confirming limit dilution in these assays (Fig. 4 *h* and *i*). Similar analysis of clonal cell lines generated *in vitro* from the spleens of primary leukemic mice demonstrated that *BCR/ABL* and *NUP98/HOXA9* fusion genes were present in the same cell (data not shown). Taken together, these data provide convincing evidence for cooperativity between BCR/ABL and NUP98/HOXA9 in a cotransduction system that allows for biological selection for rare doubly transduced murine bone marrow cells.

***TEL/PDGFβR* and *NUP98/HOXA9* also Cooperate to Induce an Acute Myeloid Leukemia.** To determine whether the cooperation between the constitutively activated BCR/ABL tyrosine kinase and NUP98/HOXA9 could be generalized to other tyrosine kinase fusions, similar cotransduction experiments were performed using a *TEL/PDGFGβR* retrovirus. *TEL/PDGFβR* cotransduced with *NUP98/HOXA9*, using a strategy similar to that described above, also resulted in a myeloid blast crisis phenotype indistinguishable from the *BCR/ABL* + *NUP98/HOXA9* phenotype (Figs. 1 *b*, *e*, and *f*, and 2 *b* and *d*). These data indicate that the paradigm for cooperation between *BCR/ABL* and *NUP98/HOXA9* can be extended to other tyrosine kinase fusion genes.

BCR/ABL-NUP98/HOXA9 Leukemic Cells Retain Sensitivity to STI571. Progression of CML to blast crisis is usually a fatal complication of the disease. There are few effective therapies, and even intensive chemotherapy and allogeneic stem cell transplantation are usually ineffectual. To determine whether cells in the murine model of CML blast crisis remained dependent on the ABL kinase despite acquisition of the additional *NUP98/HOXA9* mutation, we assayed the ability of STI571, an ABL kinase inhibitor of the 2-phenylaminopyrimidine class (35, 36), to impair growth of BCR/ABL-NUP98/HOXA9 leukemic cells. As shown in Fig. 5*a*, these cells demonstrated sensitivity to STI571 in methylcellulose colony forming assays. Control experiments demonstrated that cells cotransduced with a constitutively activated mutant of STAT5 (37), which is not inhibited by STI571, and NUP98/HOXA9 were insensitive to STI571. *Ex vivo* and *in vivo* experiments demonstrated that treatment of BA/NH leukemic cells with STI571 abrogated leukemogenic potential in secondary transplant recipients (Fig. 5 *c–e*). AML could be abrogated in animals transplanted with 10^4 BA/NH leukemic cells treated with STI571 (Fig. 5*e*). Growth analysis of the

BA/NH cells in the presence of STI571 confirmed that majority of the BA/NH leukemic cells were sensitive to STI571 treatment (Fig. 5*b*). However, we observed that higher doses of cells treated with STI571 still caused AML in secondary recipients (Fig. 5 *c* and *d*). For example, 10^6 cells transplanted after STI571 treatment were fully capable of inducing AML (Fig. 5*c*). Similarly, in the four recipient mice that were first transplanted with BA/NH leukemic cells and subsequently treated with STI571 by oral gavage, one mouse succumbed to AML, whereas the disease was abrogated in the others (Fig. 5*f*).

Discussion

Chronic myelogenous leukemia invariably progresses to acute leukemia, but little is understood about the mechanisms of progression. However, in some cases secondary cytogenetic abnormalities have been identified that are associated with disease progression, including the NUP98/HOXA9 fusion. We have demonstrated that BCR/ABL cooperates with NUP98/HOXA9 to cause an acute leukemia phenotype in a murine model that recapitulates the essential features of CML blast crisis in humans.

These data support a model in which two mutations are required for acute leukemia phenotypes. One class of mutations, exemplified by BCR/ABL, confers proliferative and/or survival advantage to cells but does not affect differentiation. A second class of mutations, exemplified by NUP98/HOXA9, confers a subtle growth advantage to hematopoietic cells, but serve primarily to impair hematopoietic differentiation. Previous literature has indicated that cooperation between oncogenes may contribute to leukemic phenotypes (38–40), but this is the first direct demonstration that two oncogenes associated with progression of human CML to blast crisis cooperate to cause acute leukemia. Furthermore, these experiments indicate that although acute leukemia cells harbor more than one mutation, they retain some level of dependence on BCR/ABL. Similar conclusions were reached in a tetracycline-regulated murine model of BCR/ABL disease (41).

In our experiments, BA/NH leukemia cells retained sensitivity to the ABL inhibitor STI571 in cell culture systems. Pretreatment of BA/NH cells with1 μM STI571 was sufficient to prevent leukemia in secondary recipients that received 1×10^4 cells. However, at higher doses of cells treated with the same concentration of STI571 (1×10^5 or 1×10^6 cells, respectively) AML still developed in secondary recipient mice. We also observed that one-fourth of secondary recipients of BA/NH cells treated with twice daily oral gavage with STI571 also developed AML. It is possible that BA/NH cells may have escaped STI571-induced cell death because of pharmacokinetic considerations in these experiments. Alternatively, these cells may have developed STI571 resistance. Further experiments will be required to test these possibilities. It has recently been reported that a significant proportion of CML myeloid blast crisis patients respond to STI571, although resistance and relapse of disease occurs in the majority of responders (42). Resistance to STI571 has also recently been shown to be associated with *BCR/ABL* amplification or point mutations in the kinase domain of BCR/ABL oncoprotein that impair STI571 binding (43). The availability of a murine model of CML blast crisis that recapitulates the human response to the drug may provide a useful platform for analysis of mechanisms of resistance to STI571 and to test the efficacy of STI571 in combination with other agents that might circumvent resistance mechanisms. These data also suggest that it may be possible to treat *de novo* AML with tyrosine kinase inhibitors. Recent data indicates that both FLT3 and c-KIT may be attractive therapeutic targets based on activating mutations in these receptor tyrosine kinases in a significant proportion of AML patients (44).

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