

Stromal Niche Cells Protect Early Leukemic FLT3-ITD⁺ Progenitor Cells against First-Generation FLT3 Tyrosine Kinase Inhibitors

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Abstract

Targeting constitutively activated FMS-like tyrosine kinase 3 [(FLT3); FLT3-ITD] with tyrosine kinase inhibitor (TKI) in acute myeloid leukemia (AML) leads to clearance of blasts in the periphery but not in the bone marrow, suggesting a protective effect of the marrow niche on leukemic stem cells. In this study, we examined the effect of stromal niche cells on CD34⁺ progenitors from patients with FLT3-ITD⁺ or wild-type FLT3 (FLT3-WT) AML treated with the TKIs SU5614 or sorafenib. TKIs effectively and specifically inhibited FLT3 and increased the fraction of undivided progenitors in both FLT3-ITD⁺ and FLT3-WT samples. Treatment with SU5614 and sorafenib also reduced the number of mature leukemic progenitors, whereas contact with stroma protected against this cell loss. In contrast, primitive long-term progenitors from both FLT3-ITD⁺ and FLT3-WT AML were resistant to TKIs. Additional contact with niche cells significantly expanded long-term FLT3-ITD⁺ but not FLT3-WT progenitors in the presence of SU5614 but not that of sorafenib. Thus, TKIs with first-generation inhibitors fail to eradicate early leukemic stem/progenitor cells in FLT3-ITD⁺ AML. Further, we defined a specific interaction between FLT3-ITD⁺ progenitors and niche cells that enables the maintenance of leukemic progenitors in the presence of TKI. Collectively, our findings suggest that molecular therapy may have unpredicted effects on leukemic progenitors, underscoring the necessity of developing strategies to selectively eliminate the malignant stem cell clone. *Cancer Res*; 71(13); 4696–706. ©2011 AACR.

Introduction

Acute myeloid leukemia (AML) is organized as a hierarchy resembling normal hematopoiesis with leukemic stem cells (LSC) responsible for producing the bulk of leukemic blasts and sustaining the disease by their ability to self-renew (1, 2). Conventional chemotherapy induces high rates of remission but cures only a small percentage of patients with AML (3). The persistence of LSCs in the bone marrow after chemotherapy is thought to be responsible for the high rate of relapse (4, 5). Therefore, new strategies to eradicate residual LSCs are urgently needed. Targeting key signaling pathways with small molecule inhibitors is one therapeutic approach currently being evaluated.

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FMS-like tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase overexpressed on leukemic blasts in almost all cases of AML (6). Activating mutations in the *FLT3* gene in the form of internal tandem duplications (FLT3-ITD) can be identified in one third of AML patients and are associated with poor prognosis and increased relapse rates (7, 8). These mutations induce constitutive tyrosine kinase activity in the absence of FLT3 ligand (FL) and confer growth factor independence, proliferation, and survival to myeloid cells in mouse models. Introduction of FLT3-ITD into murine bone marrow induces myeloproliferative disease, indicating the importance of *FLT3* mutations in malignant transformation (9–11). *FLT3-ITD* mutations were shown to be present in primitive human CD34⁺CD38[−] cells, showing that the mutation can occur within the hematopoietic stem cell compartment (12). Moreover, detection of FLT3-ITD in the CD34⁺CD33[−] stem/progenitor cell fraction in children with FLT3-ITD⁺ AML was associated with a particularly poor prognosis (13). Thus, targeting FLT3-ITD may improve prognosis by enabling eradication of leukemic CD34⁺ stem/progenitor cells. Indeed, inhibition of constitutively active FLT3 has been shown to prolong survival in a mouse model of FLT3-ITD⁺ leukemia (14, 15) and several tyrosine kinase inhibitors (TKI) have entered clinical trials (16–18). However, although inhibition of mutant FLT3 leads to clearance of leukemic blasts in the periphery, the bone marrow often remains unchanged and remissions are usually short-lived

(17, 19), raising the question whether a protective effect of the marrow niche on LSCs exists.

The stem cell niche of the bone marrow provides a supportive microenvironment for normal hematopoietic stem cells (20, 21) and regulates the balance between self-renewal and differentiation in the stem cell pool (20). Newer data suggest that LSCs are also protected by the stem cell niche and may even manipulate the microenvironment to their advantage (21, 22). If so, the interaction between leukemic FLT3-ITD⁺ stem/progenitors and the niche may influence the efficacy of TKI on these cells. We addressed this question by investigating the effects of the TKIs SU5614 and sorafenib on leukemic CD34⁺ stem/progenitor cells from AML patients with wild-type (FLT3-WT) or mutated (FLT3-ITD) FLT3

receptor in the presence or absence of niche cells. The murine embryonic stromal cell line EL08-ID2 was used as an *in vitro* model for the stem cell niche (23).

Materials and Methods

Bone marrow samples

Bone marrow samples were obtained from patients recruited to the German AMLSG trials between 2002 and 2010 (Table 1). Written informed consent in accordance with the Declaration of Helsinki was obtained from all patients prior to bone marrow aspiration according to a protocol approved by the local Ethics Committee. All patients were newly diagnosed and untreated. Bone marrow samples

Table 1. AML sample characteristics

ID	Age, y	Karyotype	CD34, % blasts	FAB	FLT3 status	FLT3-ITD ratio	Additional molecular aberrations
1	62	46, XY t(5;6)	65	M1	ITD 105 bp	0.341	
2	43	46, XX -5 -22	81	M1	ITD 42/57 bp	0.394	<i>MLL-PTD</i>
3	23	46, XY t(6;14)	53	M1	ITD 57 bp	0.884	
4	31	46, XY t(6;9)	2	M5	ITD 57 bp	1.48	
5	56	46, XY	35	M4	ITD 39 bp	0.55	
6	42	46, XY	5	M5	ITD 27 bp	0.98	
7	51	46, XX	49	M4	ITD 15 bp	0.399	
8	33	47, XY +6	82	M1	ITD 27 bp	0.70	
9	22	46, XY	42	M4	ITD 39 bp	0.80	
10	39	46, XX	56	M1	ITD 30 bp	0.64	<i>NPM1</i>
11	65	46, XX	63	M1	ITD 57 bp	0.88	<i>NPM1</i>
12	47	46, XX	17	M5	ITD 123 bp	0.658	<i>NPM1</i>
13	71	46, XX	20	M1	ITD	0.735	
14	72	46, XX	3	M5	ITD	0.459	
15	38	46, XX	84	M2	ITD	n/a	
16	36	46, XY	n/a	n/a	ITD	n/a	
17	35	46, XY del(13)	5	M5	ITD	0.665	
18	37	46, X, -Y, t(8;21), del(9)	3	M5	ITD	0.80	
19	76	46, XX	7	M5	ITD	n/a	
20	47	46, XY	15	M5	ITD	0.658	<i>NPM1</i>
21	46	46, XX	30	M5	ITD	0.939	<i>NPM1</i>
22	58	46, XX	0	M5	WT		
23	56	46, XX	1	M5	WT		
24	44	46, XX	1	M4	WT		
25	31	46, XX	5	M5	WT		
26	68	47, XY +8	25	M2	WT		
27	76	46, XY	1	M4	WT		<i>NPM1</i>
28	61	46, XX t(1;6)	28	M4	WT		
29	46	46, XY	5	M5	WT		
30	69	46, XY	0	M5	WT		
31	26	46, XY inv(16)	53	M4	WT		
32	43	46, XY t(6;11)	75	M5	WT		
33	70	46, XY	80	M0	WT		
34	38	46, XX	90	M1	WT		

NOTE: Other molecular mutations screened for were *FLT3-TKD*, *MLL-PTD*, and *NPM1*.

Abbreviations: FAB, French-American-British classification for AML; n/a, not available.

underwent standardized processing including cytogenetics, FISH, and molecular genetics. All samples were screened for the presence of *FLT3-ITD* mutation as recently described (24) as well as for mutations in the tyrosine kinase domain of *FLT3* (*FLT3-TKD*), partial tandem duplications of the *MLL* gene (*MLL-PTD*), and mutations of the nucleophosmin gene (*NPM*).

Cell isolation, enrichment, and CFSE staining

Mononuclear cells were enriched for $CD34^+$ cells by magnetic selection as described by the manufacturer (Miltenyi Biotec). Enriched cells were analyzed for expression of *CD34* and, in some cases, *CD38* and *FLT3* (*CD135*). Purity of $CD34^+$ -enriched cells ranged from 87% to 98%. Further lineage depletion of $CD34^+$ cells was not done because of primary sample size constraints. In some experiments, $CD34^+$ -enriched cells were labeled for 10 minutes at 37°C with 2 $\mu\text{mol/L}$ of the fluorescent dye 5-(and 6-)carboxy-fluorescein succinimidyl ester (CFSE; Molecular Probes) in Iscove's modified Dulbecco's medium (GIBCO; Invitrogen), 1% fetal calf serum, and 10 mmol/L HEPES (Gibco) as described (25). To determine the location of undivided cells for cell division tracking, a control culture was set up in the same manner but was additionally supplemented with colcemid (Karyomax; Gibco) as described (26). Stromal cell lines FBMD-1 and EL08-1D2 were cultured as described (23). The RS4;11 and MV4-11 human leukemia cell lines were obtained from and propagated as suggested by the German Collection of Microorganisms and Cell Cultures (DSMZ) and were authenticated by DSMZ by DNA typing and PCR analysis as well as cytogenetic testing. Cells used for all experiments were passaged for fewer than 6 months after receipt.

Culture of $CD34^+$ progenitor cells from primary AML samples

$CD34^+$ progenitors from AML bone marrow samples were cultured in serum-free medium supplemented with 5 growth factors (5GF): kit ligand (KL), FL, thrombopoietin (TPO), interleukin (IL)-3, and Hyper-IL-6, a designer cytokine consisting of IL-6 and soluble IL-6 receptor (H-IL-6; a kind gift from S. Rose-John, Kiel, Germany; ref. 27). Cells were cultured in suspension or on confluent EL08-1D2 stromal cells and treated with SU5614, sorafenib, or dimethyl sulfoxide (DMSO) as indicated. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 . After 4 days, cells were harvested and assayed for short- and long-term hematopoietic activity.

Analysis of apoptosis in $CD34^+$ cells

$CD34^+$ bone marrow cells were cultured in serum-free medium with 5GF and DMSO or SU5614. After the indicated time, cells were harvested and stained for Annexin V and propidium iodide (PI) as described by the manufacturer (ApoTest-FITC; Becton Dickinson). Cells were washed in PBS supplemented with 1% bovine serum albumin, resuspended in PBS buffer containing RNase (100 $\mu\text{g/mL}$) and PI (50 $\mu\text{g/mL}$), followed by flow cytometry done on an Epics XL cytometer (Beckman Coulter). Acquired data were analyzed using FlowJo Software (version 8.7.3 for Macintosh).

Hematopoietic progenitor cell clonogenic assays

Mature hematopoietic progenitors were assessed by colony formation before and after 4 days of 5GF-supplemented serum-free culture. A total of 1,000 to 5,000 input cell equivalents were plated in growth factor-supplemented methylcellulose (H4435; Stem Cell Technologies) and incubated at 37°C in a humidified atmosphere with 5% CO_2 . After 14 days, colony-forming units (CFU) were scored using standard criteria.

Immature hematopoietic stem/progenitors were determined after long-term culture on the FBMD-1 stromal cell line in the presence of TPO and FL as described (25). After 6 weeks of culture, the entire culture was harvested by trypsin detachment and assayed for the presence of long-term, culture-derived colony-forming cells (LTC-CFC) in methylcellulose.

PCR for *FLT3*

AML bone marrow samples were validated for the presence of *FLT3-ITD* by PCR after isolation of mononuclear cells, as well as after both *CD34* enrichment and culture in methylcellulose. For PCR from hematopoietic colonies, single hematopoietic colonies were picked from methylcellulose. Total genomic DNA was isolated using the QiaAmp Micro Kit (Qiagen) and eluted into 30 μL TE (Tris-EDTA) buffer. Eight microliters of single colony-derived genomic DNA was amplified by *FLT3-ITD* PCR. Products were resolved on 2% agarose gels and visualized under UV light after ethidium bromide staining.

Western blot analysis

MV4-11 or RS4;11 cells or primary $CD34^+$ cells were starved overnight in suspension culture or on the stromal cell line EL08-1D2 at 37°C in 5% CO_2 . Cells were incubated with SU5614 (5 $\mu\text{mol/L}$), sorafenib (100 nmol/L), or DMSO at 37°C and 5% CO_2 for indicated time periods. Cells were placed in pre-chilled tubes containing ice-cold PBS with Na_2VO_4 (1 mmol/L; Sigma) and washed once in cold PBS with Na_2VO_4 . Cell lysis, SDS-PAGE, and immunoblotting were done as described (25). Antibodies to *FLT3* (S-18, sc-480; Santa Cruz Biotechnology) and p*FLT3* (#3461; Cell Signaling Technologies), AKT1/pS473 and AKT1/pT308 AKT (all from Cell Signaling Technologies), ERK1 (K-23; Santa Cruz Biotechnology), pY204-ERK1 (E4; Santa Cruz Biotechnology), β -actin (Sigma), pY694STAT5 and STAT5 (Cell Signaling Technologies), and phosphotyrosine (4G10 and PY20; Transduction Laboratories) were used as described in manufacturers' instructions. Signals were visualized on Kodak films, using polyclonal secondary horseradish peroxidase-labeled antibodies (Pierce) and enhanced chemoluminescence (Pierce). Band intensities were determined using the ImageJ software package (NIH).

ELISA for FL

Levels of murine and human FL in stroma coculture were measured using species-specific Quantikine ELISA kits for murine (MFK00) and human FL (DFK00) following the manufacturer's instructions (R&D Systems).

Statistics

For analysis of functional assays of patient samples, we used nonparametric tests for both unpaired (between WT and ITD)

and paired (within either WT or ITD) comparisons. For unpaired comparisons, we used the Mann–Whitney *U* test; for paired samples, the Wilcoxon matched pairs signed-rank test was used. To test for possible differences between groups in other experiments, we used the paired *t* test. Statistical testing was done using InStat software (GraphPad Software).

Results

Characteristics of AML patient samples

The characteristics of AML bone marrow samples used in this study are summarized in Table 1. A total of 21 AML samples harboring the *FLT3-ITD* mutation were analyzed and compared with 13 AML samples without the mutation (FLT3-WT). The majority of samples (23 of 34) had normal cytogenetics. With the exception of sample 2, which additionally harbored an *MLL-PTD* mutation, and samples 10 to 12 and 20, 21, 27, which were NPM mutated, none of the other molecular abnormalities routinely screened for were detected in the remaining 27 AML samples. Primary FLT3-ITD⁺ AML samples showed variable expression of CD34 (Table 1), CD38 and FLT3 receptor (CD135), as shown in Figure 1. Enrichment for CD34 by magnetic bead isolation was successful in all samples.

Cell death induction in primary leukemic CD34⁺FLT3-ITD⁺ cells by SU5614

The effect of TKI on growth factor–stimulated leukemic CD34⁺ cells was first studied using the TKI SU5614, which has been shown to effectively inhibit FLT3-ITD and activated FLT3-WT kinase (28). We confirmed the selective inhibitory effect of SU5614 in human leukemia cell lines containing either

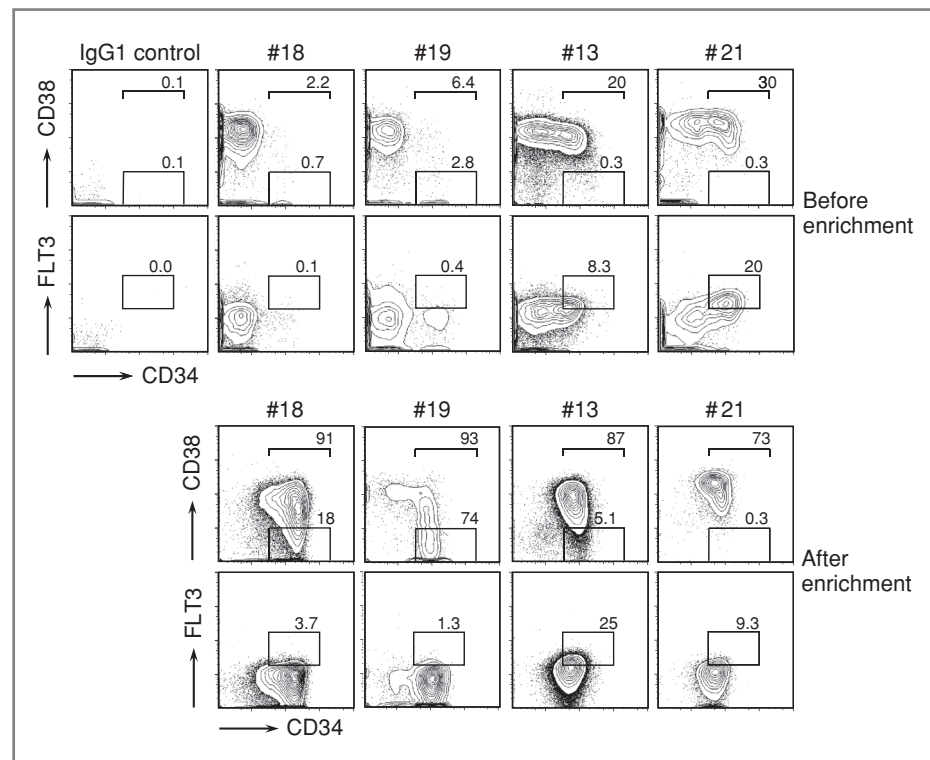
the wild-type FLT3 receptor (RS4;11) or *FLT3-ITD* (MV4-11) mutation (Fig. 2A). Proliferation of MV4-11 cells was inhibited by SU5614, whereas growth of RS4;11 cells was not affected. Ligand stimulation did not increase susceptibility of RS4;11 cells to SU5614, whereas inhibition of MV4-11 cells by SU5614 was partially overcome by stimulation with FL. Constitutively active FLT3-ITD in MV4-11 cells was completely inhibited by treatment with SU5614 (Fig. 2B). Direct contact of leukemic cells with EL08-1D2 stromal cells did not significantly influence FLT3 activation or its inhibition by SU5614.

In primary CD34⁺FLT3-ITD⁺ cells, induction of apoptosis by SU5614 was moderate (Fig. 2C) whereas apoptosis induction in CD34⁺FLT3-WT cells was not statistically significant (Fig. 2C). Culture of primary leukemic CD34⁺ cells on EL08-1D2 stroma prevented SU5614-induced apoptosis. This was not observed for MV4-11 cells (Fig. 2C).

FLT3-ITD–specific downstream signaling is uncoupled from FLT3-ITD in leukemic CD34⁺ cells

Phosphorylation of FLT3 in CD34⁺FLT3-ITD⁺ AML cells was suppressed by treatment with SU5614 (Fig. 3A), confirming inhibition of the target mutation in primary leukemic cells. Coculture on EL08-1D2 stroma partially overcame inhibition of phosphorylation by SU5614 (Fig. 3A and B). We therefore looked downstream from FLT3 to investigate whether signaling differences occurred during coculture on stroma. Of particular interest was STAT5, whose phosphorylation and subsequent activation is a hallmark of the oncogenic FLT3-ITD pathway (29, 30). In MV4-11 cells, SU5614 inhibited phosphorylation of STAT5 in suspension and when cocultured with EL08-1D2 (Fig. 3C and D). Phosphorylation of both T308

Figure 1. Expression of CD34, CD38, and FLT3 receptor before and after CD34 enrichment in FLT3-ITD⁺ primary AML samples. Bone marrow mononuclear cells from patients with FLT3-ITD⁺ AML were stained for expression of CD34, CD38 and FLT3. Each plot show the number of CD34⁺ cells (as % of total cells), and boxes represent either CD34⁺CD38[−] cells or CD34⁺FLT3⁺ cells. Results from 4 representative AML samples (#13, #18, #19, and #21; Table 1) are shown.



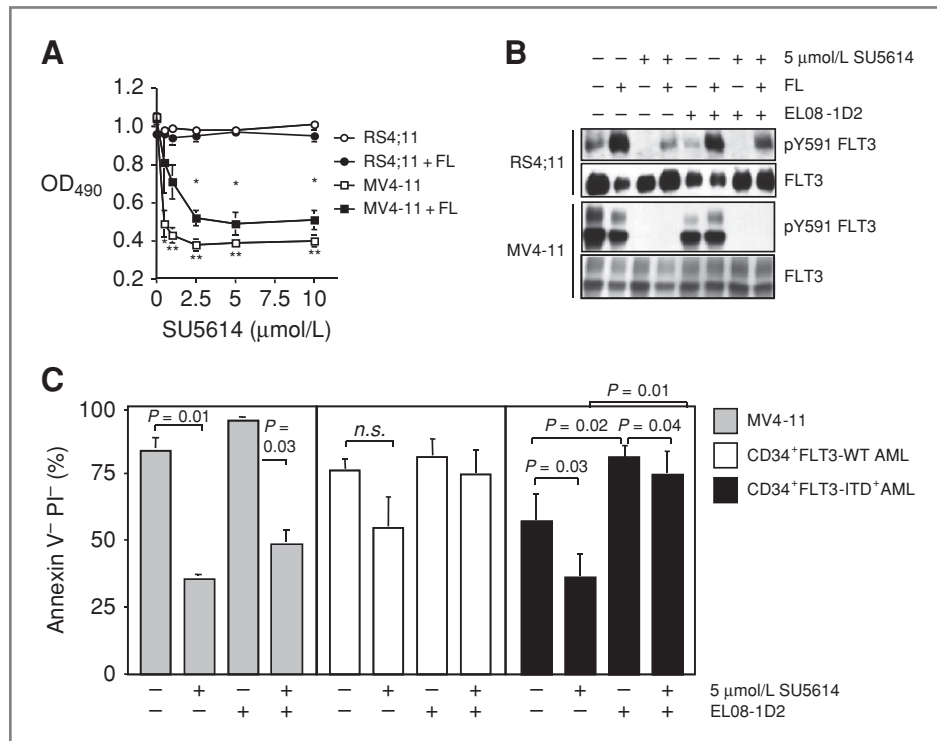


Figure 2. SU5614 induces death in MV4-11 cells and CD34⁺FLT3-ITD⁺ cells, but contact with niche cells confers protection to leukemic progenitors. **A**, dose response for MV4-11 and RS4;11 cells treated with SU5614 for 96 hours with or without FL (50 ng/mL). Proliferation was determined by MTT assay in triplicate. Results shown are mean ± SEM of 3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$. **B**, MV4-11 and RS4;11 cells were treated with SU5614 for 30 minutes with or without FL (50 ng/mL) and EL08-1D2 stroma. FLT3 was immunoprecipitated from cell lysate and resolved by SDS-PAGE. Blots were probed with anti-phospho-FLT3 antibody (591Y), and the membrane was stripped and reprobed for total FLT3 to confirm equal loading. **C**, MV4-11 cells (gray bars), CD34⁺FLT3-WT (open bars), or CD34⁺FLT3-ITD⁺ cells (black bars) were treated in serum-free medium with 5GF with SU5614 (5 μmol/L) or DMSO for 96 hours with or without coculture on EL08-1D2 stroma. Percentage of live cells (defined as Annexin V and PI negative) was determined by flow cytometry. Results are representative of 3 (MV4-11), 3 (FLT3-WT, samples 26–28), or 5 (FLT3-ITD⁺ samples 10–14) independent experiments. Error bars, SEM.

and S473 AKT and ERK was also inhibited by SU5614 (Fig. 3D). In EL08-1D2 stromal cells analyzed as a control, both AKT phosphorylation sites were found to be constitutively phosphorylated and were not inhibited by SU5614 (Fig. 3D).

In untreated AML patient samples, activated STAT5 was more readily detectable in CD34⁺FLT3-ITD⁺ cells (7 of 7 samples) than FLT3-WT cells (4 of 7 samples) and was not inhibited by SU5614 (Fig. 3C and E). In CD34⁺FLT3-ITD⁺ cells cultured in suspension, SU5614 inhibited phosphorylation of AKT (at T308) and ERK whereas phosphorylation of AKT at S473 was not affected (Supplementary Fig. S3). Coculture on EL08-1D2 cells did not influence signaling pathways downstream of FLT3 in either CD34⁺FLT3-ITD⁺ or FLT3-WT cells (Fig. 3E and Supplementary Fig. S3). Thus, while SU5614 inhibits FLT3 and T308AKT and ERK pathways, STAT5 and S473AKT phosphorylation seem to be uncoupled from FLT3-ITD activation status in primary leukemic CD34⁺ cells.

SU5614 increases the fraction of undivided leukemic CD34⁺ cells

Analysis of primary CD34⁺ AML cells revealed the majority to be in G₀-G₁ phase of the cell cycle after 4 days of growth factor stimulation (data not shown), precluding detection of an inhibitory effect of SU5614 on cell cycle. Therefore,

although one mechanism of action for TKI is cell-cycle arrest, this effect is not readily detectable in primary CD34⁺ cells. We reasoned that an analysis of cell division might provide a better discrimination of TKI effect. Cell division was indeed detectable by CFSE staining, with the majority of samples dividing once during the 4-day period. FLT3-ITD⁺ AML samples contained a significantly higher proportion of cells that did not divide during the 4-day culture than did FLT3-WT samples (Supplementary Fig. S1). Treatment with SU5614 significantly increased the fraction of nondividing cells in both FLT3-ITD⁺ and FLT3-WT samples. This effect was almost completely reversed by coculture with EL08-1D2 stroma for CD34⁺FLT3-WT cells but not for CD34⁺FLT3-ITD⁺ cells (Supplementary Fig. S1C).

TKI eradicates short-term committed leukemic progenitor cells, but stromal contact counteracts this effect

Next, we investigated hematopoietic activity of primary CD34⁺ AML bone marrow cells cultured for 4 days in the presence or absence of SU5614 with or without EL08-1D2 cells. The 4-day incubation period was chosen, as this is the time period within which progenitors undergo at least 1 division but do not lose CD34 expression, as we have shown for normal

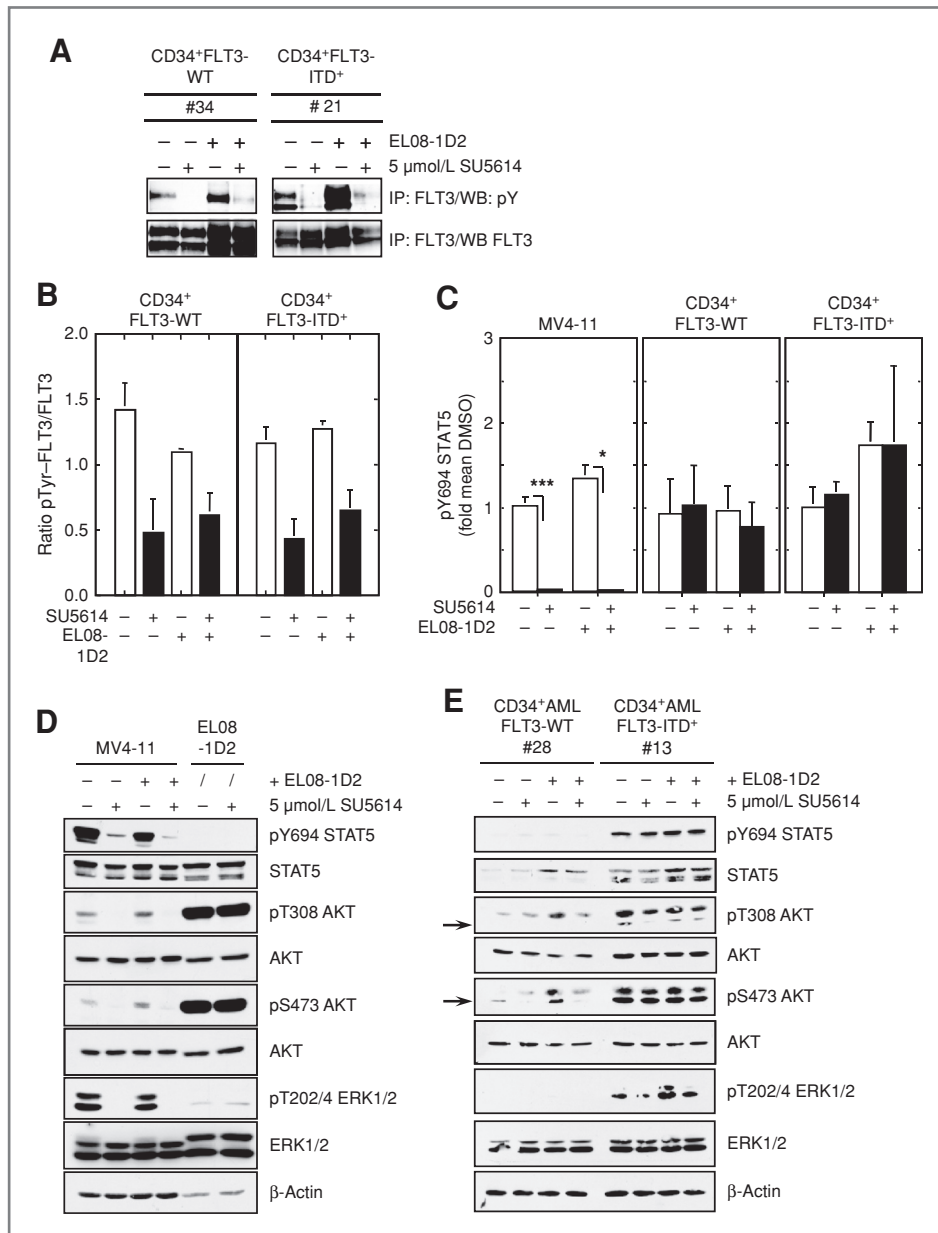


Figure 3. SU5614 inhibits phosphorylation of FLT3, AKT, and ERK but not of STAT5 in CD34⁺ FLT3-ITD⁺ cells. **A**, FLT3 phosphorylation in primary CD34⁺ cells following treatment with SU5614. CD34⁺ cells were incubated in serum-free medium with 5GF overnight in suspension or cocultured with EL08-1D2 stromal cells as indicated. Cells were incubated with DMSO or SU5614 (5 μmol/L) for 30 minutes. FLT3 was immunoprecipitated with FLT3 antibody (S-18). Polyvinylidene difluoride membranes were probed with anti-phosphotyrosine antibodies (4G10 and YP20), stripped, and reprobed for FLT3 to confirm equal loading. Results of 2 representative samples (#21 and #34) are shown. IP, immunoprecipitation; WB, Western blotting. **B**, quantitation of FLT3 inhibition by SU5614 in primary CD34⁺ AML cells treated as in **A**. Western blots were quantitated using ImageJ. Results shown are SEM from 3 individual blots, each for FLT3-WT (samples 28, 31, and 34) and FLT3-ITD (samples 12, 13, and 21). **C**, quantitation of STAT5 phosphorylation in MV4-11 cells and primary CD34⁺ cells from FLT3-ITD⁺ or FLT3-WT AML following treatment with SU5614. Cells were treated as in **A**. Lysed samples were subjected to immunoblotting with anti-phospho-STAT5. Quantitation of Western blots was done with ImageJ. Results are representative of 7 experiments with MV4-11, 7 FLT3-ITD⁺ AML samples (#12–17, and #21), and 7 FLT3-WT AML samples (#28–34, only those samples with detectable phosphorylation of STAT5 were used for quantitation: #29–31, #34). Error bars, SEM. ***, $P = 0.005$; *, $P = 0.04$. **D**, analysis of signaling pathways downstream of FLT3. MV4-11 cells were treated as in **A**. Lysed samples were subjected to immunoblotting with antibodies as indicated. EL08-1D2 cells were starved overnight in serum-free medium, incubated with DMSO or SU5614 (5 μmol/L) for 30 minutes, and subjected to immunoblotting. Polyvinylidene difluoride membranes were probed with anti-phosphotyrosine antibodies, stripped, and reprobed for total protein. **E**, analysis of signaling pathways downstream of FLT3 in primary AML cells. CD34⁺FLT3-ITD⁺ and CD34⁺FLT3-WT cells were treated as in **A** and subjected to immunoblotting as in **D**. Shown are blots prepared from 1 representative patient sample, each for CD34⁺FLT3-WT (sample 28) and CD34⁺FLT3-ITD⁺ (sample 13) AML.

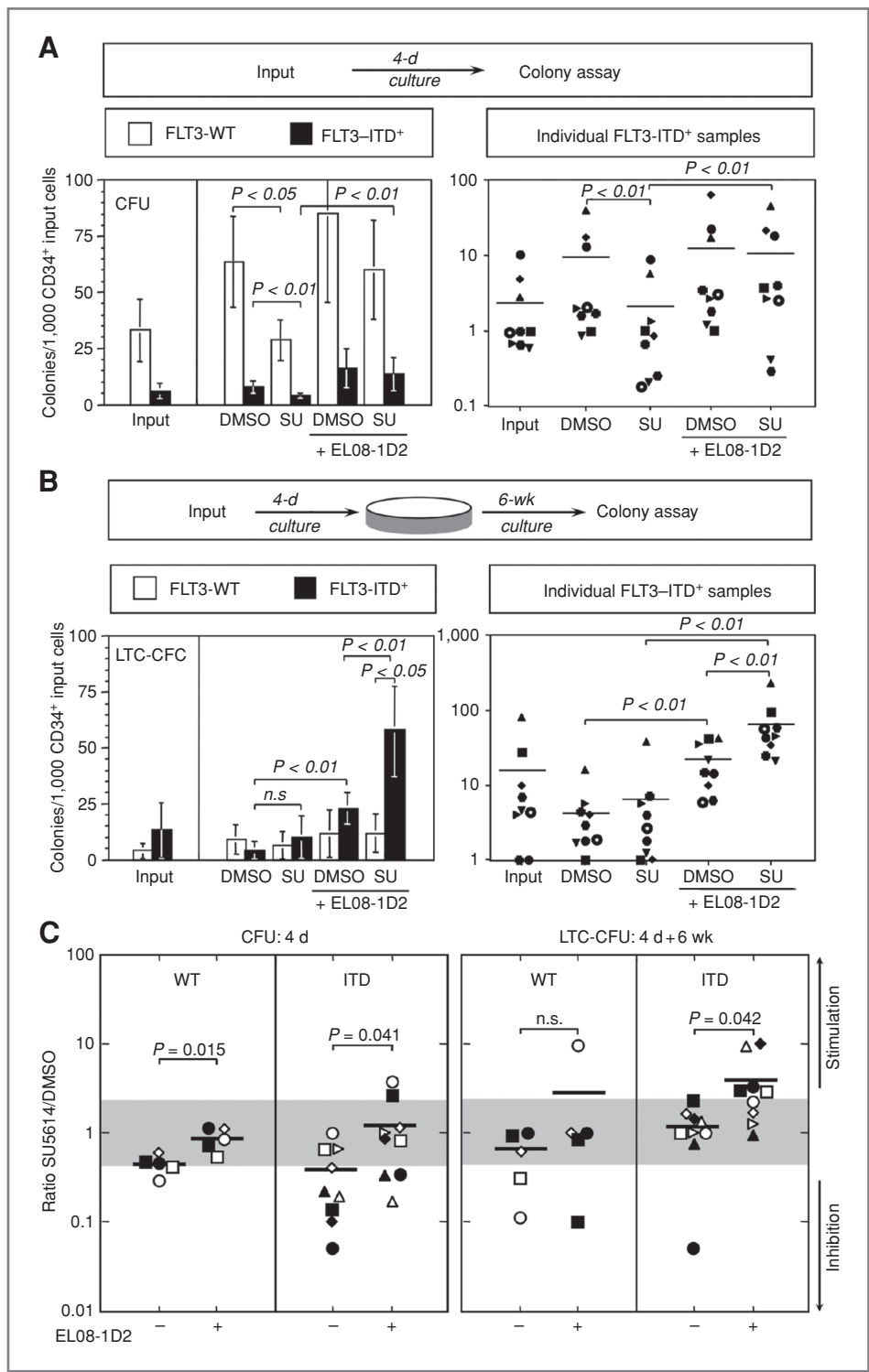


Figure 4. Effect of stromal niche cells on progenitor cell activity of CD34⁺ AML cells treated with SU5614. **A**, short-term colony-forming assay. CD34⁺FLT3-WT or CD34⁺FLT3-ITD⁺ AML cells were incubated for 4 days in serum-free medium with 5GF and DMSO or SU5614 (SU; 5 μmol/L) in suspension or cocultured with EL08-1D2 stroma as indicated. After 4 days, cells were harvested and plated in methylcellulose to determine CFUs. Colonies were scored after 14 days by using standard criteria. Input, colony-forming assay of untreated CD34⁺ cells on day 0. Results are shown as colony number per input cell number at day 0. Left, mean number of CFUs from 5 individual samples for FLT3-WT AML (open bars, samples 22–26) and 9 individual samples for FLT3-ITD⁺ AML (black bars, samples 1, 2, 4, 5, and 8–12). Error bars, SEM. Right, individual results for FLT3-ITD⁺ AML samples. Each icon represents a separate patient sample. **B**, long-term colony-forming assay. CD34⁺FLT3-WT or CD34⁺FLT3-ITD⁺ AML cells treated as in **A**. After 4 days, cells were harvested and subjected to long-term culture on FBMD-1 stromal cells in the presence of TPO and FL. After 6 weeks, cells were harvested and plated in methylcellulose to determine the number of LTC-CFCs. Left, the mean number of LTC-CFCs from same samples as in **A**. Error bars, SEM. Right, the distribution of individual FLT3-ITD⁺ AML samples. Each icon represents a separate patient sample. SU, SU5614. **C**, to determine the contribution of stroma support to leukemic colony growth, a ratio of SU5614/DMSO colony numbers was obtained. Values below the shaded area indicate inhibition, and values above the shaded area stimulation of colony growth for the conditions indicated. Each icon represents an individual patient sample.

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hematopoiesis (31) and have confirmed by our CFSE experiments for leukemic progenitors. All of the 5 FLT3-WT AML samples studied yielded colony growth at day 0 (i.e. input) and after the 4-day culture. However, 3 of the 12 FLT3-ITD⁺ AML samples (samples 3, 6, and 7) used for progenitor cell assays

did not form colonies either at day 0 or after 4 days of *in vitro* culture (Table 1 and Supplementary Table S1). Overall, untreated CD34⁺FLT3-ITD⁺ progenitors produced significantly fewer colonies than CD34⁺ cells from FLT3-WT AML samples (Fig. 4A and Supplementary Table S1), in accordance

with published observations (26). Treatment with SU5614 reduced committed progenitors in suspension culture by 77% for FLT3-ITD⁺ and 44% for FLT3-WT samples, showing that SU5614 targets committed leukemic progenitors (Fig. 4A and Supplementary Table S1). Direct contact with EL08-1D2 did not significantly enhance expansion of committed CFU in either FLT3-ITD⁺ or FLT3-WT AML samples. However, culture with EL08-1D2 stromal cells completely abrogated the inhibitory effect of SU5614 on leukemic progenitor cell growth (Fig. 4A and Supplementary Table S1). Production of FL by either EL08-1D2 stromal cells or autocrine secretion of FL by AML cells was not responsible for the protective effect of stroma, as neither murine nor human FL was secreted at detectable levels in coculture (Supplementary Fig. S2).

Inhibition of activated FLT3 by SU5614 does not eradicate primitive leukemic progenitors in FLT3-ITD⁺ or FLT3-WT AML

To address the question whether inhibition of activated FLT3 can effectively target the most primitive leukemic progenitor cell population, we conducted long-term *in vitro* culture experiments, resulting in a functional readout for the early progenitor cell fraction (31). As shown in Figure 4B and Supplementary Table S2, the number of more primitive LTC-CFCs was very heterogeneous within untreated FLT3-ITD⁺ and FLT3-WT samples and not statistically different between the two groups. Exposure to SU5614 over 4 days in suspension culture did not eliminate primitive leukemic progenitors, either in FLT3-ITD⁺ or FLT3-WT samples (Fig. 4B and C, Supplementary Table S 2).

Early FLT3-ITD⁺ stem/progenitor cells protected by stromal niche cells are amplified by treatment with SU5614

To determine the contribution of stromal support on maintenance of leukemic LTC-CFCs, we assessed the effect of SU5614 on primitive CD34⁺ progenitors cultured on EL08-1D2 stroma. We have previously shown that this murine embryonic stromal cell line supports long-term production of both mature and immature human hematopoietic progenitors and can therefore mimic the stem cell niche *in vitro* (23, 32). Culture on EL08-1D2 effectively prevented loss of FLT3-ITD⁺ LTC-CFCs during the 4-day *in vitro* culture (Fig. 4B). In contrast, there was no significant difference in the number of LTC-CFCs between suspension cultures and stromal supported cultures for FLT3-WT samples (Fig. 4B). Absolute colony numbers from all bone marrow samples are summarized in Supplementary Table S2.

An unexpected finding was the expansion of LTC-CFCs in the context of stromal support and treatment with SU5614 in FLT3-ITD⁺ AML samples. As depicted in Figure 4B, LTC-CFCs were expanded 3.5-fold compared with day 0 in the presence of EL08-1D2 and SU5614. Compared with progenitors cultured on stroma without TKI, expansion of LTC-CFCs was 2.6-fold in the presence of SU5614. The increase in LTC-CFCs on stroma in the presence of SU5614 was observed for 8 of 9 FLT3-ITD⁺ patient samples, ruling out the possibility of singular outliers distorting the overall results. To better assess the contribution

of EL08-1D2 stroma to stimulation of leukemic progenitors, a ratio between colony numbers for SU5614-treated versus DMSO-treated cultures was formed. As shown in Figure 4C, stromal support led to significant stimulation of colony growth in FLT3-ITD⁺ progenitors whereas this was not the case for FLT3-WT progenitor cells, indicating that stromal support is necessary for expansion of FLT3-ITD⁺ progenitors.

Expanded LTC-CFCs are of leukemic origin

Because primitive AML progenitors are thought to have a growth advantage over their normal counterparts (33), we sought to ascertain whether expanded LTC-CFCs from FLT3-ITD⁺ samples were of leukemic origin or whether stromal contact conferred a survival advantage to healthy progenitor cells. PCR for FLT3-ITD in individual hematopoietic colonies from methylcellulose confirmed the presence of the *FLT3* mutation in LTC-CFCs from all patient samples (Supplementary Fig. S4), indicating persistence of early leukemic progenitors.

Confirmatory studies with sorafenib

To confirm the general applicability of our findings, we repeated key experiments with a second TKI, sorafenib. This compound is a more selective and potent inhibitor of FLT3 than SU5614 and is currently widely used in the clinic (34–36). As shown in Figure 5A, sorafenib effectively inhibited activated FLT3 in WT as well as FLT3-ITD⁺ AML. Inhibition of FLT3 phosphorylation by sorafenib was not influenced by stromal contact. As with SU5614, STAT5 signaling downstream of FLT3-ITD was not inhibited by sorafenib (Fig. 5B), confirming uncoupling of FLT3-ITD and STAT5 in primary CD34⁺FLT3-ITD⁺ AML cells.

Colony-forming assays revealed that similar to SU5614, treatment with sorafenib did not eliminate short- or long-term leukemic progenitors (Fig. 5C). Coculture with EL08-1D8 stromal cells again abolished the inhibitory effect of sorafenib, although expansion of progenitor cells was not statistically significant. Analysis of colonies by PCR again confirmed that a substantial number of colonies recovered from short- and long-term cultures contained the *FLT3-ITD* mutation despite treatment with sorafenib (Supplementary Fig. S4E).

Discussion

Despite entry of FLT3 inhibitors into clinical trials for FLT3-ITD⁺ AML, it has so far not been established whether inhibition of aberrant FLT3 signaling can actually eradicate the earliest stem/progenitor cells responsible for propagating the disease. We showed, using the FLT3 inhibitors SU5614 and sorafenib as proof of concept, in primary bone marrow samples from patients with newly diagnosed FLT3-ITD⁺ AML that treatment with TKI does not eliminate early leukemic CD34⁺FLT3-ITD⁺ stem/progenitor cells. In addition, we show a protective effect of the stromal microenvironment on these cells, conferring a growth advantage to FLT3-ITD⁺ leukemic progenitors over normal ones in the presence of TKI.

We show that primary early leukemic CD34⁺FLT3-ITD⁺ progenitors are insensitive to the cytotoxic effects of TKI. The

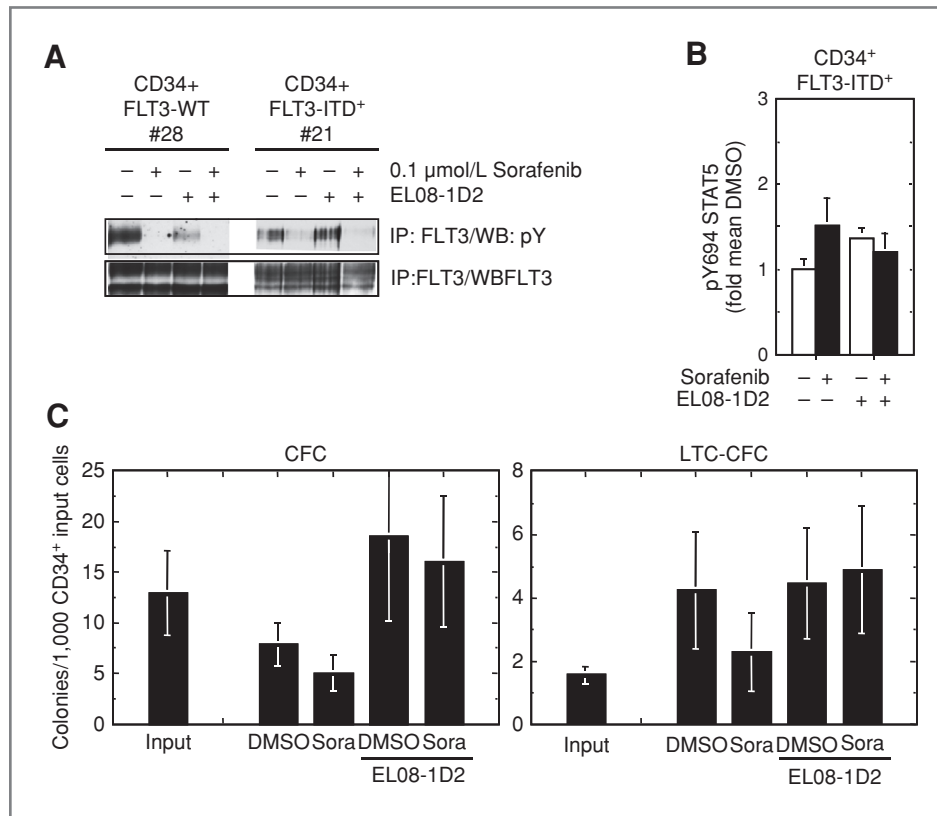


Figure 5. Confirmatory studies with sorafenib. **A**, FLT3 phosphorylation in primary CD34⁺FLT3-ITD⁺ cells following treatment with sorafenib. CD34⁺ cells were incubated in serum-free medium with 5GF overnight in suspension or cocultured with EL08-1D2 stroma as indicated. Cells were incubated with DMSO or sorafenib (100 nmol/L) for 30 minutes. FLT3 was immunoprecipitated with FLT3 antibody (S-18). PVDF membranes were probed with anti-phosphotyrosine antibodies (PY20 and 4G10), stripped, and reprobed for FLT3 to confirm equal loading. Results of 2 representative samples (#21 and #28) are shown. IP, immunoprecipitation; WB, Western blotting. **B**, quantitation of STAT5 phosphorylation in primary CD34⁺FLT3-ITD⁺ cells following treatment with sorafenib. Cells were treated as in **A**. Quantitation of Western blots was done with ImageJ. Results shown are representative of 3 individual samples (#13, #16, and #21). Error bars, SEM. **C**, short- and long-term colony-forming assays. CD34⁺FLT3-ITD⁺ cells were incubated for 4 days in serum-free medium with 5GF and DMSO or sorafenib (100 nmol/L) in suspension or cocultured with the adherent stromal cell line EL08-1D2 as indicated. After 4 days, cells were harvested and assayed as in Figure 4. Left, mean number of CFU from 5 individual samples (#13, #16-18, and #21). Right, the mean number of LTC-CFCs from same samples. Error bars, SEM. Sora, sorafenib.

majority of CD34⁺FLT3-ITD⁺ cells divided upon cytokine stimulation *in vitro*, with 50% of cells undergoing at least 1 cell division within 4 days. Treatment with SU5614 significantly increased the fraction of undivided cells, suggesting that the predominant effect of the inhibitor on this population is a decrease in cell division and not induction of cell death.

The inherent unresponsiveness of early FLT3-ITD⁺ leukemic progenitors to TKI may be due to the fact that they are not dependent on mutant FLT3 signaling for survival. In contrast to chronic myeloid leukemia (CML), more than 1 genetic alteration is necessary to cause AML. Therefore, inhibiting activated FLT3 kinase may not be sufficient to eliminate the stem cell fraction or FLT3-ITD may not be the relevant target in these early cells. For CML, it has been elegantly shown that primitive CD34⁺ stem/progenitors are insensitive to first- and second-generation Bcr-Abl inhibitors and are instead induced into quiescence by treatment with TKI (37, 38). Our results suggest that this principle may also be true for primitive FLT3-ITD⁺ progenitors from AML. This counterproductive effect on leukemic stem/progenitor cells has also been reported for

other novel treatment strategies in AML, such as the histone deacetylase inhibitor valproate (39).

An alternative explanation may be that current TKIs are not effective enough to completely prevent constitutive activation of FLT3. Because it has been suggested that complete and enduring inhibition of FLT3 phosphorylation is critical for achieving clinical efficacy (40, 41), failure to completely inhibit FLT3 may contribute to persistence of leukemic progenitors after treatment. However, to our knowledge, it has not yet been definitively shown that complete inhibition of FLT3 actually translates into improved clinical outcome in FLT3-ITD⁺ AML. Increased allelic ratios of FLT3-ITD to WT receptor have also been linked to poor prognosis (42). In our small cohort, we could not observe a correlation between allelic ratio and outcome of progenitor assays.

In addition, continued activation of signaling pathways downstream of FLT3 through other mechanisms may contribute to TKI resistance in CD34⁺FLT3-ITD⁺ progenitors. Our results show uncoupling of FLT3-ITD from STAT5 signaling in primary CD34⁺FLT3-ITD⁺ cells in the presence of TKI. Activation of

STAT5 by FLT3 has been shown to be dependent on intracellular localization of the receptor (30), thus restricted to mutated FLT3 and not found in FLT3-WT signaling. In addition, FLT3-ITD itself has recently been shown to be differentially phosphorylated on different tyrosine residues in a compartment-dependent manner (30). In this context, our results suggest that inhibition of mutated FLT3 by TKI in primary patient samples may take place mainly at the plasma membrane, allowing intracellular activation of STAT5 to persist.

Our data extend findings previously obtained using leukemic cell lines with acquired resistance to TKI showing continued STAT5 activation in resistant cells (43, 44). Prolonged exposure of leukemic cell lines or AML blasts to TKI opts for cells that are FLT3 independent, leading to pharmacologic resistance (35, 43, 44). However, our results suggest that at least primitive CD34⁺FLT3-ITD⁺ progenitors are insensitive to TKI from the onset of treatment. The inability of TKI to sustain suppression of leukemic blasts may therefore be due not only to outgrowth of resistant blasts but also to the fact that the more primitive leukemic stem/progenitors maintaining the disease persist despite treatment with TKI. Thus, our data offer an additional explanation to the transient clinical responses seen so far with TKI in FLT3-ITD⁺ AML. However, these results should be interpreted with caution, as third-generation FLT3 inhibitors with improved pharmacokinetic properties now entering clinical trials (45) may yield different results.

Finally, we show that interaction of CD34⁺FLT3-ITD⁺ progenitors with stromal niche cells mimicking the bone marrow environment protects these cells from the effects of TKI. Because FL was not significantly produced by either stromal cells or AML cells during coculture, this protective effect is not dependent on FL in our *in vitro* system, as has been observed *in vivo* in response to chemotherapy (46). Although similar protective effects of stroma have been reported for normal progenitors, our observation that the combination of niche cells and concomitant TKI actually may, in some instances, even lead to expansion of malignant progenitors is unexpected. This effect was specific for FLT3-ITD⁺ progenitors and not observed for FLT3-WT cells, pointing to a differential response of FLT3-ITD⁺ cells to the niche. However, we did not observe this expansion when leukemic progenitors were treated with sorafenib. Because SU5614 and sorafenib show differential inhibitory effects on

FLT3-WT and FLT3-ITD, the difference in observed effects on long-term CFC-producing cultures may be due to the weaker efficiency of SU5614 in inhibiting FLT3 or to an altered balance between inhibition of WT and mutant FLT3. The mechanistic basis for our finding is still unclear but suggests that the niche generates specific self-renewal or survival signals, perhaps in reaction to leukemic cells themselves, to which FLT3-ITD⁺ progenitors can uniquely respond.

Taken together, these data highlight the fact that molecular therapy may have unpredicted effects on leukemic stem/progenitor cells and underscores the importance of developing strategies to selectively eliminate the malignant stem cell clone. Our data point to an altered interaction between FLT3-ITD⁺ stem/progenitors and the stem cell niche. To efficiently target FLT3-ITD⁺ stem/progenitor cells in AML, future investigations should focus on how the bone marrow microenvironment regulates these cells. Our results suggest that combining inhibitors to additionally block downstream pathways (e.g., STAT5) or adding agents that disrupt the interaction between LSCs and niche (e.g., CXCR4 antagonists) may be necessary to overcome the unresponsiveness of FLT3-ITD⁺ leukemic stem/progenitor cells to TKI (44, 47). Finally, our findings have potentially important clinical implications for the use of TKI to treat FLT3-ITD⁺ AML, as they raise the possibility of unwittingly amplifying leukemic stem/progenitor cells.

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest to disclose.

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