

Induction of Vascular Endothelial Growth Factor Secretion by Childhood Acute Lymphoblastic Leukemia Cells via the FLT-3 Signaling Pathway

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Abstract

Human leukemia cells secrete VEGF, which can act in a paracrine manner within the bone marrow microenvironment to promote leukemia cell survival and proliferation. The FLT-3 receptor tyrosine kinase plays an essential role in regulating normal hematopoiesis, but its constitutive activation via mutation in acute leukemias is generally associated with poor outcome. The aim of this study was to investigate interactions between the FLT-3 and VEGF signaling pathways in acute leukemia using cell lines and *ex vivo* cultures of pediatric acute lymphoblastic leukemia cells following expansion of direct patient explants in immunodeficient mice. Different xenograft lines exhibited variable cell surface FLT-3 expression, as well as basal and FLT-3 ligand-induced VEGF secretion, whereas the MV4;11 cell line, which expresses constitutively active FLT-3, secreted high levels of VEGF. The FLT-3 inhibitor, SU11657, significantly reduced VEGF secretion in three of six xenograft lines and MV4;11 cells, in conjunction with inhibition of FLT-3 tyrosine phosphorylation. Moreover, exposure of xenograft cells to the FLT-3–blocking antibody, D43, also reduced VEGF secretion to basal levels and decreased FLT-3 tyrosine phosphorylation. In terms of downstream signaling, SU11657 and D43 both caused dephosphorylation of extracellular signal-regulated kinase 1/2, with no changes in AKT or STAT5 phosphorylation. Finally, partial knockdown of FLT-3 expression by short interfering RNA also resulted in inhibition of VEGF secretion. These results indicate that FLT-3 signaling plays a central role in the regulation of VEGF secretion and that inhibition of the FLT-3/VEGF pathway may disrupt paracrine signaling between leukemia cells and the bone marrow microenvironment. *Mol Cancer Ther*; 11(1); 183–93. ©2011 AACR.

Introduction

VEGF-A is an integral component of both neovascularization and normal hematopoiesis (1). Neovascularization is a tightly controlled process, which is disrupted during tumor growth to promote malignancy, particularly in the lung, breast, and prostate. VEGF enhances the migration, permeability, and mitogenic activity of endothelial cells to facilitate tumor vascularization and metastasis (2). While the important role VEGF plays in the progression and invasiveness of solid tumors has been widely documented, its potential function in hematologic malignancies has received less attention. Previous studies have reported that human leukemia cells produce and secrete VEGF, which may act in a paracrine manner within the bone

marrow microenvironment to promote the survival and proliferation of leukemia cells (3). The production of VEGF by leukemias, lymphomas, and myelodysplastic syndromes can also result in increased vascularity within the bone marrow, which has been observed in acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), B-cell non-Hodgkin lymphoma, and chronic lymphocytic leukemia (4). High serum VEGF levels have also been associated with poor prognosis in certain leukemias (5). Accordingly, one study has shown that standard-risk pediatric patients with ALL who have high VEGF levels after induction therapy relapse earlier than those with low levels (6).

The FMS-like tyrosine kinase-3 (FLT-3) is a member of the class III receptor tyrosine kinase (RTK) family, sharing structural homology with other members, such as KIT and FMS. FLT-3 is expressed by primitive CD34⁺ hematopoietic cells, dendritic cells, B-progenitors, and natural killer cells, as well as in neural tissues, the gonads, and placenta (7). In addition to regulating the expansion of normal hematopoietic progenitors, FLT-3 is also highly expressed in several hematologic malignancies, including AML and ALL (8). In addition, FLT-3 mutation in AML is associated with poor prognosis (9).

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The binding of FLT-3 to its ligand (FL) causes receptor dimerization, tyrosine kinase activation, and receptor autophosphorylation, initiating the phosphorylation of downstream signaling proteins (10). Wild-type FLT-3 transduces its signaling cascade principally via the phosphatidylinositol-3 kinase (PI3K) and Ras pathways, leading to activation of AKT (protein kinase B) and the extracellular signal-regulated kinase-1 and -2 (ERK1/2). Mutant FLT-3 has also been reported to activate STAT5 (11, 12). FLT-3 has been an intense focus of drug development in recent years primarily due to its high expression and/or mutation in leukemia.

In this study, we investigated the relationship between FLT-3 and VEGF and showed that FL stimulated the secretion of VEGF in *ex vivo* cultured ALL xenograft cells. Moreover, the role of FLT-3 signaling in VEGF secretion was confirmed by pharmacologic intervention, FLT-3-blocking antibodies, and short interfering RNA (siRNA) knockdown of FLT-3 expression. We also investigated the mechanism by which FL induced VEGF secretion and showed that this occurred primarily via the ERK1/2 pathway. These findings provide additional insight into the interactions between FLT-3 and VEGF in leukemia cells and may result in improved strategies to treat the disease.

Materials and Methods

In vitro cell culture

Childhood ALL xenograft cells were cultured, harvested, and characterized as previously described (13, 14). For all experiments, xenograft cells were retrieved from cryostorage and resuspended at a density of 2×10^6 cells/mL in QBSF-60 medium (Quality Biological, Inc.), supplemented with penicillin (100 U/mL), streptomycin (100 μ g/mL), and L-glutamine (2 mmol/L; PSG). FL (kindly provided by Amgen) was added at 20 ng/mL. Cell viability was determined by trypan blue exclusion. The principal cell line used in this study, MV4;11, was obtained from American Type Culture Collection (ATCC), tested by ATCC using short tandem repeats, and passaged in the laboratory for less than 6 months in Iscove's Modified Dulbecco's Medium supplemented with 20% fetal calf serum (FCS), PSG, and insulin, transferrin, and selenium (Invitrogen). RS4;11 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), tested by DSMZ using multiplex PCR of minisatellite markers, and passaged in the laboratory for less than 3 months in α MEM with 10% FCS plus PSG. The NB4, NALM6, and HL60 cell lines were general laboratory stocks maintained in RPMI-1640 plus 10% FCS and PSG. All cells were cultured at 37°C and 5% CO₂. Where specified, cells were incubated with SU11657, SU5416, and SU6668 (kindly provided by Pfizer), FLT-3-blocking antibodies EB10 and D43 (kindly provided by ImClone) or commercially available inhibitors: KDR inhibitor (KDRi), U0126, and PD98059 (Merck); LY294002 and wortmannin (Sigma; Supplementary Fig. S1).

ELISA

VEGF secreted into cell culture media was quantified by human VEGF-specific ELISA, as detailed by the manufacturer (R&D Systems). VEGF was expressed as picograms per 1 million viable cells, with a detection limit of 10 pg/mL VEGF.

Flow cytometry

Viability of CD45⁺ xenograft cells was determined by propidium iodide exclusion (15). Cell surface FLT-3 was quantified using phycoerythrin-conjugated anti-human FLT-3, and the relative fluorescent intensity (RFI) quantified with respect to isotype control antibody. All antibodies were from Becton Dickinson, and data acquired using FACSCalibur (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson).

Immunoprecipitation and immunoblot analysis

Cells were treated for 2 hours with inhibitors, with ALL xenografts treated for an additional 15 minutes with FL (20 ng/mL). Lysates were prepared at 10^8 cells/mL in 200 mmol/L Tris-Cl pH7.4, 150 mmol/L NaCl, 0.2% NP-40, 10 mmol/L EDTA, 100 mmol/L NaF, 1 mmol/L Na₃VO₄ supplemented with protease inhibitor cocktail (Sigma). Insoluble materials were removed by centrifugation at $10,000 \times g$ for 10 minutes at 4°C, and supernatants stored at -80°C. Total protein concentration was quantified by the bicinchoninic acid assay (Pierce) using a BSA standard. For immunoprecipitation, ALL xenograft (500 μ g) or MV4;11 (1 mg) protein lysates were incubated overnight with FLT-3 antibody (Santa Cruz Biotechnology Inc.), then captured using protein-A Sepharose beads for 2 hours at 4°C. Lysates or immunoprecipitates were separated in 4% to 12% Bis-Tris PAGE and electrotransferred to polyvinylidene difluoride membrane (Immobilon-P), then probed with antibodies against phosphotyrosine (Millipore), phospho-STAT5, phospho-AKT (S473), or phospho-ERK1/2 (Cell Signaling). Secondary antibodies used were horseradish peroxidase conjugates of either anti-mouse or anti-rabbit IgG (Pierce). Blots were subsequently stripped with a commercial stripping buffer (Pierce) and reprobed with antibodies against FLT-3, STAT5, AKT, or ERK1/2. Following incubation with HRP-conjugated secondary antibodies, proteins were visualized by autoradiography of secondary antibody-HRP chemiluminescence and quantified by phosphoimaging using VersaDoc 5000 Imaging System (BioRad). Data were analyzed using QuantityOne software (BioRad).

siRNA knockdown

Xenograft cells and the MV4;11 cell line were transfected with Amaxa Nucleofector (Cologne) according to the manufacturer's instructions. For xenograft cells, the Primary B-Cell Kit was used and Kit-L for the MV4;11 cell line. FLT-3 siRNA (On-Target Plus) and the scrambled controls were from Dharmacon. The siRNA (2 μ g per well) was used for the experiments. After transfection, cells were cultured for 72 hours and harvested for analysis.

Statistics

Quantitative data were compared using the nonparametric Mann–Whitney *U* test (GraphPad Prism). Experimental data are expressed as the mean \pm SEM. *P* values less than 0.05 were considered significant. All experiments were carried out in a minimum of triplicates. The Spearman rank correlation coefficient was used to determine the rank correlation of VEGF secretion and phosphorylation.

Results

FLT-3 expression and VEGF secretion by leukemia cells

To explore the possible relationship between FLT-3 and VEGF, we assessed cell surface FLT-3 and FL-induced VEGF expression in a series of *ex vivo* cultured pediatric ALL xenograft lines (13, 14). In addition, the myelomonocytic leukemia cell line, MV4;11, which has a constitutively active FLT-3 with an internal tandem duplication (ITD), was also used (16). The ALL xenograft cells showed varying levels of FLT-3 cell surface expression (Table 1). Four xenografts showed very high FLT-3 expression, with RFI values of 5.2, 6.8, 4.7, and 7.9 for ALL-2, ALL-3, ALL-17, and P-14, respectively. Conversely, FLT-3 expression in ALL-4 and -7 cells was relatively low (RFI, 2.8 and 1.9, respectively). An additional 6 xenografts expressed low levels of FLT-3 (RFI < 2.0) and were not used in subsequent experiments (data not shown).

To determine whether these lines expressed VEGF, we analyzed VEGF media levels after 72 hours of culture with and without FL (Table 1). In the absence of exogenous FL, VEGF was secreted in 6 xenograft lines (ALL-2, ALL-3, ALL-4, ALL-7, and ALL-17, as well as the MLL xenograft

P-14). With the exception of ALL-7, the xenograft lines with the highest expression of FLT-3 also secreted the highest amounts of VEGF. The effects of FL on VEGF secretion were also assessed: FL increased VEGF secretion by ALL-3 and P-14 by 3.7- ($P = 0.0002$) and 2.3 ($P = 0.008$)-fold, respectively. FL also increased VEGF secretion in the remaining 4 lines that expressed detectable basal levels of VEGF, although the difference was not statistically significant. Both basal ($R^2 = 0.7$, $P = 0.02$) and FL-induced ($R^2 = 0.7$, $P = 0.011$) VEGF secretion correlated with FLT-3 surface expression levels (RFI) in the xenograft cells. No consistent or significant differences in cell viability were detected with the addition of FL (data not shown). No secretion of basic fibroblast growth factor (bFGF), another potent angiogenic growth factor shown to be expressed by leukemia cells (17), was detected using the above models (data not shown).

Inhibition of VEGF secretion with the FLT-3 small-molecule inhibitor, SU11657

The relationship between VEGF and FLT-3 was explored further using the small-molecule RTK inhibitor, SU11657, which inhibits FLT-3, KIT, VEGFR2, and platelet-derived growth factor receptor (PDGFR). Exposure of ALL-3 cells to sublethal concentrations (100 nmol/L and 1 μ mol/L) of SU11657 significantly reduced FL-induced VEGF secretion by 62% and 74%, respectively ($P = 0.037$ and $P = 0.012$; Fig. 1A). In ALL-2, SU11657 significantly reduced VEGF secretion in cells cultured both with and without FL (Fig. 1B). P-14 xenograft cells showed a similar SU11657 response compared with ALL-3, significantly reducing FL-induced VEGF secretion at 1 μ mol/L (Fig. 1C). No statistically significant decreases in VEGF secretion by ALL-4, -7, and -17 were detected after exposure to SU11657, both with and without FL (data not

Table 1. Characteristics of pediatric ALL xenografts used in the study

Xenograft	Subtype	FLT-3 expression	VEGF secretion (pg/million cells)		<i>P</i>
			–FL	+FL	
ALL-2	BCP-ALL	5.2	1,060 \pm 311	1,456 \pm 556	0.6
ALL-3	BCP-ALL	6.8	1,220 \pm 206	4,458 \pm 680 ^a	0.0002
ALL-4	BCP-ALL	2.8	213 \pm 78	242 \pm 141	0.1
ALL-7	BCP-ALL	1.9	3,776 \pm 713	4,584 \pm 797	0.4
ALL-8	T-ALL	1.7	nd	nd	
ALL-10	BCP-ALL	1.9	nd	nd	
ALL-11	BCP-ALL	1.7	nd	nd	
ALL-16	T-ALL	1.2	nd	nd	
ALL-17	BCP-ALL	4.7	1,052 \pm 315	1,334 \pm 273	0.49
ALL-18	BCP-ALL	1.9	nd	nd	
ALL-19	BCP-ALL	2.5	nd	nd	
P-14	MLL	7.9	878.1 \pm 187.3	1,979.4 \pm 479.4 ^a	0.008

Abbreviation: BCP-ALL, B-cell precursor ALL; T-ALL, T-lineage ALL; nd, not detected.

^aSignificant increase with the addition of FL.

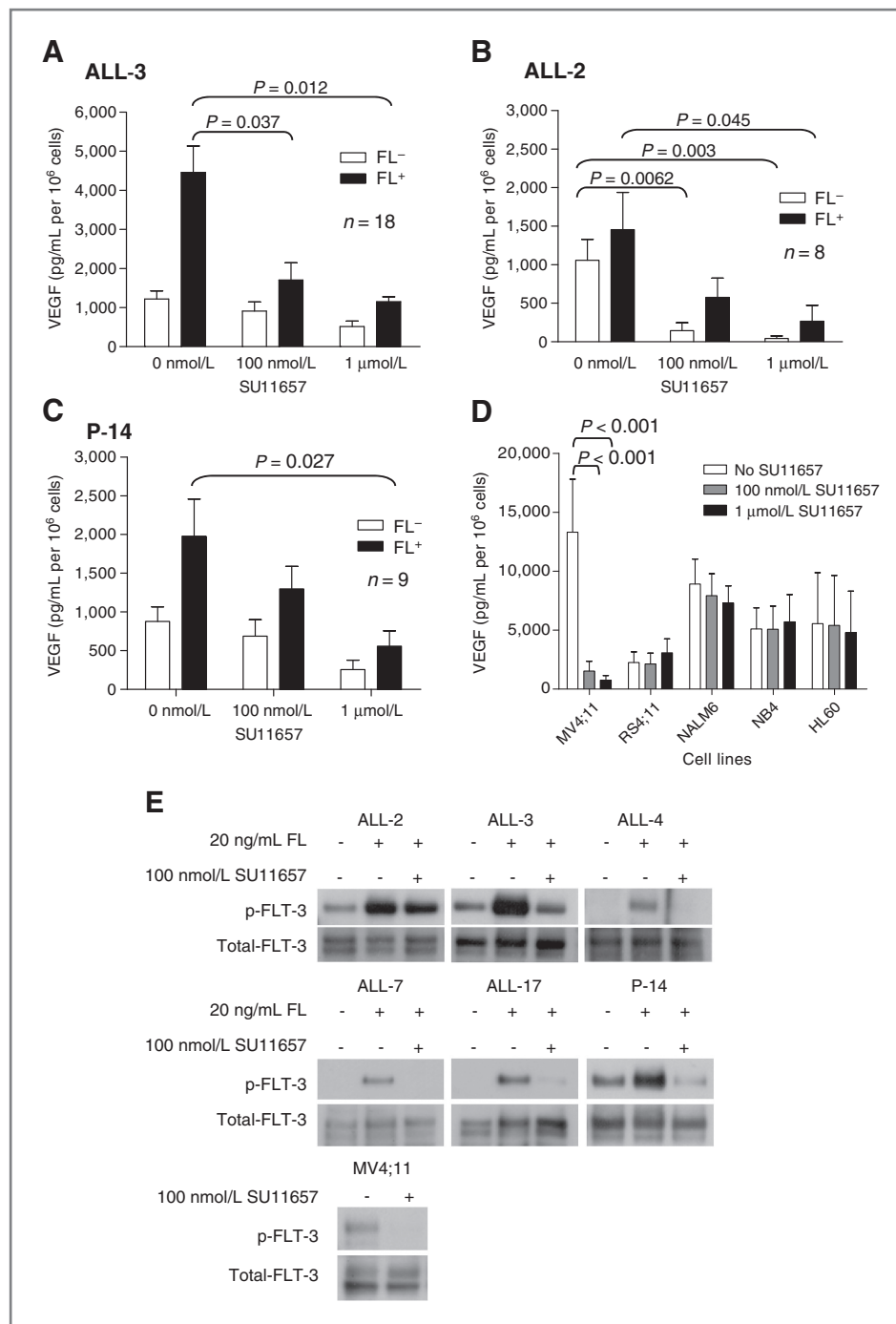


Figure 1. Effects of SU11657 on VEGF secretion (A–D) and FLT-3 phosphorylation (E). Modulation of VEGF secretion by SU11657 in ALL-3 (A), ALL-2 (B), P-14 (C), and leukemia cell lines (D) was measured by ELISA 72 hours after seeding cell cultures. Results are the mean \pm SE of at least 3 separate experiments. E, phospho- and total-FLT-3 immunoprecipitated from whole-cell lysates of ALL xenograft cells and the MV4;11 cell line.

shown), indicating an alternative mechanisms compared with ALL-2, -3, and -14. MV4;11 cells exhibit constitutively high levels of VEGF secretion, which was significantly inhibited by SU11657 exposure by more than 89% ($P < 0.001$; Fig. 1D). This effect was not observed in the established leukemia cell lines NALM6 and HL60, which did not express detectable cell surface FLT-3, or NB4 (RFI 7.1) and RS4;11, which do not have a characterized FLT-3 mutation.

SU11657 inhibits FLT-3 phosphorylation and downstream signaling pathways

To elucidate the pathway between FLT-3 activation and VEGF secretion, the phosphorylation status of FLT-3 and SU11657-mediated downstream effects were examined. Among the 6 xenografts examined, because of expression of FLT-3 and secretion of VEGF, basal phosphorylation of FLT-3 could be detected in ALL-2, -3, and P-14 (Fig. 1E). The addition of FL (20 ng/mL) increased

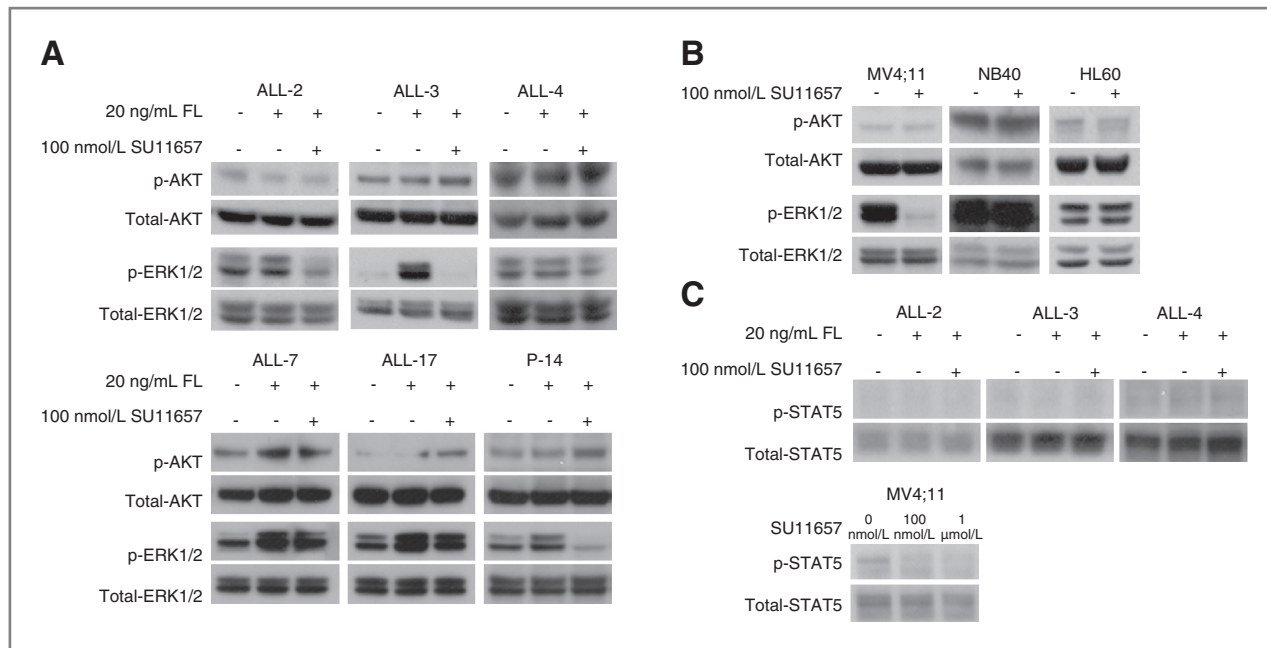


Figure 2. Effects of SU11657 on the FLT-3 signaling pathway. Cells were treated with 100 nmol/L SU11657 for 2 hours, FL for another 15 minutes, and then immunoblotted for the indicated proteins.

the phosphorylation of FLT-3 receptor in all 6 xenograft lines. The xenografts with the highest levels of FLT-3 expression (ALL-2, -3, and P-14; Table 1) showed the most pronounced phosphorylation. SU11657 (100 nmol/L) reduced the FL-induced phosphorylation of FLT-3 in all ALL xenograft lines. In ALL-3, -4, -7, -17, and P-14, a reduction to basal phosphorylation levels was observed, whereas an approximate 60% reduction in FLT-3 phosphorylation was achieved in ALL-2. In the case of MV4;11 cells, SU11657 also inhibited FLT-3 phosphorylation to undetectable levels (Fig. 1E). The effects of SU11657 on downstream targets of FLT-3 were then explored. FLT-3 activation results in signal transduction via the AKT and mitogen-activated protein kinase (MAPK) pathways (18). As shown in Fig. 2A, phosphorylation of AKT was unaffected by either FL or SU11657 across 6 xenograft lines. In contrast, basal and FL-induced phosphorylation of ERK1/2 varied substantially between xenografts. The most profound effects of FL stimulation were observed in ALL-3 and P-14, with smaller increases observed in ALL-2 and -17. ALL-4 showed no change in basal levels of ERK phosphorylation. The addition of 100 nmol/L SU11657 produced varying changes to ERK1/2 phosphorylation, with minor reductions in ALL-4, -7, and -17, contrasting with reductions to basal levels in ALL-2, -3, and P-14. The extent of inhibition of ERK1/2 phosphorylation was commensurate with the reduction in VEGF secretion by xenografts (see Fig. 1).

Similar to the ALL xenograft cells, MV4;11, HL60, and NB4 cell lines exhibited detectable basal AKT phosphorylation with no reduction upon addition of 100 nmol/L SU11657 (Fig. 2B). ERK1/2 was endogenously activated in

MV4;11 cells, and the addition of SU11657 abolished ERK1/2 phosphorylation. This observation for MV4;11 cells is consistent with the results for both phospho-ERK1/2 suppression and inhibition of VEGF secretion in ALL-2, -3, and P-14.

Because FLT-3 with an ITD has also been reported to signal through the STAT pathway (19), activation of STAT5 was also examined. No changes in phospho-STAT5 were detected in any of the ALL xenograft lines tested (Fig. 2C), which have been previously shown to lack ITDs (data not shown). The positive control (ITD) MV4;11 cells exhibited the expected phospho-STAT5 and subsequent reduction to below detectable levels upon the addition of SU11657 (Fig. 2C).

Effects of RTK and signaling pathway inhibitors on the FLT-3/VEGF relationship

To further examine the FLT-3 signaling pathway with respect to VEGF secretion in leukemia, alternative RTK and signaling inhibitors were used (Supplementary Table S1 and Fig. S1). Across all the inhibitors tested against MV4;11 cells, the effects of SU11657 were distinctly the most potent on VEGF secretion (Fig. 3A). Even at 10 nmol/L, there was a 43% ($P = 0.038$) decrease in VEGF secretion. At higher concentrations of SU11657 (100 nmol/L and 1 μmol/L), VEGF secretion was decreased by 63% ($P = 0.0008$) and 84% ($P < 0.0001$), respectively. At 10 nmol/L, the other inhibitors tested did not show any decreases in VEGF secretion. A 27% decrease in VEGF secretion occurred at 100 nmol/L SU5416, with a further decrease to 50% at 1 μmol/L. The other RTK inhibitors showed observable effects

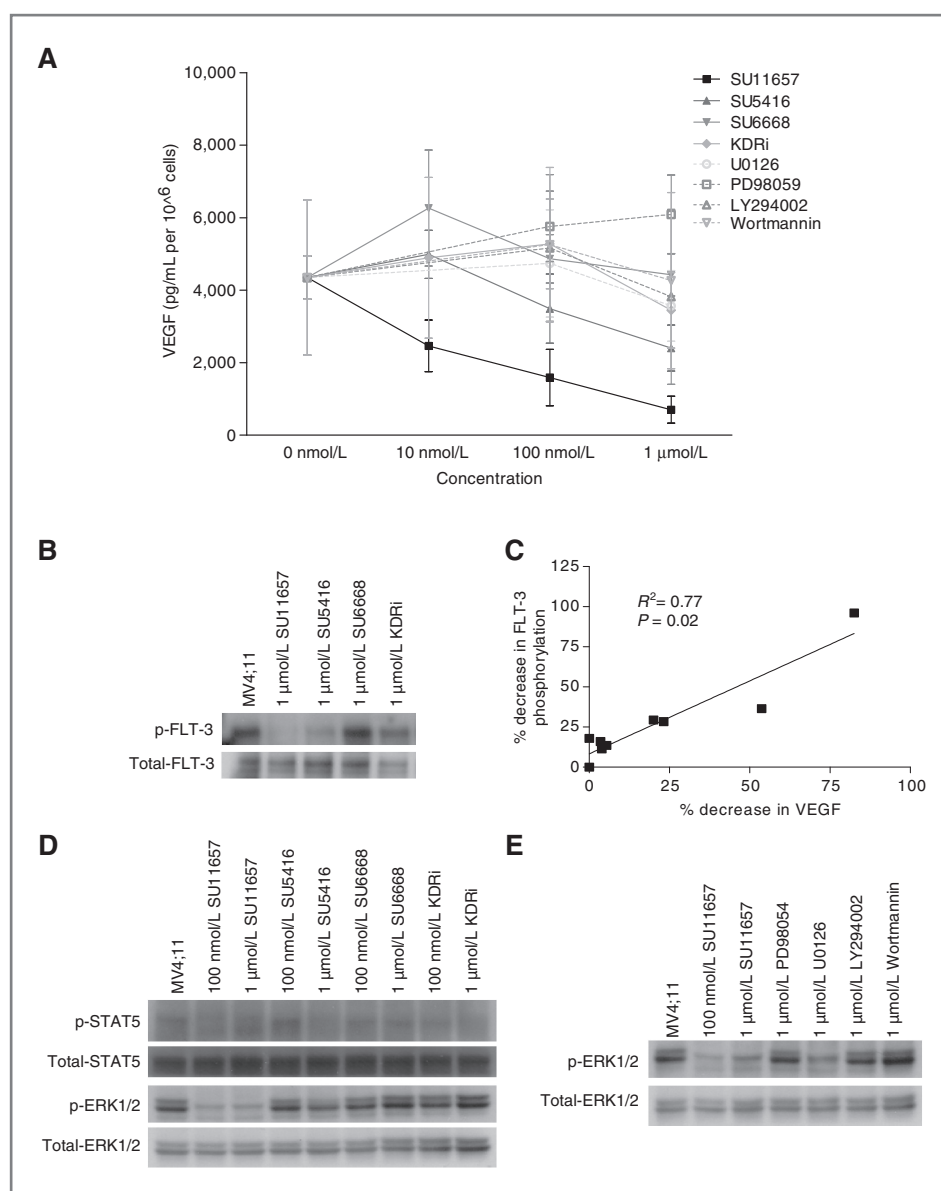


Figure 3. Pharmacologic dissection of the FLT-3/VEGF signaling pathway in MV4;11 cells. Effects of signaling inhibitors on VEGF secretion (A) and phospho-FLT-3 (B). C, correlation between inhibition of phospho-FLT-3 and VEGF secretion. D, effects of RTK inhibitors on phosphorylation of STAT5 and ERK1/2. E, effect of signaling inhibitors on ERK1/2 phosphorylation.

only at 1 μmol/L. Overall, SU11657 showed the strongest inhibition of VEGF secretion followed by SU5416, compared with SU6668 (7%) and KDRi (28%). The specificities of the inhibitors are shown in Supplementary Table S1.

The effects of these inhibitors were subsequently tested on FLT-3 and its downstream mediators. The decrease in VEGF secretion caused by SU11657 in MV4;11 (Fig. 3A) corresponded with a comparable decrease in FLT-3 receptor phosphorylation by these compounds (Fig. 3B). There proved to be a significant correlation between the decrease in VEGF secretion by the inhibitors (100 nmol/L and 1 μmol/L) and the decrease in the tyrosine phosphorylation of the FLT-3 receptor, $R^2 = 0.77$ and $P = 0.02$ (Fig. 3C). A comparison of the effects of these RTK inhibitors on phosphorylation of downstream signaling mediators

ERK1/2 and STAT5 is shown in Fig. 3D. The phosphorylation of STAT5 varied in response to the different inhibitors, with the greatest inhibition occurring with SU11657, followed by SU5416 (at 1 μmol/L). As was shown previously, the endogenous activation of AKT was not attenuated by SU11657, nor any of the other RTKs examined (data not shown). In terms of the MAPK pathway, ERK1/2 phosphorylation was dramatically decreased by SU11657 (100 nmol/L and 1 μmol/L), which was the only inhibitor to markedly decrease the phosphorylation of ERK1/2, as only minor decreases were caused by 1 μmol/L SU5416 and SU6668 (22% and 15%, respectively). No changes to the phosphorylation status of either STAT5 or AKT were observed with any of the pathway inhibitors (data not shown). The MAPK inhibitor, U0126, caused a decrease (45%) in ERK1/2

phosphorylation (Fig. 3E). This however, did not translate to a dramatic decrease in VEGF secretion (Fig. 3A). Thus, overall the effects observed with SU11657 were the most potent compared with other inhibitors assessed and all the parameters tested, on the MV4;11 cell line.

The effects of these inhibitors were tested on ALL-3 xenograft cells. At equivalent concentrations (1 $\mu\text{mol/L}$), SU11657 had the highest potency (80%) in the reduction of VEGF secretion (Fig. 4A). A smaller reduction (35%–40%) in VEGF secretion was observed with SU5416, SU6668, and KDRi. The effects of ERK (PD98059, U0126, and MEKi) and AKT (LY294002 and wortmannin) inhibitors on VEGF secretion were also tested. A small reduction ranging from 25% to 40% was observed, with the MEK inhibitor having the most pronounced effect (42% reduction). However, the only statistically significant reduction in VEGF secretion occurred with SU11657 at both 100 nmol/L (59% reduction) and 1 $\mu\text{mol/L}$ (80%; $P = 0.032$ and $P = 0.015$, respectively).

As observed with VEGF secretion, the addition of SU11657 (at both 100 nmol/L, $P = 0.032$ and 1 $\mu\text{mol/L}$, $P = 0.015$), significantly decreased the FL-induced phosphorylation of FLT-3 (Fig. 4B). The other RTK inhibitors SU5416, SU6668, and KDRi, which had previously caused minor decreases in VEGF secretion (Fig. 4A), induced concomitant moderate changes in FLT-3 as well as ERK1/2 phosphorylation (Fig. 4B). As was the case in the MV4;11 cell line, the decrease in VEGF secretion caused by different RTK inhibitors significantly correlated with their effects on FLT-3 activation ($P = 0.017$, Fig. 4C).

Inhibitors (PD98059, U0126, MEKi, LY294002, and wortmannin) were also used to clarify the downstream signaling of FLT-3. In accordance with the results shown above, no changes in AKT phosphorylation were observed. However, ERK1/2 phosphorylation was decreased by the addition of U0126 (45%) and to an even greater extent by the MEK inhibitor (61%), both at 1 $\mu\text{mol/L}$ as shown in Fig. 4D.

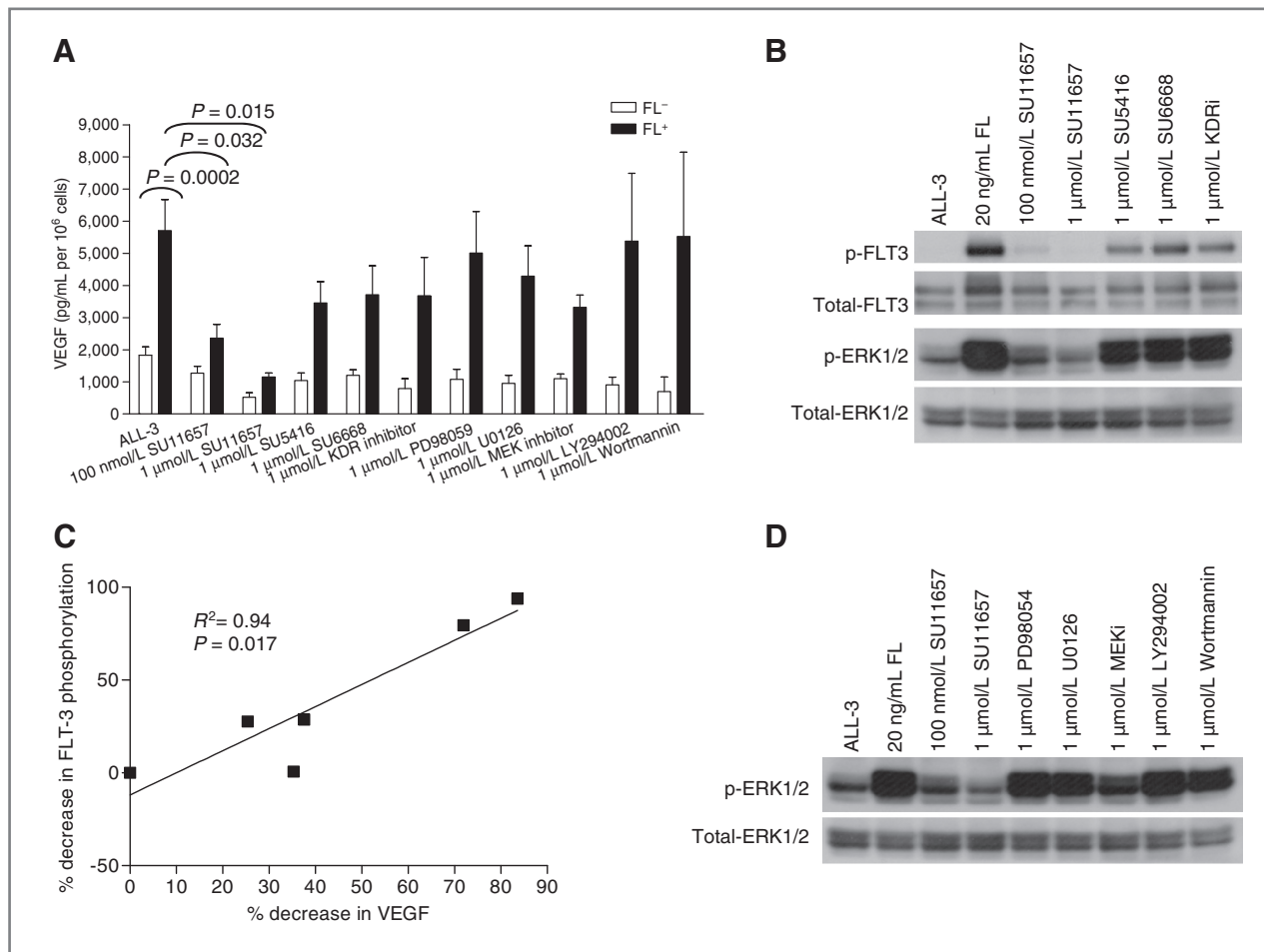


Figure 4. Effect of signaling inhibitors on VEGF secretion (A) and FLT-3 signaling (B) in ALL-3 xenograft cells. C, correlation between inhibition of FLT-3 phosphorylation and decrease in VEGF secretion. The decrease in VEGF and phospho-FLT-3 was calculated relative to the FL-induced VEGF secretion. D, effect of signaling pathway inhibitors on phospho-ERK1/2 in ALL-3 xenograft cells.

Verification of VEGF secretion via the FLT-3 signaling pathway

FLT-3-specific blocking antibodies (EB10 and D43) were used to block FL-induced phosphorylation of FLT-3 (20). ALL-3 xenograft cells were used to examine the effect of these antibodies on FLT-3 phosphorylation, along with their impact on downstream targets of FLT-3 signaling. The MV4;11 cell line, with its constitutively active receptor, could not be included in this set of experiments. Humanized nonspecific antibodies were used for controls in all experiments. The EB10 antibody exerted a concentration-dependent inhibition of VEGF secretion by ALL-3 cells, whereas D43 caused complete inhibition at

both concentrations tested (Fig. 5A). Furthermore, the ability of both antibodies to inhibit FLT-3 and ERK1/2 phosphorylation were consistent with their effects on VEGF secretion at both concentrations tested (Fig. 5B).

In addition to inhibition of FLT-3 by pharmacologic means as well as neutralizing antibodies, FLT-3 siRNA was used in ALL-3 xenograft cells and the MV4;11 cell line. Representative immunoblots shown in Fig. 5C indicate 40% to 45% FLT-3 knockdown. Consistent with the degree of FLT-3 knockdown, VEGF secretion at 72 hours posttransfection was also decreased by approximately 30% in FLT-3 siRNA-transfected cells compared with controls (Fig. 5D).

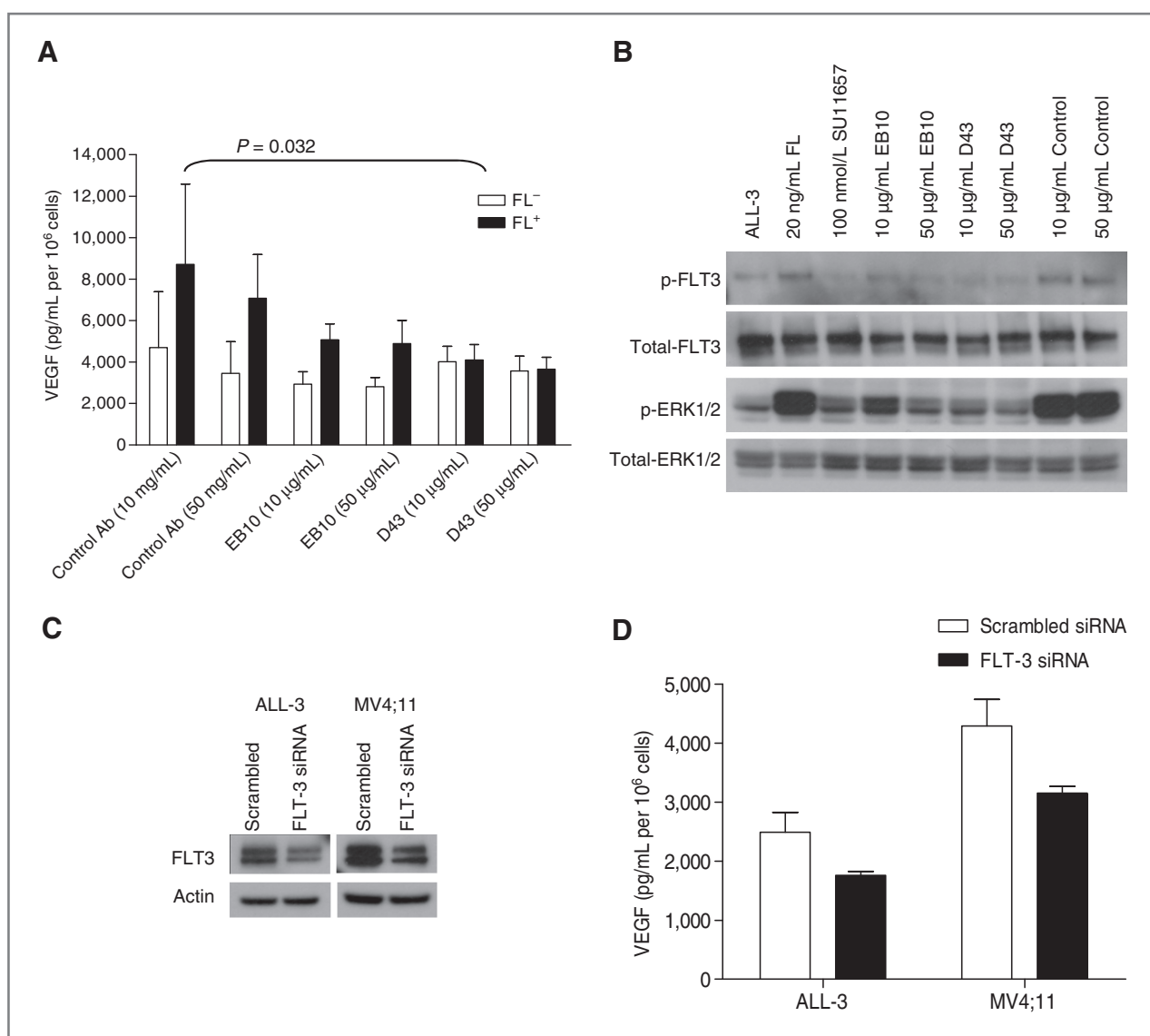


Figure 5. The effects of FLT-3–blocking antibodies and FLT-3 knockdown on VEGF secretion. **A**, cells were cultured for 72 hours with EB10 and D43 ± FL and VEGF measured by ELISA. **B**, ALL-3 cells were incubated for 2 hours with the appropriate antibody, 20 ng/mL FL added for an additional 15 minutes, and then cells were harvested for immunoblot analysis as described above. FLT-3 expression (**C**) and VEGF secretion (**D**) assessed following 72 hours of culture.

Discussion

While increased VEGF has been detected in the serum of patients with various hematologic malignancies (21), it may not necessarily be cancer derived, as other cells such as platelets and megakaryocytes are a potential source (22). The use of pediatric ALL xenograft cells in this study supports previous observations that directly show VEGF secretion by leukemia cells (21). The secretion of endogenous VEGF by leukemia cells infers an explicit alteration of the bone marrow microenvironment by these cells, analogous to the invasion and metastasis of solid tumors [as reviewed by Folkman (23)]. Although capillary formation by endothelial cells in the bone marrow has been reported in leukemia (17), its role has not been as widely established compared with its solid tumor counterparts. While other studies have shown the induction of VEGF by growth factors such as IGF-I (24), GM-CSF, and IL-5 (25), the results from this study are the first to show secretion of VEGF via the activation of FLT-3 pathway. Aside from the novel finding of FL-induction of VEGF in childhood ALL, FL is also secreted by bone marrow stromal cells (26) suggesting a paracrine interaction between the bone marrow microenvironment and leukemia cells.

The panel of ALL xenograft cells used in this study is representative of the heterogeneity of this disease in terms of ALL subtype and clinical outcome of the patients from whom they were derived (13). The 2 xenografts that secreted the largest amounts of VEGF also exhibited the highest cell surface FLT-3 expression (ALL-3 and P-14). Notably, these 2 xenografts harbor translocations involving the mixed-lineage leukemia (*MLL*) gene at 11q23 [ALL-3 has a t(11;19) translocation, and P-14 xenograft has a non-classical, more complex translocation (14)], which is consistent with the high FLT-3 expression identified in the *MLL* subtype by gene expression array studies (27).

Although, FLT-3 is expressed in leukemias of both lymphoid and myeloid lineage (28), the development of small-molecule FLT-3 inhibitors was driven by the predominance of FLT-3 mutations in AML and their association with a poor prognosis for both adult (29) and pediatric patients (30, 31). Results from experiments involving inhibitor, SU11657, in this study provided evidence of a relationship between FLT-3 activation and VEGF secretion. Notably, the addition of FL induced significant increases in VEGF secretion in both ALL-3 and P-14 xenograft cells. Furthermore, inhibition of the FLT-3 receptor with SU11657, reversed the FL-induced effect in both cases. These results strengthen the evidence indicating that activation of FLT-3, by its ligand, induces the secretion of VEGF in these ALL xenograft cells.

In ALL-2 and P-14, SU11657 caused a reduction in VEGF secretion regardless of the presence or absence of FL. Such a finding indicates that FLT-3 signaling may still play a role in VEGF secretion in these xenograft cells. Because there was no observed increase in VEGF secretion

upon the addition of FL to cultures of ALL-4, -7, and -17 xenograft cells, it was expected that there would be no corresponding change with the addition of SU11657, which proved to be the case. It is likely that the secretion of VEGF in these xenografts is via an alternative pathway. Moreover, the effects of SU11657 on basal VEGF expression in ALL-2 and P-14 coincide with detectable basal FLT-3 phosphorylation in these xenografts, which was not apparent in ALL-4, -7, or -17.

Several other RTK inhibitors were also used to explore the FLT-3 signaling pathway and its induction of VEGF. Their effects were compared with SU11657, which showed strong inhibitory effects against VEGF secretion. The alternative RTK inhibitors were not as effective as SU11657 in reducing VEGF secretion, and the minor decreases observed could be accounted for by the concurrent decrease in FLT-3 phosphorylation itself, which is consistent with published data on the inhibitory effects of these RTKs on FLT-3 phosphorylation (Supplementary Table S1). As these RTK inhibitors did not exert significant inhibitory effects, it appears that the activation of FLT-3, rather than VEGFRs most likely underpins the secretion of VEGF in ALL cells. The deactivation of ERK1/2 by U0126 and a MEK inhibitor showed a corresponding decrease in VEGF secretion, which indicates that the FLT-3 signal proceeds through the MAPK pathway in these cells.

Wild-type FLT-3 is known to transduce its signaling cascade principally via ERK1/2 (32), as well as through the PI3K pathway, leading to activation of AKT. However, it has also been reported that AKT is activated only by FLT-3 with ITDs in AML cells (33). Our results show that after activation of the FLT-3 receptor with FL, the MAPK signaling pathway, but not the AKT pathway, was specifically activated. These results provide evidence that the pathway by which FLT-3 induces VEGF secretion is more likely to occur through ERK1/2 phosphorylation rather than AKT. This possibly is further supported by the observed concomitant decrease in ERK1/2 deactivation with SU11657. Such an observation would indicate that AKT signaling is not primarily involved in FLT-3 induction of VEGF, and is confirmed with the lack of alteration detected in either ALL xenograft cells or the MV4;11 cell line. FLT-3-ITD initiates the activation of Ras/MAPK and AKT, in a similar manner to the wild-type receptor (11). However, STAT5 also plays a role in FLT-3-ITD signaling (19). Our results show that 100 nmol/L SU11657 reduced phospho-STAT5 in MV4;11 cells to below detectable levels. In contrast, the ALL xenograft cells had no detectable phospho-STAT5, which is consistent with wild-type receptor signaling (34). Thus, when taken together, our results suggest that STAT5 does not play a direct role in the signaling leading to VEGF secretion.

SU11248 (sunitinib) is an analogue of SU11657 that has progressed to clinical trials (35). SU11248 decreased VEGF secretion by AML cells and the MV4;11 cell line (36). The *in vivo* single agent efficacy of SU11248 has been previously tested in our xenograft model and significantly

delayed the progression of ALL-2 (37). It remains to be determined whether the *in vivo* effect of SU11248 on ALL-2 is related to the almost complete reduction of VEGF secretion caused by SU11657 *in vitro* in these cells.

The FLT-3–blocking antibodies tested in this study (EB10 and D43), which block ligand binding and negate FLT-3 activity, were previously shown to significantly decrease engraftment of AML cells in NOD/SCID mice, and prolong the survival of mice engrafted with ALL cell lines and primary cells (20). Our results, showing that these FLT-3–blocking antibodies also reduce FL-induced VEGF secretion, are consistent with the SU11657 results. To confirm the contribution of FLT-3 activity to VEGF secretion, the receptor was down-regulated with siRNA.

In summary, this study provides 3 lines of evidence of an intricate relationship between VEGF secretion and FLT-3 activity in ALL xenograft cells. VEGF was previously shown to be a downstream target of Src with the signaling cascade mediated through the MAPK pathway (38). Our research clearly shows that FLT-3 stimulation of VEGF secretion involves the MAPK pathway through its activation of ERK1/2 and not via the PI3K/AKT pathway. This contrasts with the findings of Zhang

and colleagues (39) who showed the dominance of the PI3K pathway in FLT-3 signaling. In addition to the work of O'Farrell and colleagues (36), our research also shows that VEGF secretion can be enhanced in ALL cells with the exogenous addition of FL. Considering the clinical relevance of FLT-3 and VEGF in leukemia, this novel finding provides additional rationale for the inclusion of FLT-3 inhibitors in the treatment of ALLs expressing high levels of FLT-3.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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