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FGF2 from marrow microenvironment promotes resistance to FLT3 inhibitors in acute myeloid leukemia

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Abstract

Potent FLT3 inhibitors such as quizartinib (AC220) have shown promise in treating acute myeloid leukemia (AML) containing FLT3 internal tandem duplication (ITD) mutations. However, responses are not durable and resistance develops within months. In this study, we outline a two-step model of resistance whereby extrinsic microenvironmental proteins FLT3 ligand (FL) and fibroblast growth factor 2 (FGF2) protect FLT3-ITD+ MOLM14 cells from AC220, providing time for subsequent accumulation of ligand-independent resistance mechanisms. FL directly attenuated AC220 inhibition of FLT3, consistent with previous reports. Conversely, FGF2 promoted resistance through activation of FGFR1 and downstream MAPK effectors; these resistant cells responded synergistically to combinatorial inhibition of FGFR1 and FLT3. Removing FL or FGF2 from ligand-dependent resistant cultures transiently restored sensitivity to AC220, but accelerated acquisition of secondary resistance via reactivation of FLT3 and RAS/MAPK signaling. FLT3-ITD AML patients treated with AC220 developed increased FGF2 expression in marrow stromal cells, which peaked prior to overt clinical relapse and detection of resistance mutations. Overall, these results support a strategy of early combination therapy to target early survival signals from the bone marrow microenvironment, in particular FGF2, to improve the depth of response in FLT3-ITD AML.

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Introduction

Fms-like tyrosine kinase 3 (FLT3) is a class III receptor tyrosine kinase expressed on early hematopoietic stem and progenitors cells, and is important for hematopoiesis. FLT3 is activated by its ligand, FLT3 ligand (FL), which promotes receptor dimerization and growth via activation of STAT5, Ras/MAPK and Akt pathways (1). FLT3 is frequently mutated in acute myeloid leukemia (AML). The most common genetic alteration is the internal tandem duplication (ITD) in the juxtamembrane domain of the protein (2), which disrupts the autoinhibitory mechanism of the juxtamembrane domain and results in chronic FLT3 activation (3). FLT3-ITDs are present in ~20% of newly diagnosed AML and lead to increased risk of relapse (4,5). FLT3 is also frequently mutated by point mutations in the activation loop of the protein, most commonly at D835, and is present in ~10% of newly diagnosed AML (6,7). However the prognostic significance of these kinase domain mutations is less clear.

The success of tyrosine kinase inhibitors (TKIs) such as imatinib in chronic myeloid leukemia (CML) generated considerable enthusiasm for FLT3 inhibitors in AML. Early generation FLT3 inhibitors only achieved a transient reduction in peripheral blasts, whereas bone marrow blasts were less affected (8,9). Recently, quizartinib (AC220), a more potent FLT3 inhibitor, was developed and demonstrated a 50% composite complete remission rate in phase II clinical trials (10). However, despite a higher response rate, the durability of AC220 response is still quite limited, and most patients develop resistance after a few months of therapy. Similar to resistance in CML, point mutations in FLT3 have been shown to decrease affinity of AC220 for its target, leading to clinical resistance (11–13). In particular, mutations of the “gatekeeper” residue F691L and mutations in the activation loop (the region around D835) confer resistance to AC220 both *in vitro* and *in vivo* (12). However, resistance also occurs in the absence of FLT3 mutations, although the nature of this resistance is less clear.

Recently, the ligand for FLT3 (FL) was also found to promote resistance to FLT3 inhibitors (14). FL ligand expression increases during therapy with FLT3 inhibitors, providing one potential explanation of why leukemia cells in the bone marrow are relatively more resistant to FLT3 inhibitors (14). However, FL is likely not the only component of the bone marrow microenvironment that provides sanctuary to leukemia cells. We hypothesized that other proteins from the bone marrow microenvironment would promote resistance to FLT3 inhibitors and tested known microenvironmental cytokines, growth factors, and soluble proteins for their ability to protect the FLT3-ITD AML cell line, MOLM14, from AC220 treatment. Fibroblast growth factor 2 (FGF2) was one of the most effective proteins in promoting survival of MOLM14 cells in the presence of AC220, and led to resistance in extended cultures. FGF2 is highly expressed by bone marrow stromal cells and plays an active role in hematopoietic colony formation in vitro (15,16), and hematopoiesis in vivo (17,18). Here, we determine the mechanism of FGF2-mediated resistance in FLT3-ITD AML and contrast it with resistance induced by FL.

Methods

Cell lines

MOLM14 cells were generously provided by Dr. Yoshinobu Matsuo (Fujisaki Cell Center, Hayashibara Biochemical Labs, Okayama, Japan) (19). BAF3 cells with retrovirally introduced FLT3-ITD were created as previously described (20), and then retrovirally transfected with empty pMX vector or human FGFR1 in pMX. The transfected cells were selected with 1μg/ml puromycin. The human stromal cell lines HS-5 and HS-27 were kindly provided by Dr. Beverly Torok-Storb (Fred Hutchinson Cancer Research Center, Seattle, WA). Cell lines were maintained in RPMI1640 media supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin/100 μg/mL streptomycin and 2 mM L-glutamine (R10) at 37°C in 5% CO_2, except MOLM13 which was grown in RPMI supplemented with 20% FBS (R20). All cell lines were authenticated by extensive functional and genetic analysis in our lab.

Viability assays

MOLM14 cells were incubated in media supplemented with recombinant proteins obtained from Peprotech (Rocky Hill, NJ, USA) at indicated concentrations and treated as described. Viability was assessed with MTS reagent, CellTiter 96 AQueous One Solution Cell Proliferation Assay from Promega Corporation (Madison, WI, USA), or with Guava ViaCount reagent and cytometer (Millipore, Billerica, MA, USA).

FGF2- and FL-dependent resistant cultures

10^7 MOLM14 cells in 10 ml of R10 media, or supplemented with FGF2 or FLT3 ligand (FL) at 10 ng/ml, were cultured with 10 nM quizartinib. Media, recombinant protein, and quizartinib were replaced every 2–3 days. Cell viability was evaluated every 2–3 days using Guava ViaCount reagent.

Tyrosine kinase inhibitors

Quizartinib (AC220) and sorafenib were purchased from LC labs (Woburn, MA, USA). PD173074 was purchased from Selleck (Houston, TX, USA). Ponatinib was provided by Ariad Pharmaceuticals (Cambridge, MA). Crenolanib was provided by AROG pharmaceuticals (Dallas, TX).

siRNA and Kinase Inhibitors

siRNAs were purchased from Thermo Fisher Scientific Dharmacon RNAi Technologies (Waltham, MA, USA). MOLM14 cells were washed in PBS, resuspended in siPORT (120 mM Trehalose, 20 mM HEPES, 1 mM Myo-Inositol, 1 mM KCl, 1 mM $MgCl_2$, 1 mM K_2HPO_4, 0.4 mM KH_2PO_4, 2.14 mM KOH and 1 mM Glutathione) (21) at 1:6 dilution, and electroporated using a square wave protocol (350V, 1.5 seconds, 2 pulses, 0.1s interval) in a BioRad Gene Pulser XCell (Hercules, CA, USA). After 48 hours, FGF2 and quizartinib were added and viability was assessed with MTS after another 48 hours.

Primary AML samples

Frozen viable cells from FLT3-ITD AML patients were washed in IMDM with 20% heat-inactivated FBS and then cultured overnight in RPMI with 25% FBS, 100 U/mL penicillin/100 μg/mL streptomycin, 2 mM L-glutamine, 10 ng/ml IL-3, 10 ng/ml SCF, 10 ng/ml G-CSF, and 10 ng/ml GM-CSF (22). The cells were washed the following day. Fresh FLT3-ITD AML mononuclear cells were purified over Ficoll gradient as described (23). 7×10^6 primary cells were plated in 96-well plates with R10 alone or supplemented with FGF2; and then treated with AC220. Viability was measured after 72 hours by MTS.

Immunoblot analysis

Cells were treated as described, washed in PBS, and lysed in lysis buffer (Cell Signaling, Danvers, MA, USA) supplemented with Complete protease inhibitor (Roche, Indianapolis, IN, USA) and phosphatase inhibitor cocktail-2 (Sigma-Aldrich, St. Louis, MO, USA). Equal amounts of protein were fractionated on 4–15% Tris-glycine polyacrylamide gels (Criterion gels, Bio-Rad), transferred to PVDF membranes, and probed with antibodies against FGFR1, pSTAT5, STAT5, pMEK1/2, MEK1/2, pERK1/2, ERK1/2, pS6, and S6 (Cell Signaling, Danvers, MA, USA). Other Antibodies: actin (MAB1501, Millipore), FLT3 (S-18, Santa Cruz Biotechnology, Dallas, TX, USA), FGF2 (SC-79, Santa Cruz Biotechnology), and FL (EP1140Y, AbCam, Cambridge, MA, USA).

Sequencing

Primers were used to amplify the FLT3 kinase domain and for sequencing as described (12). Next-generation sequencing of resistant MOLM14 cells was performed as previously described (24).

Transwell assays

10^5 HS-5 and HS-27 cells were plated in 6-well plates in 2 ml media. 1 μm transwells were then placed over the stromal cells and 10^5 MOLM14 cells were plated in 2 ml media (4 ml total). The co-cultures were then treated with AC220 10 nM and PD173074 250 nM individually or in combination. After two days, the transwells with MOLM14 were removed and 2 ml fresh media and inhibitor added to stroma below. The co-cultures were incubated for another 2 days and MOLM14 cells assessed for annexin-5 staining by GuavaNexin (Millipore). The underlying stromal cultures were evaluated by MTS assay for viability. Each condition was performed in triplicate.

Immunohistochemistry

All patient specimens were obtained after informed consent on protocols approved by the Institutional Review Board of Oregon Health & Science University. Immunohistochemistry for FGF2 was performed as previously described (25). Slides were scanned with an Aperio ScanScope AT microscope using 20X objective (Vista, CA, USA). The FGF2 staining was then quantitated with Aperio ImageScope software using a macro kindly provided by Dr. Brian Ruffell in Dr. Lisa Coussens' laboratory at OHSU.

Statistical methods

Graphical and statistical data were generated using either Microsoft Excel or GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Continuous variables were compared by Student's *t*-test for two independent samples using Microsoft Excel. A P value <0.05 was considered significant.

Results

FGF2 protects MOLM14 cells from AC220

To screen for microenvironmental factors that protect AML cells from FLT3 inhibitors, we cultured MOLM14 cells with graded concentrations of selected proteins expressed in the bone marrow microenvironment (Agarwal et al., manuscript in preparation) in the presence of 10 nM AC220. Viability was assessed after two days by MTS assay and averaged over all concentrations (1, 10, and 100 ng/ml). FL and FGF2 were the only proteins that increased viability >2 standard deviations and are highlighted in red (Figure 1A). The most protective molecule in this screen was FL, which has been previously reported to attenuate the effect of FLT3 inhibitors (14), providing an internal positive control. We confirmed the results using lower concentrations (1 ng/ml and 0.1 ng/ml, see Supplement for discussion of physiologic concentration) and found again that FL and FGF2 were the most protective overall (Figure 1B). We focused our attention on FGF2, which is highly expressed in bone marrow (25), and FL as two distinct mechanisms of ligand-mediated resistance.

To test the protective effects of FGF2 and FL over a longer duration, MOLM14 cells were cultured over many weeks with 10 nM AC220, media, FL, and FGF2 replaced every 2–3 days. Addition of FL or FGF2 promoted outgrowth in all replicates (N=4) at around 6 weeks (Figure 1C). In contrast, only 2/4 replicates cultured in media without FGF2 or FL developed resistance, and it took nearly 12 weeks, providing strong evidence that continuous exposure to protective ligand accelerates development of resistance.

FGF2 activates FGFR1 to protect FLT3-ITD AML cells

To test if FGF2 protects cells through activation of an FGF receptor (FGFR), we treated MOLM14 cells with the pan-FGFR inhibitor PD173074 in the presence of FGF2 and AC220. Even at doses as low as 25 nM, PD173074 was able to attenuate the protective effect of FGF2 (Figure 2A). Since FGF2 is capable of binding multiple FGFRs (26), we measured the relative expression of FGFRs 1–4 by Taqman qPCR and found that FGFR1 was the most highly expressed FGFR in MOLM14 cells (Supplemental Figure 1A). siRNA targeting blocked the protective effect of FGF2 in the presence of AC220 (Figure 2B), and efficiently reduced mRNA and protein expression (Supplemental Figure 1B and Figure 2C). In contrast, siRNAs targeting FGFR2, FGFR3, and FGFR4 had no effect on FGF2 protection (Figure 2B and Supplemental Figure 1). To further test the role of FGFR1, we took advantage of the fact that BaF3 cells do not express FGFRs 1–4 (27). BaF3 cells expressing human FLT3-ITD (20) were subjected to a second retroviral infection with either an empty pMX vector or pMX containing human FGFR1. Expression of FGFR1 was confirmed by Western blot (Figure 2D). FGF2 was only protective against AC220 when FGFR1 was also expressed (Figure 2E,F2F). We tested whether FGF2 protected two other FLT3-ITD AML

cell lines (MOLM13 and MV-4-11) with lower FGFR expression from AC220 (Supplemental Figure 2A–D), but found no significant protective effect in these lines, indicating that FGFR expression is a prerequisite for FGF2-mediated protection.

FGFR1 is the most highly expressed FGFR in cytogenetically normal AML, with expression comparable to FLT3 and AXL, another receptor tyrosine kinase reported to mediate resistance in FLT3-ITD AML (28,29) (Supplemental Figure 2E). To test if FGF2 protects primary FLT3-ITD AML samples from AC220, we cultured fresh and thawed frozen viable primary AML cells with media alone (no ligand), or supplemented with FGF2, and treated with a gradient of AC220. FGF2 was highly protective of a subset of primary AML samples (example shown in Figure 2G), and enhanced viability in a dose-dependent and statistically significant manner (Figure 2H).

FGF2-mediated resistance is overcome with combined FGFR and FLT3 inhibition

MOLM14 cells grown in continuous 10 nM AC220 plus FGF2 or FL that developed resistance (Figure 1C) are henceforth referred to as **FGF2- or FL-dependent resistant cultures**. We tested the sensitivity of these resistant cells to multiple combinations of AC220 and the FGFR inhibitor PD173074 by creating a 64-well matrix with a gradient of AC220 on one axis, and overlaying a gradient of PD173074 on the other axis. The results are shown as a surface plot, with corresponding linear graphs of monotherapy AC220, monotherapy PD173074, and an equimolar combination of AC220 and PD173074 to highlight synergy (Figure 3A–C). Naïve MOLM14 cells were exquisitely sensitive to AC220 at low nM concentrations but were not affected by PD173074 in the absence of FGF2 (Figure 3A, see also Supplemental Figure 3). In contrast, FGF2-dependent resistant cultures were protected from AC220 at higher AC220 concentrations, and the addition of PD173074 was highly synergistic in overcoming this protection (Figure 3B). FL-dependent resistant cultures had a shifted AC220 dose-response curve (Figure 3C), but resistance was overcome by higher doses of AC220 (>10 nM) and PD173074 had minimal additional effect.

FGF2-dependent resistant cultures re-activate the MAPK pathway

To explore the mechanism of resistance in FGF2- and FL-dependent resistant cultures, we first evaluated the FLT3 gene for known resistance mutations in the activation loop (12). A single Y842C mutation was found at 4 months in one FGF2-supplemented culture, but the remainder of the FGF2-dependent resistant cultures did not have detectable resistance mutations. Phosphorylated FLT3 (pFLT3) and the MAPK pathway, which is downstream of both FLT3 and FGFR1, were then analyzed by Western blot. AC220 treatment of naïve MOLM14 cells effectively abrogated pFLT3 at all phosphorylation sites, pSTAT5, and pERK1/2 (lanes 1 and 2, Figure 4A). The single FGF2-dependent resistant culture with a Y842C mutation had partial reactivation of FLT3 despite the presence of AC220 (FGF2 R-1, lane 3). FL-dependent resistant cultures also had partial restoration of pFLT3, consistent with previous reports (14) (lanes 7–10). In contrast, FGF2-dependent resistant cultures R-2 to R-4 maintained phosphorylation of ERK but there was little pFLT3. To test if alternative kinase pathways might be altered in either FGF2- or FL-dependent resistant cultures, we used a phospho-kinase array to assess 46 phosphorylated proteins, but found that only the

MAPK signaling pathway was consistently restored in all FGF2- and FL-dependent resistant cultures (Supplemental Figures 4, 5).

Removing FGF2 or FL from resistant cultures leads to ligand-independent resistance and increased frequency of FLT3 mutations

To evaluate whether FGF2- and FL-dependent resistant cultures remained dependent on ligand after four months, we split the FGF2- and FL-dependent cultures and removed ligand from one subset of cultures (Figure 5B–C). The time when cells resumed exponential growth is indicated in gray and plotted on a timeline to allow direct comparison with Figure 1C. The single FGF2-dependent culture with the FLT3 Y842C mutation resumed exponential growth within a week after FGF2 was removed, while the remainder of FGF2- and FL-dependent cultures resumed exponential growth after about one month (Figure 5B,C). Of note, resistance after removal of FGF2 or FL developed more rapidly than initial resistance (~2 months) in the presence of FGF2 or FL. The cultures were evaluated for FLT3 activation loop mutations by Sanger sequencing at 4 and 6 months (Supplemental Figure 6), and then again at 8 months using a next-generation sequencing panel of 42 genes frequently mutated in AML (24) (Supplemental Table 2). Most of the FGF2- and FL-supplemented cultures did not have detectable resistance mutations during initial resistance, but removing FGF2 or FL greatly accelerated the acquisition of mutations. In addition, we identified frequent activating mutations of the RAS pathway, suggesting a strong selective pressure to reactivate RAS/MAPK signaling (Figure 5). The frequency of the KRAS G13D mutation was particularly notable but we suspect this mutation is present at a low level in the naïve MOLM14 cells, since 2/659 reads contained this exact mutation, although this is below the sensitivity of the assay to call a true mutation.

To confirm that the observed FLT3 mutations drive resistance, we compared the sensitivity of these cultures to multiple FLT3 inhibitors, including crenolanib, a FLT3 inhibitor that is not blocked by mutations in the activation loop (30). D839V and Y842C mutated cultures were resistant to AC220, sorafenib, and ponatinib, but remained sensitive to crenolanib (Figure 5D). In contrast, resistant cultures without FLT3 mutation remained resistant to crenolanib (single example shown, FL R-1). All resistant cultures remained sensitive to higher doses of FLT3 inhibitors, suggesting the continued importance of partial FLT3 signaling (Figures 3 and 5). In agreement with this result, most resistant cultures had partial restoration of FLT3 phosphorylation and reactivation of MAPK signaling by Western blot (Supplemental Figure 7), providing further evidence that the primary routes of resistance are mediated by FLT3 and RAS/MAPK.

FGFR inhibitors overcome paracrine protection of FGF2-expressing stroma and inhibit FGF2 autocrine stromal growth

FGF2 is expressed in normal bone marrow stroma (25,31). We evaluated if FGFR inhibitors could block the protective effects of FGF2-expressing stroma in co-culture with MOLM14 to overcome FGF2-mediated resistance. We used the human bone marrow stromal cell lines HS-5 and HS-27, which are derived from the same person but have distinct functional characteristics (32). HS-5 also expresses much higher amounts of FGF2 compared to HS-27 (Figure 6A). We cultured MOLM14 cells in media alone, with 10 ng/ml FGF2, or in

transwells above HS-5 and HS-27. The cells were then treated with 10 nM AC220, 250 nM PD173074, or a combination and assessed for viability after four days. PD173074 alone had no effect on MOLM14 cell viability (data not shown). MOLM14 cells cultured with recombinant FGF2 or in transwells over HS-5 stroma were protected from effects of AC220 treatment, but protection could be overcome by concomitant PD173074 treatment. In contrast, the protective effect of HS-27 was less substantial than HS-5, and unchanged by addition of PD173074 (Figure 6B). Since FGF2 is also reported to be an autocrine growth factor for stromal cells (17,18), we removed the transwells and assessed viability of the underlying HS-5 and HS-27 cells. Only HS-5 growth was significantly attenuated by addition of PD173074 (Figure 6C). In summary, addition of FGFR inhibitor overcomes protective effects of FGF2-expressing stromal co-cultures and attenuates autocrine growth of FGF2-expressing stroma (25).

FGF2 increases in marrow stroma during development of resistance to AC220

To test expression of FGF2 in FLT3-ITD AML patients, bone marrow core biopsies from patients on the AC220 trial at our institution were collected prior to treatment, during response to AC220, and at development of resistance (clinical characteristics in Supplemental Table 3). The marrow biopsies were stained for FGF2 by immunohistochemistry (IHC). A normal bone marrow biopsy from a surgical hip repair is shown for comparison in Figure 7A and a representative patient series is shown in Figure 7B, with the peripheral blood and bone marrow leukemia burden shown above the stained bone marrow biopsies. Of note, this patient acquired a FLT3 D835 mutation that was detected on day 158. FGF2 staining for all 10 patients was quantitated by Aperio ImageScope software (Figure 7C). FGF2 increased significantly during response to AC220, peaked with early resistance, and then decreased again after overt clinical resistance. FGF2 was primarily expressed in marrow stroma and not hematopoietic cells (CD45+) by immunofluorescence (Supplemental Figure 8). We investigated FLT3 mutations at the time of resistance: three of five patients acquired FLT3 resistance mutations during therapy; one patient had a D835 mutation prior to AC220; and three patients underwent allogeneic transplant prior to resistance (Figure 7C). Since FL expression is reported to be increased with FLT3 inhibitor treatment (14), we also attempted FL staining by IHC with two different antibodies but were unable to obtain reliable staining.

Discussion

Activation of FGFRs is known to be a driver in numerous malignancies including FGFR1 fusions in 8p myeloproliferative neoplasms (33,34); t(4;14) translocation with FGFR3 overexpression in multiple myeloma (35,36); FGFR3 mutations in bladder carcinoma (37); and both FGFR1 and FGFR3 fusions in glioblastoma (38), to name a few. However, it is becoming apparent that ligand-activation of FGFR is also an important mechanism of resistance in cancers with primary activation of other oncogenic kinases. For example, FGF2 activation of FGFR1 or FGFR3 has been shown to promote resistance to EGFR inhibitors in lung cancer (39,40), B-RAF inhibitors in melanoma (41), BCR-ABL inhibitors in CML (25), and KIT inhibitors in gastrointestinal stromal tumors (42,43).

FGF2 is not uniquely capable of promoting resistance however. A recent screen tested the ability of different ligands to promote resistance to kinase inhibitors in cancer cell lines and found that FGF2, hepatocyte growth factor (HGF), and neuregulin 1 (NRG1) were the most "broadly active" ligands capable of promoting resistance to kinase inhibitors (44). HGF mediates paracrine resistance to vemurafenib in B-RAF-mutated melanoma and resistant patients were found to have increased serum HGF (44), as well as increased HGF expression in the stromal cells adjacent to the melanoma (45). Growth arrest-specific 6 (GAS6) ligand activation of the receptor AXL is another mechanism of resistance that has recently been described (46), including in FLT3-ITD AML (28,29). Primary AML cells strongly express FGFR1, and to a lesser extent AXL (Supplemental Figure 2), suggesting that different ligands may be capable of protecting receptive subsets of FLT3-ITD AML cells. We found that 6/23 primary FLT3-ITD AML samples were highly responsive to FGF2 ligand stimulation (Figure 2H), and anticipate that FGF2-mediated resistance would be important in these patients. In summary, our results support the importance of ligand-RTK interaction as a general mechanism of kinase resistance; and FGF2 in particular, as an important member of a class of ligands expressed in the tumor microenvironment that promote resistance.

Although potent FLT3 inhibitors such as AC220 induce a rapid clearance of leukemia blasts from the peripheral blood, the leukemic blasts within the bone marrow respond more slowly (Figure 7), providing strong clinical evidence that the leukemia cells in the marrow are relatively protected from AC220. This protective effect has been reproduced *in vitro* using either marrow stromal cells (11–13) recombinant FL (14), and now recombinant FGF2. Of note, FGF2 and FL initially promoted resistance largely in the absence of mutations, but continued culture or removal of ligand greatly accelerated development outgrowth of resistance mutations (Figure 5). This is analagous to the residual AML blasts that persist at low levels in the marrow *in vivo,* and over time develop additional mutational or non-mutational resistance leading to clinical relapse (Figure 7B, marrow relapse day 133). Taken together, our data supports a two-step model of resistance where ligands from the microenvironment initially blunt the effect of FLT3 inhibitors allowing leukemia cells time for survival and adaptation, which is then followed by microenvironment-independent resistance.

Although recombinant FGF2 and FL promote resistance, the mechanism of protection is distinct. FGF2 bound to FGFR1 (Figure 2) and FGF2/FGFR1 signaling led to increased dependence upon the FGFR1/MAPK signaling pathway for cell survival, as evidenced by the strong synergy between AC220 and the FGFR inhibitor PD173074 (Figure 3B). We investigated if there might be a direct interaction between FGFR1 and FLT3 using immunoprecipitation, but found no significant interaction (Supplemental Figure 9). In contrast, FL-dependent resistant cultures partially restored FLT3 and downstream MAPK signaling despite AC220 (Figure 3C and Figure 4). Removal of FGF2 or FL from ligand-dependent cultures accelerated both mutational and non-mutational activation of FLT3 and Ras/MAPK pathway (Figure 5 and Supplemental Figure 7). Recently, spleen tyrosine kinase (SYK) was found to interact directly with FLT3 and modulate response to FLT3 inhibitors (47) so we also investigated the potential role of SYK in FGF2- and FL-dependent resistant cultures, but found that pSYK and total SYK protein were comparable to naïve MOLM14

cells (Supplemental Figures 5 and 10). In addition, FGF2- and FL-dependent resistant cultures were not more sensitive to the selective SYK inhibitor PRT062607 (Supplemental Table 4), suggesting that there is not an increased dependence upon SYK signaling to maintain pFLT3 in our system. Taken together, either partial restoration of FLT3 signaling or activation of the MAPK pathway through a compensatory pathway is sufficient to restore signaling homeostasis and initial cell survival (14,48–50). Continued treatment with FLT3 inhibitors then leads to stable restoration of FLT3 and MAPK signaling through either mutational and/or non-mutational mechanisms. The variety and predominance of FLT3 and RAS mutations in our resistant cell lines was striking, indicating a strong selective pressure to restore FLT3 and MAPK signaling. That being said, the frequency of the KRAS G13D mutation was likely due to a pre-existing subclone of our MOLM14 cells, since this exact mutation was also found in 2/659 sequencing reads of naïve MOLM14 (not found with other mutations). Since NRAS mutations are more common in AML overall, we suspect that NRAS mutations will be more common in resistant AML patients.

Our results also suggest that targeting early survival pathways in the microenvironment is more likely to reduce residual leukemia, and thus decrease the opportunity for subsequent development of resistance, including acquisition of mutations. Early interruption of FGF2 signaling is also likely to be important since FGF2 expression in the bone marrow stroma increases with AC220 treatment (Figure 7C), providing even more external survival cues over time. Expansion of FGF2-expressing stroma is consistent with the recent discovery that FGF2 is an autocrine growth factor for marrow stroma that is normally activated during stress hematopoiesis (17,18). Our results suggest that this stress response is hijacked by residual AML cells for survival. Thus, FGFR inhibition can block both FGF2 paracrine survival signals to leukemia cells and FGF2 autocrine signals to stroma, making the marrow stroma itself a therapeutic target by remodeling the leukemia cell niche (see model in Figure 7D). Optimally, clinical trials using a combination of FGFR inhibitors and novel FLT3 inhibitors that remain active against the common FLT3 activation loop mutations would 1) overcome paracrine FGF2-mediated resistance, 2) attenuate autocrine-stimulated expansion of FGF2-expressing stroma, and 3) circumvent development of the most common mutations that lead to resistance and relapse. This strategy has the potential to improve the durability of response to FLT3 inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Lyman SD, James L, Vanden Bos T, de Vries P, Brasel K, Gliniak B, et al. Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. Cell. 1993; 75(6):1157–67. [PubMed: 7505204]
2. Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia. 1996; 10(12):1911–8. [PubMed: 8946930]
3. Griffith J, Black J, Faerman C, Swenson L, Wynn M, Lu F, et al. The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. Molecular cell. 2004; 13(2):169–78. [PubMed: 14759363]
4. Abu-Duhier FM, Goodeve AC, Wilson GA, Gari MA, Peake IR, Rees DC, et al. FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. British journal of haematology. 2000; 111(1):190–5. [PubMed: 11091200]
5. Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001; 98(6):1752–9. [PubMed: 11535508]
6. Abu-Duhier FM, Goodeve AC, Wilson GA, Care RS, Peake IR, Reilly JT. Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia. British journal of haematology. 2001; 113(4):983–8. [PubMed: 11442493]
7. Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood. 2001; 97(8): 2434–9. [PubMed: 11290608]
8. Stone RM, DeAngelo DJ, Klimek V, Galinsky I, Estey E, Nimer SD, et al. Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. Blood. 2005; 105(1):54–60. [PubMed: 15345597]
9. Knapper S, Burnett AK, Littlewood T, Kell WJ, Agrawal S, Chopra R, et al. A phase 2 trial of the FLT3 inhibitor lestaurtinib (CEP701) as first-line treatment for older patients with acute myeloid leukemia not considered fit for intensive chemotherapy. Blood. 2006; 108(10):3262–70. [PubMed: 16857985]
10. Cortes J, Tallman M, Schiller G, Trone D, Gammon G, Goldberg SL, et al. Results Of a Phase 2 Randomized, Open-Label, Study of Lower Doses Of Quizartinib (AC220; ASP2689) In Subjects With FLT3-ITD Positive Relapsed Or Refractory Acute Myeloid Leukemia (AML). Blood (ASH Annual Meeting Abstracts). 2013; 122(21)
11. Man CH, Fung TK, Ho C, Han HH, Chow HC, Ma AC, et al. Sorafenib treatment of FLT3-ITD(+) acute myeloid leukemia: favorable initial outcome and mechanisms of subsequent nonresponsiveness associated with the emergence of a D835 mutation. Blood. 2012; 119(22): 5133–43. [PubMed: 22368270]
12. Smith CC, Wang Q, Chin CS, Salerno S, Damon LE, Levis MJ, et al. Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia. Nature. 2012; 485(7397):260–3. [PubMed: 22504184]
13. Alvarado Y, Kantarjian HM, Luthra R, Ravandi F, Borthakur G, Garcia-Manero G, et al. Treatment with FLT3 inhibitor in patients with FLT3-mutated acute myeloid leukemia is associated with development of secondary FLT3-tyrosine kinase domain mutations. Cancer. 2014; 120(14):2142–9. [PubMed: 24737502]
14. Sato T, Yang X, Knapper S, White P, Smith BD, Galkin S, et al. FLT3 ligand impedes the efficacy of FLT3 inhibitors in vitro and in vivo. Blood. 2011; 117(12):3286–93. [PubMed: 21263155]
15. Allouche M. Basic fibroblast growth factor and hematopoiesis. Leukemia. 1995; 9(6):937–42. [PubMed: 7596180]
16. de Haan G, Weersing E, Dontje B, van Os R, Bystrykh LV, Vellenga E, et al. In vitro generation of long-term repopulating hematopoietic stem cells by fibroblast growth factor-1. Developmental cell. 2003; 4(2):241–51. [PubMed: 12586067]

17. Itkin T, Ludin A, Gradus B, Gur-Cohen S, Kalinkovich A, Schajnovitz A, et al. FGF-2 expands murine hematopoietic stem and progenitor cells via proliferation of stromal cells, c-Kit activation, and CXCL12 down-regulation. Blood. 2012; 120(9):1843–55. [PubMed: 22645180]
18. Zhao M, Ross JT, Itkin T, Perry JM, Venkatraman A, Haug JS, et al. FGF signaling facilitates postinjury recovery of mouse hematopoietic system. Blood. 2012; 120(9):1831–42. [PubMed: 22802336]
19. Matsuo Y, MacLeod RA, Kojima K, Kuwahara K, Sakata A, Drexler HG, et al. A novel ALL-L3 cell line, BALM-16, lacking expression of immunoglobulin chains derived from a patient with hypercalcemia. Leukemia. 1997; 11(12):2168–74. [PubMed: 9447837]
20. Yee KW, Schittenhelm M, O'Farrell AM, Town AR, McGreevey L, Bainbridge T, et al. Synergistic effect of SU11248 with cytarabine or daunorubicin on FLT3 ITD-positive leukemic cells. Blood. 2004; 104(13):4202–9. [PubMed: 15304385]
21. Tyner JW, Walters DK, Willis SG, Luttropp M, Oost J, Loriaux M, et al. RNAi screening of the tyrosine kinome identifies therapeutic targets in acute myeloid leukemia. Blood. 2008; 111(4): 2238–45. [PubMed: 18025156]
22. Degnin M, Tarlock K, Agarwal A, Meshinchi S, Tognon CE, Druker BJ. Novel Method to Perform Functional Drug Screens on Frozen Primary Leukemia Samples. Blood (ASH Annual Meeting Abstracts). 2015; 126(23)
23. Tyner JW, Yang WF, Bankhead A 3rd, Fan G, Fletcher LB, Bryant J, et al. Kinase pathway dependence in primary human leukemias determined by rapid inhibitor screening. Cancer research. 2013; 73(1):285–96. [PubMed: 23087056]
24. Reinig E, Yang F, Traer E, Arora R, Brown S, Rattray R, et al. Targeted Next-Generation Sequencing in Myelodysplastic Syndrome and Chronic Myelomonocytic Leukemia Aids Diagnosis in Challenging Cases and Identifies Frequent Spliceosome Mutations in Transformed Acute Myeloid Leukemia. American journal of clinical pathology. 2016; 145(4):497–506. [PubMed: 27124934]
25. Traer E, Javidi-Sharifi N, Agarwal A, Dunlap J, English I, Martinez J, et al. Ponatinib overcomes FGF2-mediated resistance in CML patients without kinase domain mutations. Blood. 2014; 123(10):1516–24. [PubMed: 24408322]
26. Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine & growth factor reviews. 2005; 16(2):139–49. [PubMed: 15863030]
27. Ornitz DM, Leder P. Ligand specificity and heparin dependence of fibroblast growth factor receptors 1 and 3. The Journal of biological chemistry. 1992; 267(23):16305–11. [PubMed: 1379594]
28. Ben-Batalla I, Schultze A, Wroblewski M, Erdmann R, Heuser M, Waizenegger JS, et al. Axl, a prognostic and therapeutic target in acute myeloid leukemia mediates paracrine crosstalk of leukemia cells with bone marrow stroma. Blood. 2013; 122(14):2443–52. [PubMed: 23982172]
29. Park IK, Mishra A, Chandler J, Whitman SP, Marcucci G, Caligiuri MA. Inhibition of the receptor tyrosine kinase Axl impedes activation of the FLT3 internal tandem duplication in human acute myeloid leukemia: implications for Axl as a potential therapeutic target. Blood. 2013; 121(11): 2064–73. [PubMed: 23321254]
30. Zimmerman EI, Turner DC, Buaboonnam J, Hu S, Orwick S, Roberts MS, et al. Crenolanib is active against models of drug-resistant FLT3-ITD-positive acute myeloid leukemia. Blood. 2013; 122(22):3607–15. [PubMed: 24046014]
31. Yoon SY, Tefferi A, Li CY. Bone marrow stromal cell distribution of basic fibroblast growth factor in chronic myeloid disorders. Haematologica. 2001; 86(1):52–7. [PubMed: 11146571]
32. Roecklein BA, Torok-Storb B. Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. Blood. 1995; 85(4):997–1005. [PubMed: 7849321]
33. Inhorn RC, Aster JC, Roach SA, Slapak CA, Soiffer R, Tantravahi R, et al. A syndrome of lymphoblastic lymphoma, eosinophilia, and myeloid hyperplasia/malignancy associated with t(8;13)(p11;q11): description of a distinctive clinicopathologic entity. Blood. 1995; 85(7):1881–7. [PubMed: 7661940]

34. Sohal J, Chase A, Mould S, Corcoran M, Oscier D, Iqbal S, et al. Identification of four new translocations involving FGFR1 in myeloid disorders. Genes, chromosomes & cancer. 2001; 32(2): 155–63. [PubMed: 11550283]
35. Chesi M, Nardini E, Brents LA, Schrock E, Ried T, Kuehl WM, et al. Frequent translocation t(4;14)(p16.3;q32. 3) in multiple myeloma is associated with increased expression and activating mutations of fibroblast growth factor receptor 3. Nature genetics. 1997; 16(3):260–4. [PubMed: 9207791]
36. Richelda R, Ronchetti D, Baldini L, Cro L, Viggiano L, Marzella R, et al. A novel chromosomal translocation t(4; 14)(p16. 3; q32) in multiple myeloma involves the fibroblast growth-factor receptor 3 gene. Blood. 1997; 90(10):4062–70. [PubMed: 9354676]
37. Cappellen D, De Oliveira C, Ricol D, de Medina S, Bourdin J, Sastre-Garau X, et al. Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. Nature genetics. 1999; 23(1):18–20. [PubMed: 10471491]
38. Singh D, Chan JM, Zoppoli P, Niola F, Sullivan R, Castano A, et al. Transforming fusions of FGFR and TACC genes in human glioblastoma. Science (New York, NY. 2012; 337(6099):1231–5.
39. Terai H, Soejima KN, Yasuda H, Nakayama S, Hamamoto J, Arai D, et al. Activation of the FGF2-FGFR1 Autocrine Pathway: A Novel Mechanism of Acquired Resistance to Gefitinib in NSCLC Cells. Molecular cancer research: MCR. 2013
40. Ware KE, Hinz TK, Kleczko E, Singleton KR, Marek LA, Helfrich BA, et al. A mechanism of resistance to gefitinib mediated by cellular reprogramming and the acquisition of an FGF2-FGFR1 autocrine growth loop. Oncogenesis. 2013; 2:e39. [PubMed: 23552882]
41. Yadav V, Zhang X, Liu J, Estrem S, Li S, Gong XQ, et al. Reactivation of mitogen-activated protein kinase (MAPK) pathway by FGF receptor 3 (FGFR3)/Ras mediates resistance to vemurafenib in human B-RAF V600E mutant melanoma. The Journal of biological chemistry. 2012; 287(33):28087–98. [PubMed: 22730329]
42. Javidi-Sharifi N, Traer E, Martinez J, Gupta A, Taguchi T, Dunlap J, et al. Crosstalk between KIT and FGFR3 Promotes Gastrointestinal Stromal Tumor Cell Growth and Drug Resistance. Cancer research. 2015; 75(5):880–91. [PubMed: 25432174]
43. Li F, Huynh H, Li X, Ruddy DA, Wang Y, Ong R, et al. FGFR-Mediated Reactivation of MAPK Signaling Attenuates Antitumor Effects of Imatinib in Gastrointestinal Stromal Tumors. Cancer discovery. 2015; 5(4):438–51. [PubMed: 25673643]
44. Wilson TR, Fridlyand J, Yan Y, Penuel E, Burton L, Chan E, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. Nature. 2012; 487(7408):505–9. [PubMed: 22763448]
45. Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. Nature. 2012; 487(7408):500–4. [PubMed: 22763439]
46. Zhang Z, Lee JC, Lin L, Olivas V, Au V, LaFramboise T, et al. Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. Nature genetics. 2012; 44(8):852–60. [PubMed: 22751098]
47. Puissant A, Fenouille N, Alexe G, Pikman Y, Bassil CF, Mehta S, et al. SYK is a critical regulator of FLT3 in acute myeloid leukemia. Cancer cell. 2014; 25(2):226–42. [PubMed: 24525236]
48. Piloto O, Wright M, Brown P, Kim KT, Levis M, Small D. Prolonged exposure to FLT3 inhibitors leads to resistance via activation of parallel signaling pathways. Blood. 2007; 109(4):1643–52. [PubMed: 17047150]
49. Weisberg E, Ray A, Nelson E, Adamia S, Barrett R, Sattler M, et al. Reversible resistance induced by FLT3 inhibition: a novel resistance mechanism in mutant FLT3-expressing cells. PloS one. 2011; 6(9):e25351. [PubMed: 21980431]
50. Yang X, Sexauer A, Levis M. Bone marrow stroma-mediated resistance to FLT3 inhibitors in FLT3-ITD AML is mediated by persistent activation of extracellular regulated kinase. British journal of haematology. 2014; 164(1):61–72. [PubMed: 24116827]

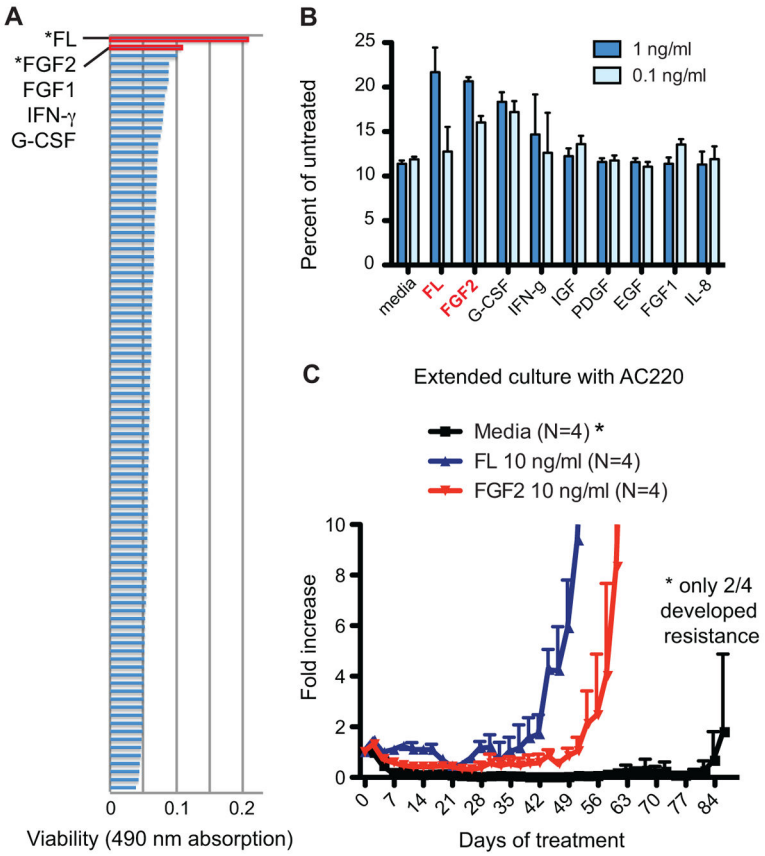

Figure 1. Microenvironmental screen identifies FL and FGF2 as protective molecules for MOLM14 cells, and they accelerate development of AC220 resistance fin extended culture

A) MOLM14 cells were added to a 384-well plate containing cytokines, chemokines, and growth factors with 10 nM AC220 (quizartinib). Viability was measured using MTS reagent, averaged over all concentrations (1, 10, and 100 ng/ml), and the results sorted according to highest average viability. Average viability >2 standard deviations above the mean are highlighted in red and by an *. **B)** MOLM14 cells were cultured in lower concentrations of recombinant proteins (1, and 0.1 ng/ml) in 96-well plates plus 10 nM AC220. Viability was assessed after 48 hours with MTS reagent and data plotted as percent of the respective untreated control. All wells were plated in triplicate with standard deviation indicated by error bars. **C)** MOLM14 cells were cultured continuously in 10 nM AC220 with FL, FGF2, or media alone as indicated (N=4 for each). Fresh media, AC220 and cytokine were replaced every 2–3 days over the indicated time period. Viable cell numbers were analyzed every 2–3 days with Guava ViaCount.

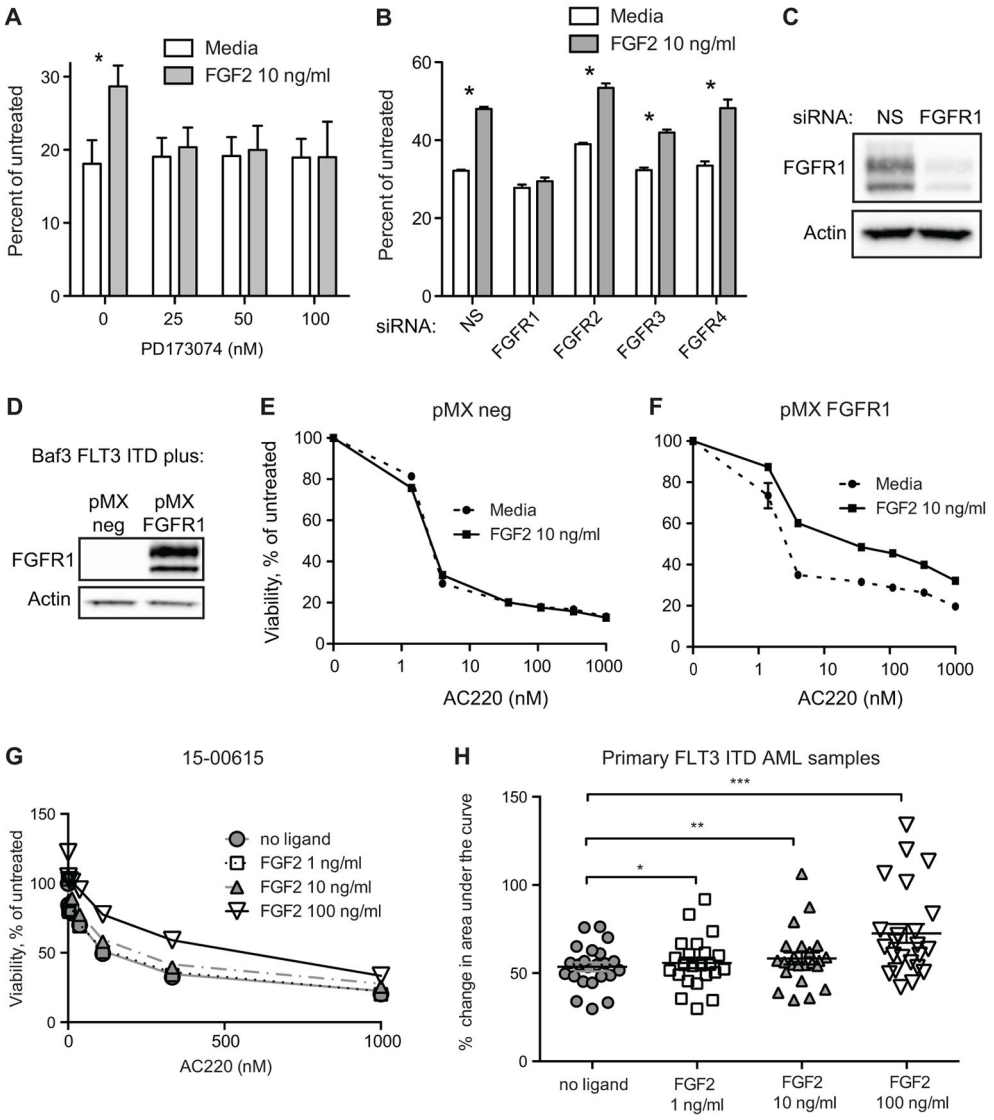

Figure 2. FGF2 protection of MOLM14 cells is mediated by FGFR1

A) MOLM14 cells were cultured in media alone or media supplemented with 10 ng/ml FGF2, treated with 10 nM AC220, and exposed to 25, 50, and 100 nM of the FGFR inhibitor PD173074. Viability was measured by MTS assay after 48 hours and normalized to their respective untreated control (media alone, FGF2, 25 nM PD173074, etc.) **B)** MOLM14 cells were electroporated with siRNAs targeting FGFR1, FGFR2, FGFR3, FGFR4 and a non-specific (NS) control. After 48 hours the cells were pelleted and resuspended in media +/− FGF2 and +/− 10 nM AC220; and viability assessed by MTS after 48 hours. * indicates $p<0.01$ by *t* test. **C)** Western blot was used to evaluate FGFR1 protein expression after siRNA (see also supplemental Figure S1). **D)** BaF3 cells expressing FLT3-ITD (20) were retrovirally transfected with either empty pMX vector (pMX neg) or vector containing FGFR1, selected with puromycin, and analyzed by Western blot to show FGFR1 expression. The FLT3-ITD Baf3 cells with **E)** empty pMX vector or **F)** pMX with FGFR1 were then treated with a gradient of AC220 in either media alone or supplemented with 10 ng/ml

FGF2. Viability was assessed after 48 hours with MTS reagent and data plotted as percent of the respective untreated control. All cell line experiments were performed in triplicate and error bars indicate standard deviation. Thawed frozen viable (see methods) and fresh primary FLT3-ITD AML samples were plated with media alone (no ligand) or with FGF2 at 1, 10 and 100 ng/ml concentration. The cells were then exposed to a gradient of AC220 and viability was measured after 72 hours by MTS. **G)** An example of *ex vivo* FGF2-mediated protection from AC220 with a primary FLT3-ITD AML sample. **H)** The area under the curve was calculated for 23 patient samples in the absence or presence of FGF2 ligand (see 2G) and graphed with mean and standard error of the mean shown. One-tailed t-tests were performed with p values indicated by *<0.05, **<0.005, and ***=0.0007.

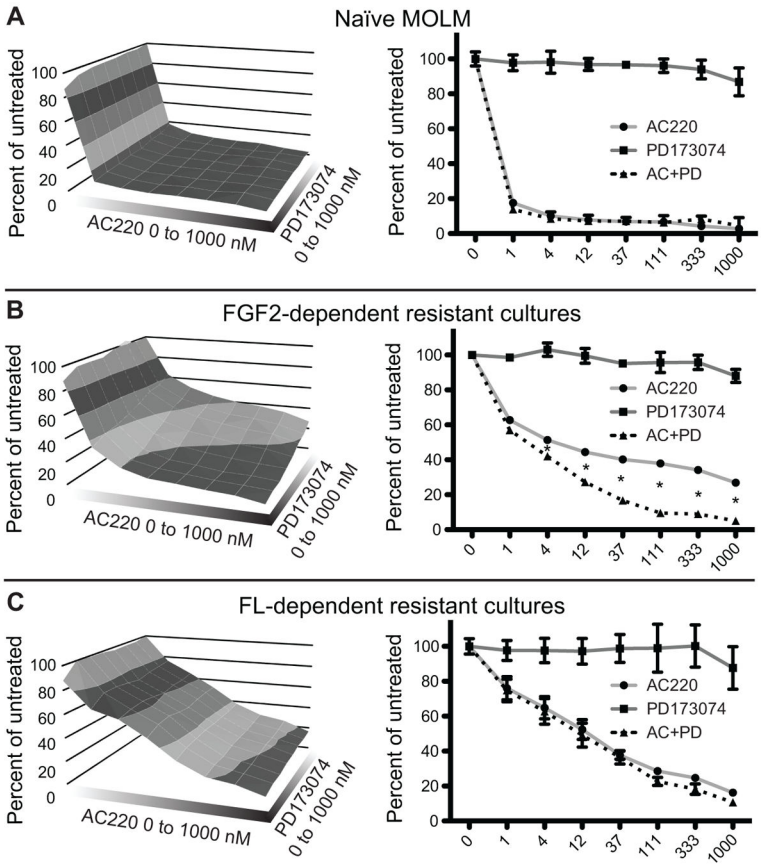

Figure 3. FGF2-dependent resistant cultures respond synergistically to combined FLT3 and FGFR inhibition

A) Naïve MOLM14 cells in media alone were exposed to a matrix of an AC220 gradient (0, 1.4, 4, 12, 37, 111, 333, 1000 nM) overlayed with the FGFR inhibitor PD173074 (0, 1.4, 4, 12, 37, 111, 333, 1000 nM) and compared to **B)** FGF2- and **C)** FL-dependent resistant MOLM14 cells (Figure 1C) in the same conditions. Viability was measured after 48 hours and average viability graphed as surface plots with gray scale denoting 20% increments. Graphs at right indicate viability with AC220 alone, PD173074 alone, and equimolar combination to highlight synergy. Calculation of the combination index is included in Supplemental Table 1 and synergy (CI<0.1) indicated by an *. All experiments were done in triplicate with average viability scaled to untreated condition (100%). Error bars represent standard deviation.

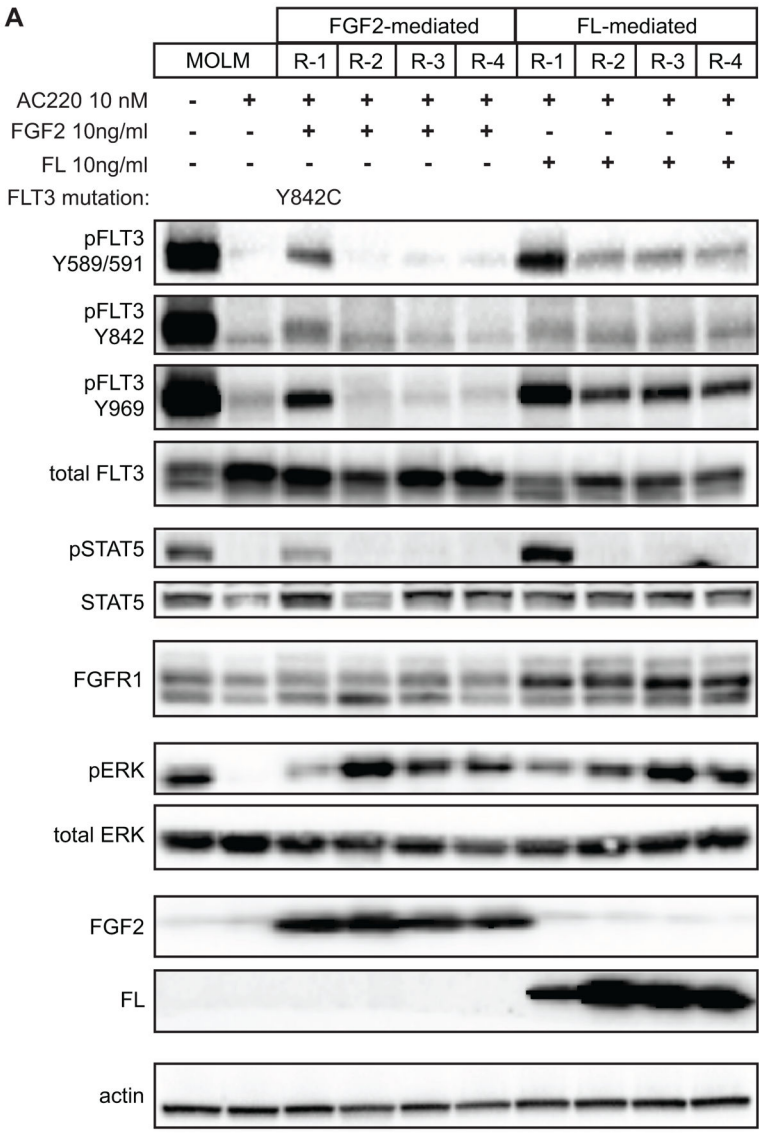

Figure 4. FGF2 and FL restore downstream FLT3 signaling, particularly the MAPK pathway

A) Naïve MOLM14 cells were treated for 24 hours −/+ 10 nM AC220 (first two lanes) and comparedto FGF2- and FL-dependent resistant cells (grown continuously in FGF2 or FL, lanes 3–10). The FGF2- and FL-dependent resistant cells were harvested 24 hours after addition of fresh media, recombinant protein and AC220. The cells were then lysed as described with Western blot analysis as per Materials and Methods. The FGF2-dependent resistant culture that developed a Y842C mutation is indicated.

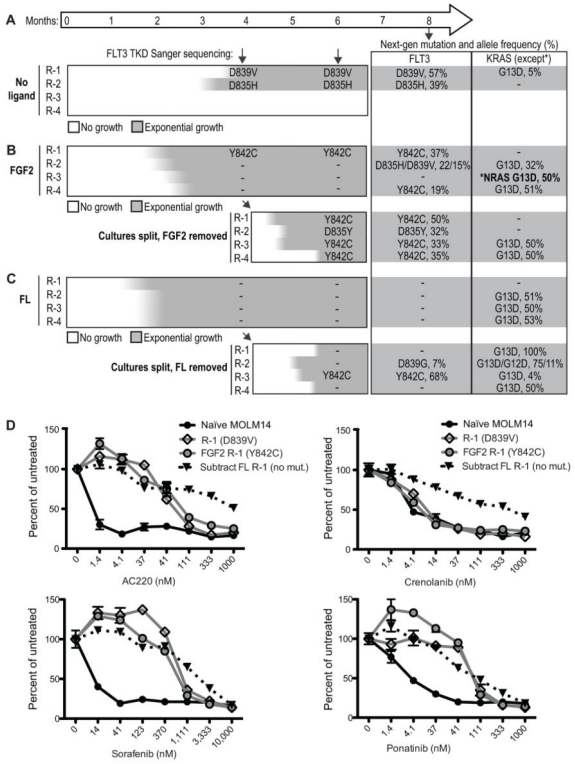

Figure 5. Removing ligand from FGF2- or FL-dependent resistant cultures results in ligand-independent resistance mediated by frequent mutation of FLT3 and Ras

The time to development of exponential growth (indicated by gray) was plotted over time for the extended cultures in Figure 1C. The timepoints at which Sanger or next-generation sequencing (at right, average read depth ~1500) was performed is indicated by arrows. If a mutation could not be detected it is indicated with "−". **A)** MOLM14 cells cultured in media and AC220 alone (no ligand) developed exponential growth later than FGF2- and FL-dependent cultures (panels B and C) and developed early resistance mutations: D839V and D835H. **B)** FGF2-dependent and **C)** FL-dependent resistant cultures (cultured continuously in 10 ng/ml FGF2 or FL plus 10 nM AC220) were split at 4 months and 1×10^7 cells placed into fresh media *without* recombinant protein with continued 10 nM AC220 treatment. Fresh media and 10 nM AC220 were replaced every 2–3 days over the indicated time period and viable cell number was analyzed using Guava ViaCount. **D)** Naïve MOLM14 cells and resistant MOLM14 cells with FLT3 D839V mutation (R-1), FLT3 D842C mutation (FGF2 R-1), and non-mutated resistant cells (FL R1 after FL subtraction) were exposed to a gradient of FLT3 inhibitors: AC220 (quizartinib), crenolanib, sorafenib, and ponatinib. Viability was measured after 48 hours by MTS assay with average viability scaled relative to untreated cells (100%). The experiment was done in triplicate and error bars represent standard deviation.

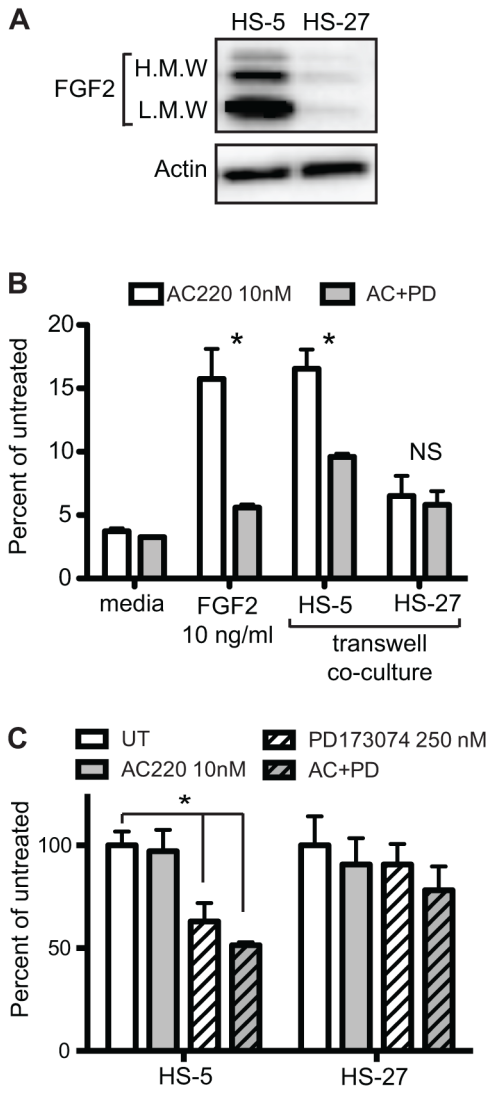

Figure 6. FGFR inhibitors overcome the protective effects of FGF2-expressing stroma (HS-5) in co-culture assays

A) The human stromal cell lines HS-5 and HS-27 were analyzed for FGF2 expression by Western blot. **B)** MOLM14 cells were cultured in media alone, 10 ng/ml FGF2, or in 1 μm transwells over HS-5 or HS-27 stromal cell lines. The cells were treated with 10 nM AC220 and/or 250 nM PD173074 as indicated and MOLM14 cells were analyzed for viable cell number after 4 days. Viable cells were plotted as percentage of untreated control. **C)** The corresponding stromal cells from the transwell co-culture experiments were also evaluated by MTS assay for viability. Results are shown relative to the untreated condition. All experiments were done in triplicate, error bars represent standard deviation, and * indicates $p<0.05$.

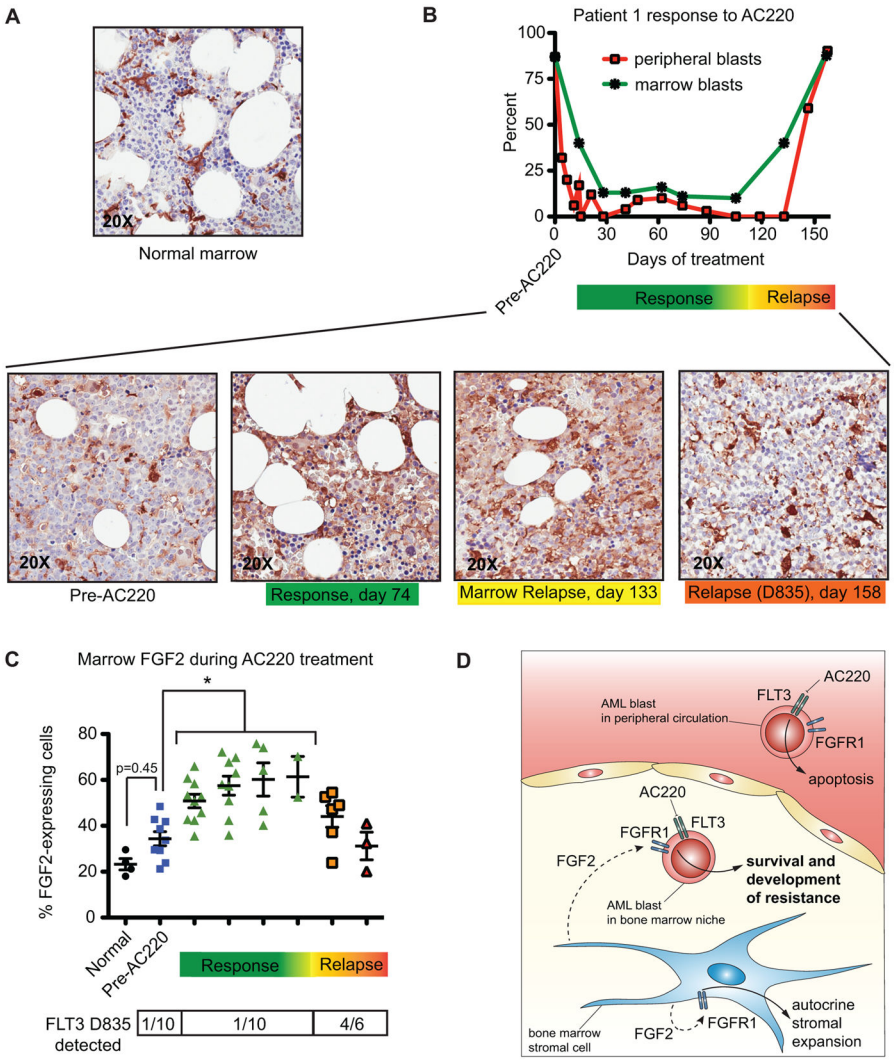

Figure 7. Bone marrow FGF2 increases during AC220 treatment and peaks prior to development of resistance

A) Normal bone marrow biopsy (hip replacement) stained by immunohistochemistry (IHC) for FGF2 as described in Materials and Methods. **B)** Graph of peripheral blood and marrow blast percentage over time of representative patient treated with AC220. Response to therapy is indicated with green, early relapse in marrow indicated in yellow/orange, and peripheral blood relapse indicated in red. Sequential bone marrow biopsies from this patient were stained for FGF2 by IHC and shown below. This patient developed a FLT3 mutation at D835, which was detected at day 158 as indicated. **C)** Quantification of marrow FGF2 by IHC of all evaluable patients treated with AC220. The marrows were analyzed with Aperio ImageScope software to quantify the percent of cells that expressed FGF2. The line indicates the median value. Statistically significant differences were evaluated using a 2-tailed *t*-test. * indicates $p<0.05$. The number of patients and the presence or absence of FLT3 mutations are indicated below. * indicates $p<0.05$. **D)** Model of FGF2 paracrine-mediated protection of leukemia cells.