



The magnitude of CXCR4 signaling regulates resistance to quizartinib in FLT3/ITD⁺ cells via RUNX1

Seiji Fukuda^{a,b,c,*}, Nozomi Matsuda^b, Tsukimi Shoji^a, Chie Onishi^d, Tomohiro Hirade^a, Takeshi Taketani^a, Louis M. Pelus^e

^a Department of Pediatrics, Shimane University School of Medicine, Japan

^b Shimane University School of Nursing, Japan

^c Sugiyama Jogakuen University School of Nursing, Japan

^d Clinical Research Center, Shimane University Hospital, Japan

^e Department of Microbiology and Immunology, and Department of Medicine, Div HEME/ONC, Indiana University School of Medicine, USA

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ABSTRACT

CXCR4 antagonists sensitize FLT3/ITD⁺ AML cells to FLT3 inhibitors; however, CXCR4 signaling can induce apoptosis in AML cells, raising the question of whether CXCR4 signaling exerts divergent effects on FLT3/ITD⁺ cells. The present study investigated the paradoxical function of CXCR4 in resistance to FLT3 inhibitors. The FLT3 inhibitor quizartinib significantly decreased the number of FLT3/ITD⁺ Ba/F3 cells, whereas 1 ng/ml CXCL12 showed a significant protective effect against quizartinib. In contrast, CXCL12 over 100 ng/ml significantly decreased FLT3/ITD⁺ cell viability with concomitant downregulation of Runx1. Moreover, the survival of FLT3/ITD⁺ Ba/F3 or MOLM13 cells with low surface CXCR4 expression incubated with quizartinib was significantly enhanced by 100 ng/ml CXCL12; however, this protective effect of CXCL12 against quizartinib was barely detected in cells with high surface CXCR4 expression. Although silencing Runx1 downregulated CXCR4 expression, RUNX1 expression levels were significantly higher in CXCR4^{LOW} FLT3/ITD⁺ Ba/F3 cells incubated with 100 ng/ml CXCL12 than in CXCR4^{HIGH} cells, coincident with an increase in FLT3 phosphorylation. Silencing RUNX1 partially abrogated resistance to quizartinib in CXCR4^{LOW} cells incubated with CXCL12, whereas ectopic RUNX1 significantly restored resistance in CXCR4^{HIGH} cells. These results indicate that CXCR4 signaling of different magnitudes paradoxically regulates resistance to quizartinib in FLT3/ITD⁺ cells via RUNX1.

1. Introduction

FLT3/ITD mutations in patients with acute myeloid leukemia (AML) are associated with poor prognosis [1,2]. Second-generation FLT3 inhibitors, type-I gilteritinib and type-II quizartinib, are used to treat patients with relapsed and refractory AML [3–5]. Although both inhibitors are effective, most patients develop resistance [4,6,7]. Resistance to quizartinib is induced by additional mutations in the *FLT3* gene [7], whereas resistance to gilteritinib was associated with mutations in other signaling molecules in 60% of patients with AML [6]. However, the mechanism(s) responsible for the resistance in the other 40% of cases remains unknown, suggesting that non-mutation-related mechanisms, such as chemokines and/or cytokines in the microenvironment, may be involved.

The chemokine CXCL12 and its receptor CXCR4 regulate the

migration and survival of hematopoietic stem cells as well as AML cells [8–16]. CXCR4 expression is associated with poor prognosis in patients with AML [17,18]. CXCR4 antagonists overcome the resistance of FLT3/ITD⁺ AML cells to FLT3 inhibitors [19–24], and these drugs demonstrate synergistic effects on apoptosis in FLT3/ITD⁺ AML cells [19]. In contrast, CXCR4 signaling induces apoptosis in AML cells [25, 26], indicating that the effect of CXCR4 on the survival and/or apoptosis of AML cells remains controversial.

Our initial data indicated that CXCL12 can inhibit the proliferation of FLT3/ITD⁺ cells, coincident with antagonistic changes in mRNAs that are regulated by FLT3/ITD, such as Runx1 (Fig. 1). While RUNX1 generally functions as a tumor suppressor, RUNX1 can also serve as an oncogene in AML [27–29]. RUNX1 expression is significantly elevated in FLT3/ITD⁺ AML cells, promotes leukemogenesis induced by FLT3/ITD [30] and enhances the resistance of FLT3/ITD⁺ cells to quizartinib [31].

* Correspondence to: Sugiyama Jogakuen University School of Nursing, 17-3, Hoshigaoka-motomachi, Chigusa, Nagoya 464-8662, Japan.

E-mail addresses: s-fukuda@sugiyama-u.ac.jp, sfukuda@med.shimane-u.ac.jp (S. Fukuda).

These findings led us to hypothesize that CXCL12/CXCR4 signaling can negatively modulate FLT3/ITD function by regulating RUNX1 expression. To understand the mechanism responsible for the resistance of FLT3/ITD⁺ cells to FLT3 inhibitors that are regulated by CXCL12/CXCR4 signaling, the present study investigated the effect of CXCL12/CXCR4 signaling on FLT3/ITD and its regulation by RUNX1.

2. Materials and methods

2.1. Reagents

Recombinant murine and human CXCL12 and PE-conjugated anti-human/mouse CXCR7 antibodies were purchased from R&D Systems (Minneapolis, MN). PE-conjugated anti-RUNX1 antibody and rat IgG2a isotype were purchased from eBioscience (San Diego, CA). APC-conjugated anti-human CXCR4 antibody and anti-mouse CXCR4 antibody were obtained from BD Bioscience (San Jose, CA). Anti-FLT3 monoclonal antibody (8F2) and anti-phospho-FLT3 antibody (Tyr591) were obtained from Cell Signaling Technology (Danvers, MA). Quizartinib and gilteritinib were from Selleckchem (Houston, TX).

2.2. Cell culture, cDNA transfection and shRNA knockdown

Ba/F cells expressing FLT3/ITD derived from two patients with AML (FLT3/ITD⁺ Ba/F3 cells, clone-1: N51 and clone-2: N78) were described previously [16,32]. The MOLM13 AML cell line harboring FLT3/ITD was purchased from DSMZ (Braunschweig, Germany) [33]. For shRNA knockdown of *Runx1*, FLT3/ITD⁺ Ba/F cells were electroporated with shRNA specific to *Runx1* cloned into the pSingle-tTS-shRNA vector (Clontech Laboratories, Mountain View, CA) using a Nucleofector Kit V (Lonza, Basel, Switzerland), as described [31]. The sequence of shRNA for RUNX1 was reported previously [31]. Stable transformants were selected with 1 mg/ml geneticin and frozen for storage. The cells were cultured in RPMI-1640 plus 10% Tet System Approved FBS (Clontech Laboratories). To activate *Runx1* shRNA, 1 µg/ml doxycycline (Sigma-Aldrich, St. Louis, MO) was added to the culture medium. The reduction in RUNX1 protein levels was validated by intracellular flow cytometry using an anti-RUNX1 antibody. To establish FLT3/ITD⁺ Ba/F3 cells expressing *Runx1* cDNA, murine *Runx1* cDNA in pcDNA3 (OMu23145C: NM_00111021.2, *Runx1* transcript variant 1, full length ORF, GeneCopoeia, Rockville, MD) was electroporated using a Nucleofector Kit V (Lonza). Stably transfected cells were selected using 400 µg/ml hygromycin B. Cells resistant to 5 nM quizartinib were established following incubation with 5 nM quizartinib for approximately one week. To generate cells resistant to gilteritinib, FLT3/ITD⁺ Ba/F3 cells were exposed to stepwise increasing concentrations of gilteritinib starting with an initial dose of 10 nM and ending with 30 nM. Similarly, MOLM13 cells that proliferated in the presence of gilteritinib were obtained after 4 weeks of incubation with 10 nM gilteritinib. MOLM13 cells refractory to quizartinib were not obtained in the present study. To determine cell proliferation, viable cells were counted with a FACSCalibur cytometer (BD Bioscience) based on light scattering.

2.3. cDNA microarray analysis and quantitative RT-PCR

The mRNA microarray data for Ba/F3 cells containing FLT3/ITD clone-1 and FLT3/ITD clone-2 treated with or without 100 ng/ml CXCL12 [16] were analyzed to identify mRNAs that are regulated by FLT3/ITD and CXCL12. Briefly, the mRNAs regulated by FLT3/ITD were identified by comparing the expression of mRNAs between FLT3/ITD⁺ Ba/F3 cells (FLT3/ITD clones 1 and 2) and those expressing wild-type FLT3. The differentially expressed mRNAs in FLT3/ITD⁺ Ba/F3 cells incubated with or without 100 ng/ml CXCL12 for 4 h were also identified. Subsequent comparison between mRNAs that are regulated by FLT3/ITD and those regulated by CXCL12 in FLT3/ITD⁺ cells identified genes upregulated or downregulated by CXCL12 among the mRNAs

upregulated or downregulated by FLT3/ITD. The microarray data were deposited into Gene Expression Omnibus (GSE176401). Quantitative RT-PCR was performed as described [31].

2.4. ELISA and Western blot analysis

For ELISA analysis, 5 million cells were lysed using 1X cell lysis buffer supplemented with 1 mM PMSF per the manufacturer's instructions (Cell Signaling Technology), and the absorbance at 450 nm was read in triplicate wells. Western blot analysis was performed as described [34].

2.5. Statistical analysis

The data are expressed as the mean ± standard error, and statistical significance was evaluated using the two-tailed Student's t test in Microsoft Excel (Microsoft Corp., Seattle, WA). Correlations between CXCR4 and RUNX1 expression levels were analyzed by Spearman's rank correlation coefficient test using SPSS Statistics for Windows (IBM, Armonk, NY, USA). Correlation coefficients ranging between 0.2 and 0.4 indicated a weak correlation, whereas those over 0.4 indicated a modest correlation between two parameters. A P value < 0.05 was considered significant.

3. Results

3.1. CXCL12 can inhibit proliferation and negatively modulate Runx1 expression in FLT3/ITD⁺ mouse hematopoietic cells

To investigate the influence of CXCL12/CXCR4 signaling on FLT3/ITD function, we examined the proliferation of Ba/F3 cells expressing FLT3/ITD (FLT3/ITD clone-1 and FLT3/ITD clone-2) incubated with CXCL12. Ba/F3 cells expressing wild-type FLT3 failed to proliferate at low serum concentrations (0.1%), whereas FLT3/ITD⁺ Ba/F3 cells continued to proliferate (Fig. 1A-Right). At a concentration of 100 ng/ml, CXCL12 significantly decreased the proliferation of FLT3/ITD⁺ Ba/F3 cells in the 0.1% serum condition (6.1 ± 2.3% and 11.4 ± 3.4% reduction in FLT3/ITD clone-1 and clone-2, respectively, P < 0.05, Fig. 1A).

The reduction in FLT3/ITD-driven proliferation by 100 ng/ml CXCL12 in FLT3/ITD⁺ cells led us to hypothesize that 100 ng/ml CXCL12 may antagonize FLT3/ITD-induced gene expression. To test this hypothesis, we examined whether CXCL12 affects genes regulated by FLT3/ITD. We investigated Ba/F3 cells expressing 2 different FLT3/ITD genes (FLT3/ITD clone-1 and FLT3/ITD clone-2) using the mRNA microarray data we previously reported (GSE176401) [16]. FLT3/ITD clone-1 differentially expressed 7675 mRNAs (>1.5-fold increase/decrease) compared to control cells expressing wild-type FLT3 [16]. Incubation with 100 ng/ml CXCL12 modulated 6509 mRNAs in cells expressing FLT3/ITD clone-1. Among the mRNAs regulated by FLT3/ITD, the expression of 240 mRNAs (3.1%) that were modulated by FLT3/ITD was augmented by 100 ng/ml CXCL12 in FLT3/ITD⁺ cells (Fig. 1B-Top-Left). In contrast, 1440 out of 7675 mRNAs (18.8%) that were regulated by FLT3/ITD were inversely modulated by CXCL12 (Fig. 1B-Top-Right).

Similarly, among the 8162 mRNAs regulated by FLT3/ITD clone-2, the expression of 126 mRNAs (1.5%) was augmented by 100 ng/ml CXCL12, whereas the expression of 1299 (15.8%) was inversely regulated by CXCL12 (Fig. 1B-Bottom).

Among the mRNAs upregulated by FLT3/ITD, *Runx1* mRNA, which is known to be upregulated by FLT3/ITD [30,31], was significantly downregulated by 100 ng/ml CXCL12 (Fig. 1C-Left). The reduction in *Runx1* mRNA was validated by Q-RT-PCR (Fig. 1C-Right). These findings indicate that 100 ng/ml CXCL12 can attenuate the proliferation of FLT3/ITD⁺ cells and the expression of mRNAs regulated by FLT3/ITD, such as *Runx1*.

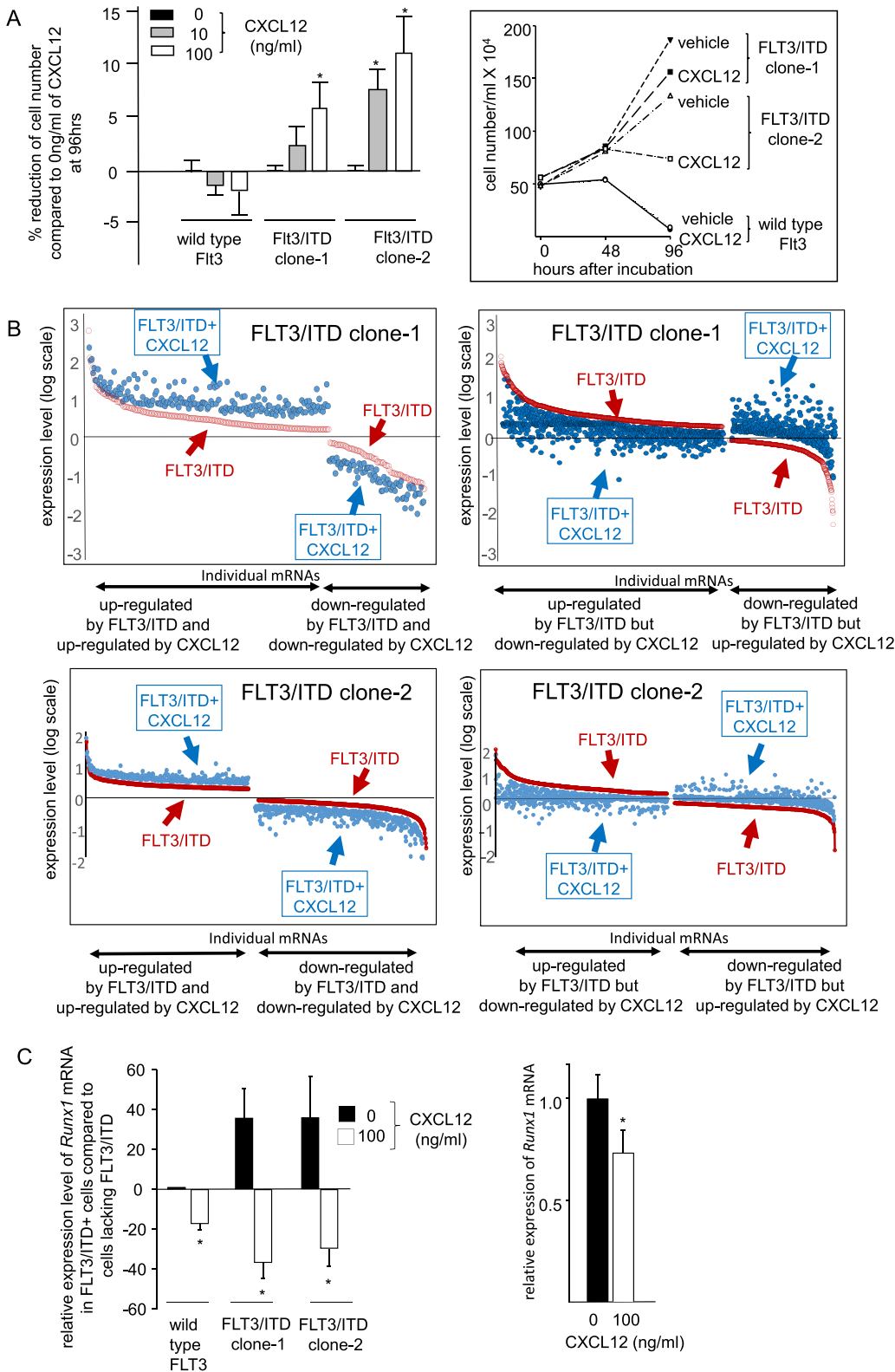


Fig. 1. CXCL12 can inhibit proliferation and negatively modulate Runx1 expression in FLT3/ITD⁺ mouse hematopoietic cells A: Percent reduction in the viable cell number of FLT3/ITD⁺ Ba/F3 cells or cells expressing wild-type FLT3 incubated in 0.1% FBS/RPMI-1640 with or without 10 or 100 ng/ml CXCL12 for 96 h. *P < 0.05 compared to 0 ng/ml CXCL12; N = 6. The right inset shows representative data of viable cell counts of the same cells incubated in 0.1% FBS/RPMI-1640 with or without 100 ng/ml CXCL12 for 96 h. B: Relative expression levels of mRNAs regulated by FLT3/ITD and modulated by 100 ng/ml CXCL12 in FLT3/ITD⁺ Ba/F3 cells. (Top-Left) Relative expression of mRNAs regulated by FLT3/ITD clone-1, open red circle) whose expression level was further upregulated (>0) or downregulated (<0) by CXCL12 in the same direction as FLT3/ITD (blue circle). Each circle represents the expression of an individual mRNA. (Top-Right) Relative expression of mRNAs regulated by FLT3/ITD (clone-1, red circle) whose expression level was inversely regulated by CXCL12 (blue circle). (Bottom) Identical analyses of the mRNAs regulated by FLT3/ITD after a 4-hour incubation with 100 ng/ml CXCL12 in Ba/F3 cells with different FLT3/ITD (clone-2). C: (Left) Relative expression of *Runx1* mRNAs determined by microarray analyses with FLT3/ITD and a 4-hour incubation with 100 ng/ml CXCL12 in FLT3/ITD⁺ Ba/F3 cells (FLT3/ITD clone-1 and clone-2). (Right) Relative expression of *Runx1* mRNA in FLT3/ITD⁺ Ba/F3 cells (FLT3/ITD clone-1) incubated with or without 100 ng/ml CXCL12 for 6 h, as determined by Q-RT-PCR. *P < 0.05 compared to 0 ng/ml CXCL12; N = 3.

3.2. The magnitude of CXCL12/CXCR4 signaling is associated with a divergent response to CXCL12 after exposure to the FLT3 inhibitor in FLT3/ITD⁺ cells

Since our data indicate that 100 ng/ml CXCL12 can antagonize FLT3/ITD function, we next examined the effect of CXCL12 on the proliferation of FLT3/ITD⁺ cells incubated with the FLT3 inhibitor quizartinib. At a concentration of 5 nM, quizartinib significantly inhibited the proliferation of FLT3/ITD⁺ Ba/F3 cells incubated in the presence of 10% serum (FLT3/ITD clone-1, Fig. 2A-Left). Proliferation of FLT3/ITD⁺ Ba/F3 cells under the 10% serum condition was unaffected by 1–500 ng/ml CXCL12 (Fig. 2A-Right). However, in the presence of 5 nM quizartinib, CXCL12 concentrations > 100 ng/ml significantly decreased the number of viable FLT3/ITD⁺ Ba/F3 cells (Fig. 2B-Left). In contrast, 1 ng/ml CXCL12 significantly prevented the inhibition of FLT3/ITD⁺ Ba/F3 cell proliferation by quizartinib (Fig. 2B-Left). Identical findings were observed in cells expressing another FLT3/ITD (clone-2, Fig. 2B-Right).

The concentration-dependent opposing effects of CXCL12 raised the hypothesis that the expression level of the cell surface receptor CXCL12 on FLT3/ITD⁺ cells might also be associated with divergent effects of CXCL12. CXCL12 primarily interacts with the CXCR4 receptor; however, it has been reported to also interact with CXCR7, which acts as a scavenger receptor for CXCL12 [35]. Analysis of cell surface expression demonstrated that only CXCR4 was expressed; surface CXCR7 protein was absent on FLT3/ITD⁺ Ba/F3 cells (Fig. 2C-Top). This led us to hypothesize that lower CXCR4 expression is associated with protection against quizartinib and that higher CXCR4 expression is linked with enhanced quizartinib activity by CXCL12. To validate this hypothesis, FLT3/ITD⁺ Ba/F3 cells with the highest 1% CXCR4 expression, those with intermediate CXCR4 expression, and those with the lowest 1% CXCR4 expression were sorted by FACS (Fig. 2C-Bottom), and sorted cells were analyzed for the effect of CXCL12 on quizartinib activity.

Five nanomolar quizartinib significantly inhibited cell proliferation in all CXCR4-expressing populations (Fig. 2D-Left-Top). CXCL12 at concentrations of 10 and 100 ng/ml significantly increased the number of viable CXCR4^{LOW} FLT3/ITD⁺ Ba/F3 cells incubated with quizartinib but significantly decreased the number of viable CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells (Fig. 2D-Right-Top). A neutralizing antibody against CXCR4 partially abrogated the effect of 100 ng/ml CXCL12 against quizartinib in CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ cells (Fig. 2D-Bottom).

Human MOLM13 FLT3/ITD⁺ AML cells also expressed CXCR4 but not the CXCR7 receptor (Fig. 2E-Top), and the CXCR4^{LOW} and CXCR4^{HIGH} populations were FACS-sorted (Fig. 2E-Bottom). Compared to 0 ng/ml CXCL12, 100 ng/ml CXCL12 significantly enhanced the viability of CXCR4^{LOW} MOLM13 cells incubated with quizartinib or another FLT3 inhibitor, gilteritinib (Fig. 2F-Left panels). In contrast, 100 ng/ml CXCL12 barely enhanced the number of viable CXCR4^{HIGH} cells (Fig. 2F-Right panels).

3.3. Decreased CXCR4 expression is associated with resistance to FLT3 inhibitors mediated by CXCL12 in FLT3/ITD⁺ cells

Since CXCL12 enhanced survival exclusively in CXCR4^{LOW} FLT3/ITD⁺ cells in the presence of quizartinib, we determined whether a reduction in CXCR4 is associated with resistance to FLT3 inhibitors. Quizartinib initially reduced the viable cell number in both CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells; however, continued incubation resulted in the emergence of cells refractory to quizartinib (Fig. 3A). Although 100 ng/ml CXCL12 did not influence the emergence of FLT3/ITD⁺ Ba/F3 cells resistant to quizartinib, CXCR4^{LOW} cells acquired resistance to quizartinib faster than CXCR4^{HIGH} cells (Fig. 3A). Colony-

formation function assays demonstrated that the number of colonies that emerged in the presence of 100 ng/ml CXCL12 incubated with quizartinib or gilteritinib was significantly higher in CXCR4^{LOW} FLT3/ITD⁺ Ba/F3 cells than in CXCR4^{HIGH} cells (Fig. 3B).

The survival of CXCR4^{LOW} FLT3/ITD⁺ Ba/F3 cells incubated with 100 ng/ml CXCL12 and quizartinib or gilteritinib was marginally but significantly higher than that of CXCR4^{HIGH} cells (Fig. 3C). Likewise, the number of viable CXCR4^{LOW} MOLM13 cells was significantly enhanced compared to that of CXCR4^{HIGH} cells after incubation with quizartinib or gilteritinib in the presence of 100 ng/ml CXCL12 (Fig. 3D). The IC50 values in CXCR4^{LOW} and CXCR4^{HIGH} MOLM13 cells after 48 h of incubation were 1.7 ± 0.1 nM and 0.6 ± 0.1 nM for quizartinib ($P < 0.005$; $N = 3$) and 10.8 ± 0.1 nM and 7.2 ± 0.1 nM for gilteritinib ($P < 0.005$; $N = 3$), respectively.

FLT3 phosphorylation relative to total FLT3 was significantly higher in CXCR4^{LOW} FLT3/ITD⁺ Ba/F3 cells than in CXCR4^{HIGH} cells in the presence or absence of 100 ng/ml CXCL12, as determined by ELISA and Western blot analyses (Fig. 3E). Likewise, FLT3 phosphorylation relative to total FLT3 was significantly higher in CXCR4^{LOW} MOLM13 cells than in CXCR4^{HIGH} cells (Fig. 3F), although CXCR4^{HIGH} MOLM13 cells harbor a higher level of total FLT3 protein and proliferate faster in the absence of FLT3 inhibitors than CXCR4^{LOW} cells.

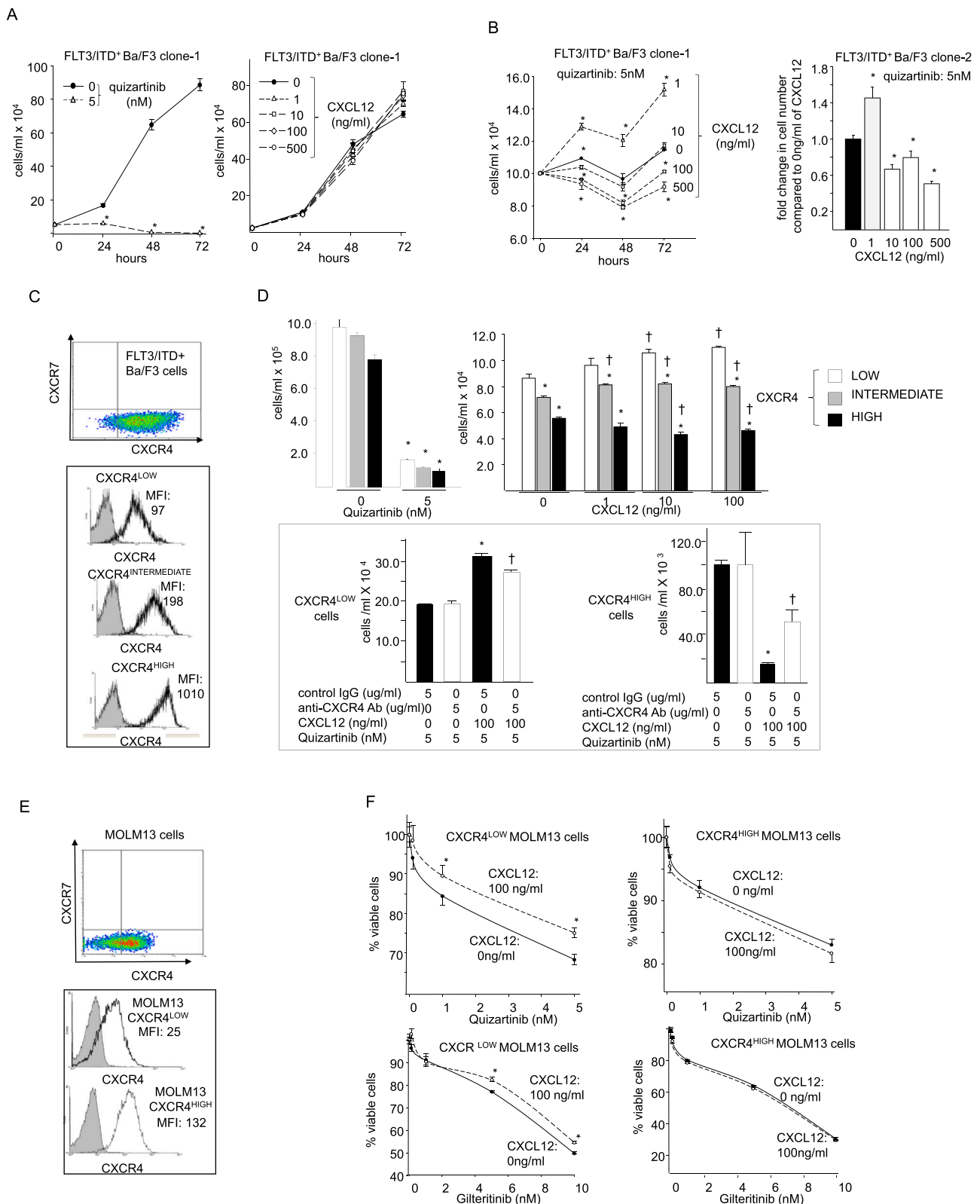
We next compared CXCR4 expression levels between FLT3/ITD⁺ cells that acquired resistance to FLT3 inhibitors and the original cells that were sensitive. CXCR4 expression was significantly downregulated in quizartinib-resistant FLT3/ITD⁺ Ba/F3 cells compared to sensitive cells (Fig. 3G-Left). CXCR7 expression remained undetectable in sensitive and resistant cells (not shown). Similarly, the CXCR4 expression level was significantly lower in gilteritinib-resistant MOLM13 cells than in sensitive cells (Fig. 3G-Right). These data suggest that a reduction in CXCR4 levels is associated with resistance to FLT3 inhibitors in FLT3/ITD⁺ cells.

3.4. The expression levels of CXCR4 and RUNX1 are inversely correlated in FLT3/ITD⁺ cells

While CXCR4 expression levels are elevated in FLT3/ITD⁺ AML cells [17,18,36], transduction of FLT3/ITD in human CD34⁺ cells and in mouse hematopoietic cells decreased CXCR4 expression [15,16,22]. Moreover, CXCR4 is transactivated by Runx1 [37], whose expression is significantly elevated by FLT3/ITD in human AML cells [30,31]. These findings suggest that while Cxcr4 expression is downregulated by FLT3/ITD, its expression can be upregulated by FLT3/ITD through RUNX1. We therefore investigated the regulation of CXCR4 expression by RUNX1 in FLT3/ITD⁺ cells. Transduction of Runx1 shRNAs downregulated CXCR4 levels in FLT3/ITD⁺ Ba/F3 cells (Fig. 4A-Left), whereas overexpressing Runx1 cDNA increased the level of CXCR4 (Fig. 4A-Right), indicating that RUNX1 upregulates CXCR4 expression, as previously reported [37].

However, RUNX1 protein and mRNA levels were significantly higher in CXCR4^{LOW} FLT3/ITD⁺ Ba/F3 cells than in CXCR4^{HIGH} cells both in the presence and absence of 100 ng/ml CXCL12 (Fig. 4B). RUNX1 protein expression was also higher in CXCR4^{LOW} FLT3/ITD⁺ Ba/F3 cells that acquired resistance to quizartinib than in CXCR4^{HIGH}-resistant cells (Fig. 4B-Bottom). Similarly, RUNX1 protein expression was significantly higher in CXCR4^{LOW} MOLM13 cells than in CXCR4^{HIGH} cells (Fig. 4C). Furthermore, RUNX1 protein or mRNA levels were negatively correlated with CXCR4 levels in human FLT3/ITD⁺ AML cells ($r = -0.378$, $P < 0.01$, $N = 78$, GSE 1159, Fig. 4D), which was not observed in FLT3/ITD⁻ AML cells ($N = 187$).

We next compared the regulation of RUNX1 expression levels by CXCL12 between CXCR4^{LOW} FLT3/ITD⁺ cells and CXCR4^{HIGH} cells



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Fig. 2. The magnitude of CXCL12/CXCR4 signaling is associated with a divergent response to CXCL12 after exposure to the FLT3 inhibitor in FLT3/ITD⁺ cells A: (Left) Viable cell number of FLT3/ITD⁺ Ba/F3 cells incubated in 10% FBS/RPMI-1640 with or without 5 nM quizartinib for 72 h. * P < 0.05 compared to 0 nM quizartinib; N = 3, (Right) Proliferation of FLT3/ITD⁺ Ba/F3 cells cultured in 10% FBS/RPMI-1640 with or without CXCL12 for 72 h; N = 3. B: (Left) Proliferation of FLT3/ITD⁺ Ba/F3 cells (FLT3/ITD clone-1) in the presence of 5 nM quizartinib with increasing doses of CXCL12 for 72 h. (Right) Fold change in viable cell number of FLT3/ITD⁺ Ba/F3 cells (FLT3/ITD clone-2) in the presence of 5 nM quizartinib with increasing doses of CXCL12 after 72 h. Quizartinib and CXCL12 were added to the culture at the initiation of incubation (time 0). *P < 0.05 compared to 0 ng/ml CXCL12; N = 3. C: (Top) Expression of surface CXCR4 and CXCR7 on FLT3/ITD⁺ Ba/F3 cells. The bar in the plot represents isotype staining levels. (Bottom) CXCR4 expression on FACS-sorted CXCR4^{LOW}, CXCR4^{INTERMEDIATE} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells. Cells were stained with an APC-conjugated anti-CXCR4 antibody, and those with the highest 1% CXCR4 expression, intermediate CXCR4 expression and the lowest 1% CXCR4 expression were FACS sorted using a BD FACS Aria II (BD Bioscience). D: (Left top) Viable cell numbers of CXCR4^{LOW}, CXCR4^{INTERMEDIATE} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells incubated with or without 5 nM quizartinib for 48 h. *P < 0.05 compared to 0 nM quizartinib; N = 3, (Right top) Cell numbers of CXCR4^{LOW}, CXCR4^{INTERMEDIATE} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells incubated for 48 h in the presence of 5 nM quizartinib with increasing doses of CXCL12 (0–100 ng/ml). *P < 0.05 compared to CXCR4^{LOW} cells; †P < 0.05 compared to 0 ng/ml CXCL12; N = 3, (Lower panel) Number of CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells incubated with 5 nM quizartinib in the presence or absence of 100 ng/ml CXCL12 and/or 5 µg/ml neutralizing antibody against mouse CXCR4 (clone 247506, R&D systems) for 48 h. * P < 0.05 compared to those without CXCL12. †P < 0.05 compared to control IgG_{2b} in the presence of 100 ng/ml CXCL12. E: (Top) CXCR4 and CXCR7 expression on MOLM13 cells. The bar in the plot represents isotype staining levels. (Bottom) CXCR4 expression on sorted CXCR4^{LOW} and CXCR4^{HIGH} MOLM13 cells. F: Comparison between 0 and 100 ng/ml CXCL12 on the survival of CXCR4^{LOW} (left) and CXCR4^{HIGH} MOLM13 cells (right) incubated with quizartinib (top) or gilteritinib (bottom) for 48 h. * P < 0.05 compared to 0 ng/ml CXCL12; N = 3.

incubated with quizartinib. In the presence of 5 nM quizartinib, CXCL12 upregulated RUNX1 mRNA and protein expression in CXCR4^{LOW} FLT3/ITD⁺ Ba/F3 cells, whereas the same treatment failed to increase RUNX1 expression in CXCR4^{HIGH} cells (Fig. 4E). These data indicate that while CXCR4 is upregulated by RUNX1, CXCR4 expression is negatively correlated with RUNX1 expression levels in FLT3/ITD⁺ cells.

3.5. RUNX1 mediates the paradoxical activity of CXCR4 signaling that alternates resistance to FLT3 inhibitors in FLT3/ITD⁺ cells with different CXCR4 levels

Since higher RUNX1 expression in CXCR4^{LOW} FLT3/ITD⁺ cells is associated with the cytoprotective effect of CXCL12 against FLT3 inhibitors, while lower RUNX1 expression levels are linked to the enhancing effect of FLT3 inhibitors by CXCL12, we investigated whether modulating RUNX1 expression reverses the response of CXCL12 to FLT3 inhibitors in FLT3/ITD⁺ cells expressing different CXCR4 levels. CXCL12 at a concentration of 100 ng/ml significantly enhanced the proliferation of CXCR4^{LOW} FLT3/ITD⁺ Ba/F3 cells incubated with quizartinib, whereas 2 different shRNAs against *Runx1* significantly decreased the enhancing effect of CXCL12 on proliferation (Fig. 5A).

Conversely, 100 ng/ml CXCL12 significantly decreased the survival of CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells incubated with quizartinib, whereas overexpressing *Runx1* cDNAs significantly enhanced their proliferation in the presence or absence of CXCL12 (Fig. 5B-Left). Moreover, overexpressing RUNX1 significantly enhanced the emergence of refractory cells that continued to proliferate in the presence of quizartinib and CXCL12 (Fig. 5B-Right). These data indicate that RUNX1 regulates the divergent activity of CXCL12, which alternates resistance to quizartinib in FLT3/ITD⁺ cells with different CXCR4 levels.

4. Discussion

This study demonstrates that CXCR4 signaling not only enhances resistance but also attenuates refractoriness against the FLT3 inhibitor quizartinib in FLT3/ITD⁺ cells by modulating RUNX1 expression. A low concentration of CXCL12 antagonized the effect of quizartinib, whereas a high dose of CXCL12 enhanced this effect. Consistently, CXCL12 enhanced resistance to quizartinib in CXCR4^{LOW} FLT3/ITD⁺ cells but enhanced the effect of quizartinib in CXCR4^{HIGH} FLT3/ITD⁺ cells. The CXCL12-mediated enhanced resistance to quizartinib in CXCR4^{LOW} FLT3/ITD⁺ cells was associated with elevated RUNX1 expression, whereas the CXCL12-mediated decreased resistance to quizartinib in CXCR4^{HIGH} cells was coincident with a reduction in RUNX1 expression.

Silencing RUNX1 significantly abrogated resistance to quizartinib in CXCR4^{LOW} FLT3/ITD⁺ cells incubated with CXCL12, whereas ectopic expression of RUNX1 restored resistance in CXCR4^{HIGH} FLT3/ITD⁺ cells. These data indicate that the divergent activity of Cxcr4 signaling, with alternating resistance to quizartinib in FLT3/ITD⁺ cells, is mediated by RUNX1.

While CXCR4 was expressed on FLT3/ITD⁺ Ba/F3 cells and MOLM13 cells, CXCR7 was absent. Moreover, a neutralizing antibody against CXCR4 partially reversed the divergent activity of CXCL12 in FLT3/ITD⁺ Ba/F3 cells with different CXCR4 levels, suggesting that the opposing activity of CXCL12 is mediated through CXCR4. Although CXCR4 signaling provides a survival signal in AML cells [20,22,24], the present study demonstrates that it can inhibit the proliferation of FLT3/ITD⁺ cells, which is consistent with a study that demonstrated CXCR4 signaling-induced apoptosis in AML cells [25,26]. Analyses of mRNAs regulated by FLT3/ITD and CXCL12 using microarrays indicated that the expression of 16–19% of mRNAs that were regulated by FLT3/ITD was inversely modulated by CXCL12. Furthermore, the inhibitory effect of CXCL12 on FLT3/ITD⁺ cell proliferation was coincident with the downregulation of Runx1 expression, which functions as an oncogene in FLT3/ITD⁺ AML cells [30,31]. These findings suggest that the apoptotic phenotype of FLT3/ITD⁺ cells induced by CXCL12 is associated with antagonistic changes in molecules downstream of FLT3/ITD, such as Runx1. However, our data demonstrated that CXCL12/CXCR4 signaling not only inhibits proliferation but also enhances proliferation of FLT3/ITD⁺ cells, which was dependent on the magnitude of CXCL12/CXCR4 signaling. The divergent effect of CXCL12 in CXCR4^{LOW} cells and CXCR4^{HIGH} FLT3/ITD⁺ cells was associated with differential regulation of RUNX1 expression levels in response to CXCL12 (Fig. 6).

While CXCR4 is transactivated by Runx1, we found that CXCR4^{HIGH} FLT3/ITD⁺ cells harbor lower RUNX1 levels, whereas CXCR4^{LOW} FLT3/ITD⁺ cells express higher levels of RUNX1. Since FLT3/ITD decreases CXCR4 [15,16,22] but increases RUNX1 expression [30,31], the inverse correlation between RUNX1 and CXCR4 likely represents FLT3/ITD function. Indeed, FLT3 phosphorylation was significantly higher in CXCR4^{LOW} FLT3/ITD⁺ cells than in CXCR4^{HIGH} cells (Fig. 6). Consistent with these findings, *RUNX1* and *CXCR4* mRNA expression levels were inversely correlated in FLT3/ITD⁺ AML samples. The data suggest that CXCR4^{LOW} FLT3/ITD⁺ cells, which harbor higher expression levels of RUNX1, represent populations that acquire resistance to FLT3 inhibitors. In this regard, low CXCR4 levels and high RUNX1 expression in FLT3/ITD⁺ AML cells may be markers to identify cells that become refractory to FLT3 inhibitors.

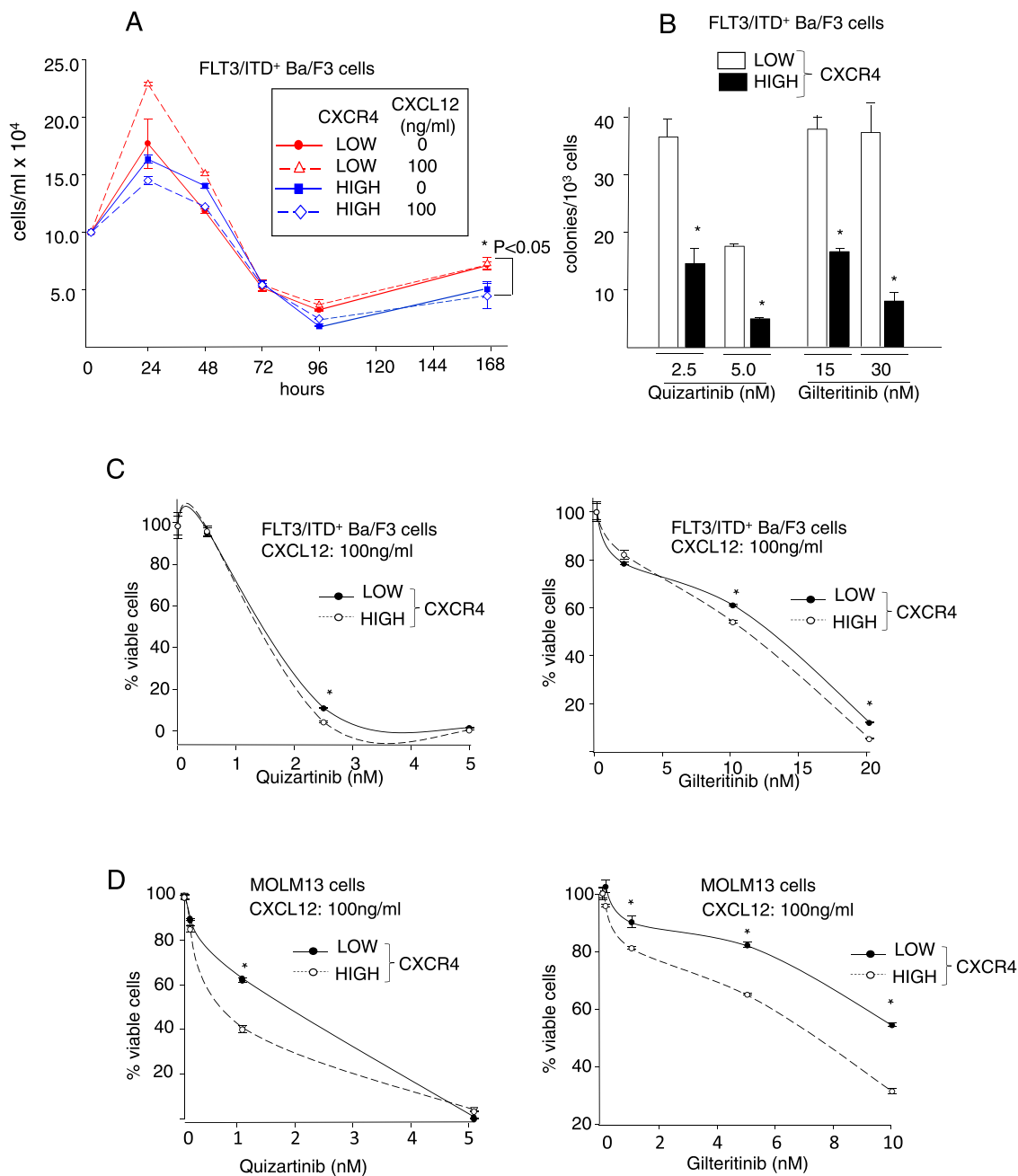


Fig. 3. Decreased CXCR4 expression is associated with resistance to FLT3 inhibitors mediated by CXCL12 in FLT3/ITD⁺ cells A: Viable cell number in CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells incubated in the presence of 5 nM quizartinib with or without 100 ng/ml CXCL12 for 7 days. *P < 0.05 compared between CXCR4^{LOW} and CXCR4^{HIGH} cells; N = 3. B: The number of colonies derived from CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells in the presence of 2.5 or 5 nM quizartinib and 15 or 30 nM gilteritinib. A total of 1 × 10⁶ cells were plated on methylcellulose medium (Methocult®, Stem Cell Technologies, VA, Canada) without any growth factors in the presence of the indicated concentrations of quizartinib or gilteritinib. Colonies were scored under a microscope on day 7. *P < 0.05 compared to CXCR4^{LOW} cells; N = 3. C: Comparison of survival between CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells incubated with 100 ng/ml CXCL12 and quizartinib (left) or gilteritinib (right) for 48 h. *P < 0.05 compared to CXCR4^{HIGH} cells; N = 3. D: Comparison of survival between CXCR4^{LOW} and CXCR4^{HIGH} MOLM13 cells incubated with quizartinib (left) or gilteritinib (right) and 100 ng/ml CXCL12 for 48 h. *P < 0.05 compared to CXCR4^{HIGH} cells; N = 3. E: (Top) Phospho-FLT3 relative to total FLT3 in CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells incubated with 5 nM quizartinib or 30 nM gilteritinib in the presence or absence of 100 ng/ml CXCL12 for 4 h, quantitated by PathScan Phospho-FLT3 (panTyr) Sandwich ELISA kits (Cell Signaling Technology, Danvers, MA). *P < 0.05 compared to CXCR4^{LOW} cells; N = 3, (Lower) Western blot of phospho- and total FLT3 in CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells. M represents the MW marker. F: (Top) Phospho-FLT3 relative to total FLT3 in CXCR4^{LOW} and CXCR4^{HIGH} MOLM13 cells incubated with 5 nM quizartinib or 30 nM gilteritinib in the presence or absence of 100 ng/ml CXCL12 for 4 h. *P < 0.05 compared to CXCR4^{LOW} cells; N = 3, (Lower) Western blot of phospho- and total FLT3 in CXCR4^{LOW} and CXCR4^{HIGH} MOLM13 cells treated in the same manner as above. G: (Left) CXCR4 expression in CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells resistant or sensitive to quizartinib. *P < 0.05 compared to sensitive cells; N = 3, (Right) CXCR4 expression on MOLM13 cells resistant or sensitive to 10 nM gilteritinib. *P < 0.05 compared to sensitive cells; N = 3.

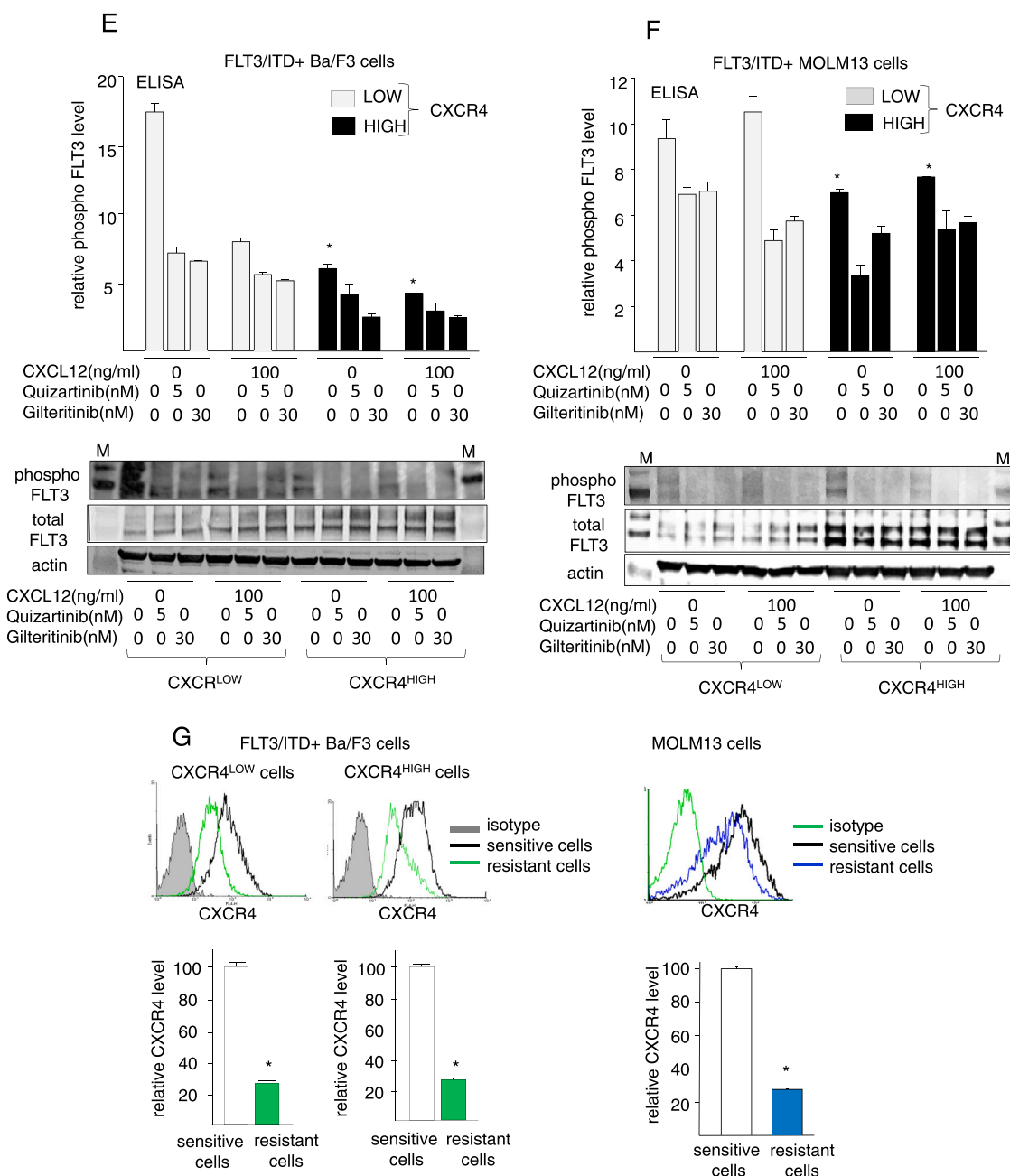


Fig. 3. (continued).

In addition, CXCR4 expression was downregulated in FLT3/ITD⁺ cells refractory to FLT3 inhibitors compared to cells sensitive to the inhibitors. The data suggest that FLT3/ITD⁺ cells that acquired resistance to FLT3 inhibitors are likely derived from the CXCR4^{LOW} population that harbors higher RUNX1 expression and that the reduction in CXCR4 expression in resistant cells is likely a consequence of preferential proliferation of CXCR4^{LOW} FLT3/ITD⁺ refractory clones. These observations are similar to a previous study demonstrating that CXCR4 expression was downregulated in the subgroup of CML stem cells resistant to a tyrosine kinase inhibitor [38].

Blocking the interaction between FLT3/ITD⁺ cells and the microenvironment using CXCR4 antagonists is a promising strategy [20,21], especially when CXCL12 provides a cytoprotective effect to FLT3/ITD⁺

cells. However, the present study demonstrates that CXCL12 is a potential apoptosis inducer and that a neutralizing antibody against CXCR4 enhances proliferation in CXCR4^{HIGH} FLT3/ITD⁺ cells incubated with quizartinib and CXCL12. These data suggest that blocking CXCR4 can abrogate the apoptotic signaling induced by CXCL12 and that antagonizing CXCR4 can enhance the survival of FLT3/ITD⁺ cells with higher CXCR4 expression levels. In this regard, antagonizing RUNX1 represents another strategy to eradicate FLT3/ITD⁺ AML cells by blocking their proliferation in the bone marrow microenvironment, where CXCR4 signaling is divergently regulated by CXCL12. Although this study demonstrates the involvement of RUNX1 in the divergent resistant phenotype by CXCR4 signaling in FLT3/ITD⁺ cells, the underlying mechanism responsible for resistance to FLT3 inhibitors

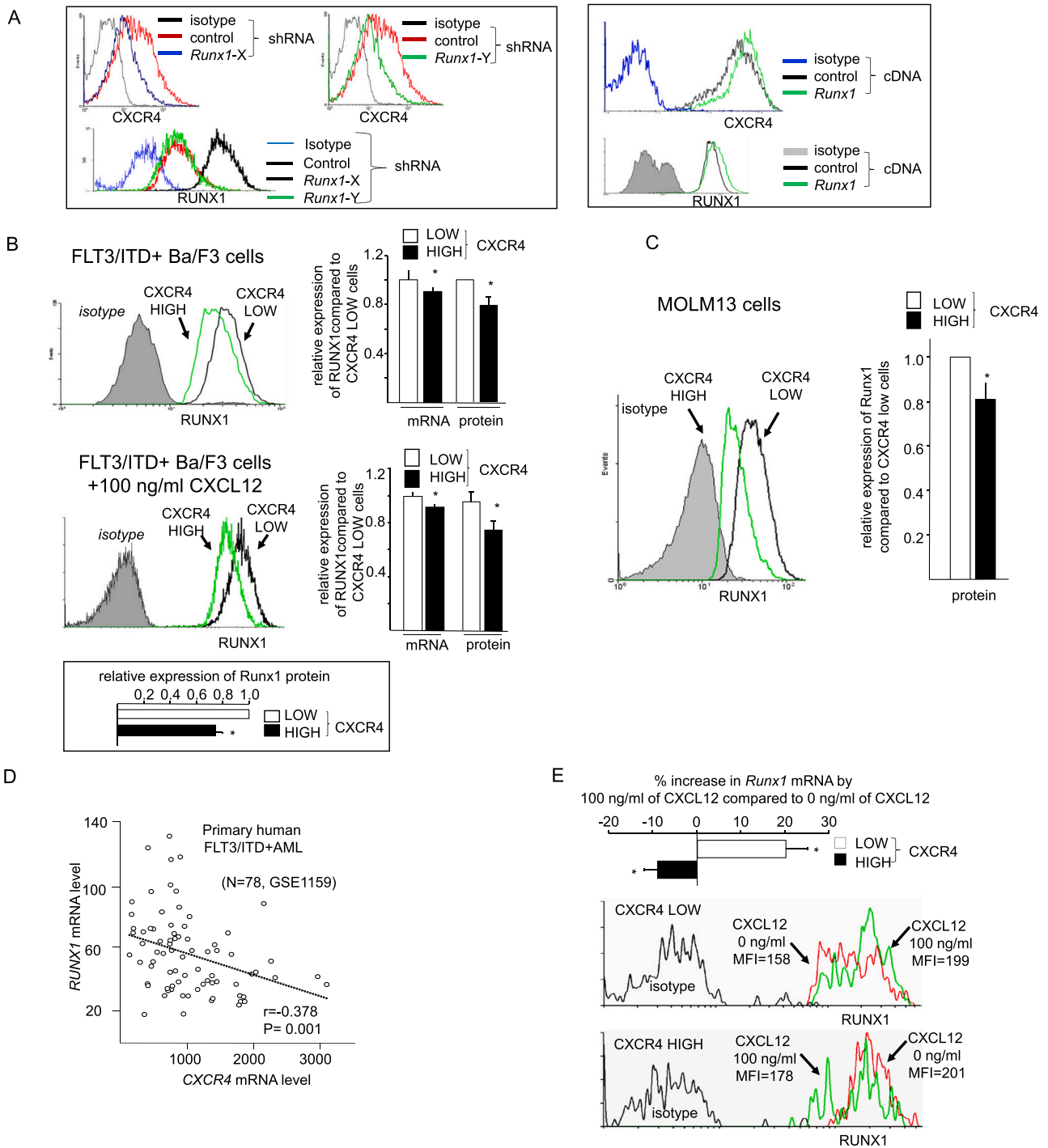


Fig. 4. The expression levels of CXCR4 and RUNX1 are inversely correlated in FLT3/ITD⁺ cells A: (Left) Comparison of CXCR4 expression between FLT3/ITD⁺ Ba/F3 cells transfected with two different *Runx1* shRNAs (shRNA-X and shRNA-Y) or control shRNA. RUNX1 expression in the transfected cells is shown beneath the plots. (Right) CXCR4 expression in FLT3/ITD⁺ Ba/F3 cells transfected with *Runx1* cDNA. RUNX1 expression in the transfected cells is shown beneath the plot. B: (Top-Left) RUNX1 protein expression in CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells. (Top-Right) Quantitative analyses of *Runx1* mRNA and protein expression levels in CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells. *P < 0.05 compared to CXCR4^{LOW} cells; N = 3. (Middle-Left) RUNX1 protein expression in CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells incubated with 100 ng/ml CXCL12 for 24 h. (Middle-Right) Comparison of *Runx1* mRNA and protein expression between CXCR4^{LOW} and CXCR4^{HIGH} Ba/F3 cells incubated with 100 ng/ml CXCL12 for 48 h. *P < 0.05 compared to CXCR4^{LOW} cells; N = 3. (Lower) Relative expression of RUNX1 protein in quizartinib-resistant CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells. *P < 0.05 compared to CXCR4^{LOW} cells; N = 3. C: (Left) Representative RUNX1 protein expression in CXCR4^{LOW} and CXCR4^{HIGH} MOLM13 cells. (Right) Quantitative analyses of RUNX1 protein expression levels in CXCR4^{LOW} and CXCR4^{HIGH} MOLM13 cells. *P < 0.05 compared to CXCR4^{LOW} cells; N = 4. D: Correlation between *CXCR4* and *RUNX1* mRNAs in primary human FLT3/ITD⁺ AML cells obtained from a public database (GSE 1159, N = 78, r = -0.378, P = 0.001). E: (Top) Percentage increase in *Runx1* mRNA in CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells incubated with 2 nM quizartinib and 100 ng/ml CXCL12 for 48 h compared with 0 ng/ml CXCL12. *P < 0.05 compared to 0 ng/ml CXCL12; N = 3. (Lower) RUNX1 protein expression in CXCR4^{LOW} and CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells incubated with 5 nM quizartinib and 100 ng/ml CXCL12 for 48 h compared with 0 ng/ml CXCL12. The figure demonstrates one of the representative results of 3 experiments with identical results.

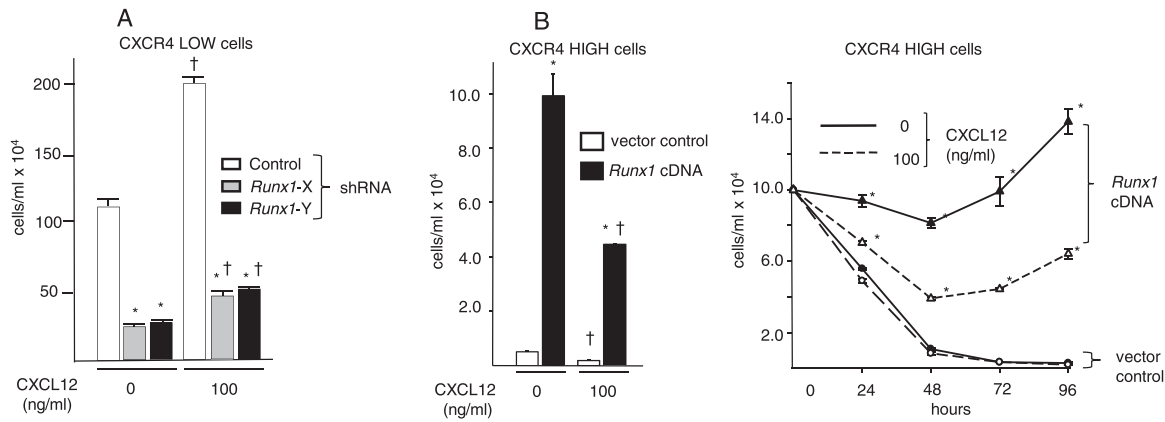


Fig. 5. RUNX1 mediates the paradoxical activity of CXCR4 signaling that alternates resistance to FLT3 inhibitors in FLT3/ITD⁺ cells with different CXCR4 levels A: Viable CXCR4^{LOW} FLT3/ITD⁺ Ba/F3 cells transfected with shRNA against *Runx1* incubated with 2 nM quizartinib in the presence or absence of 100 ng/ml CXCL12 for 48 h. *P < 0.05 compared to control shRNA; †P < 0.05 compared to 0 ng/ml CXCL12; N = 3. RUNX1 protein expression in the cells transfected with shRNAs against *Runx1* and the control is shown in Fig. 4A. B: (Left) Viable cell number in CXCR4^{HIGH} FLT3/ITD⁺ Ba/F3 cells overexpressing *Runx1* cDNA or vector control incubated with 5 nM quizartinib in the presence or absence of 100 ng/ml CXCL12 at 72 h. *P < 0.05 compared to vector control, †P < 0.05 compared to 0 ng/ml CXCL12; N = 3. RUNX1 protein expression in the cells transfected with *Runx1* cDNA is shown in Fig. 4A. (Right) Proliferation of the same cells shown in the left panel over 96 h. *P < 0.05 compared to vector control; N = 3.

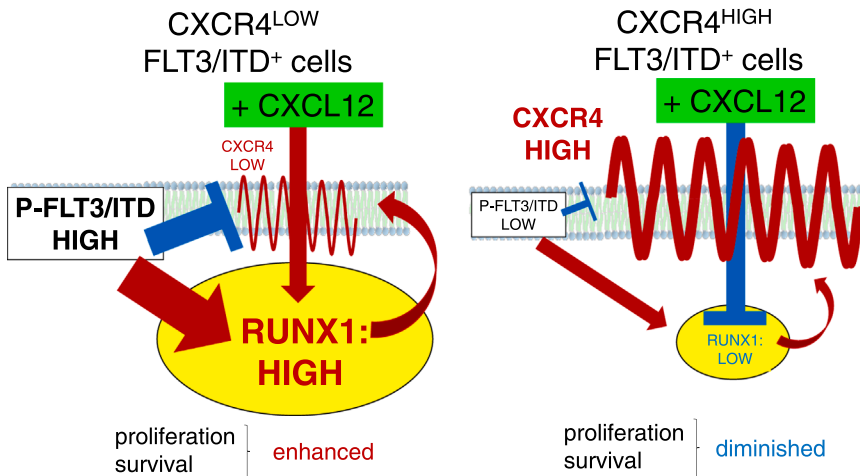


Fig. 6. CXCR4 signaling of different magnitudes paradoxically regulates resistance to quizartinib in FLT3/ITD⁺ cells via RUNX1. RUNX1 transactivates CXCR4; however, CXCR4^{LOW} FLT3/ITD⁺ cells exhibit higher RUNX1 expression, likely through enhanced phosphorylated FLT3. Resistance to FLT3 inhibitors by CXCL12 is associated with elevation of RUNX1 in CXCR4^{LOW} FLT3/ITD⁺ cells. In contrast, RUNX1 levels are low in CXCR4^{HIGH} cells, coincident with a reduction in the phosphorylation of FLT3. CXCL12-mediated decreased resistance to quizartinib was associated with a reduction in RUNX1 expression.

remains to be determined. The mechanism conferring resistance to FLT3 inhibitors may vary in different patients; therefore, the findings reported in this study may not be applicable to all cases refractory to the inhibitors.

In conclusion, the present study demonstrates that CXCL12/CXCR4 signaling can either antagonize or enhance the effects of FLT3 inhibitors, and the direction of regulation is dependent on the magnitude of CXCL12/CXCR4 signaling. The divergent effects of CXCL12/CXCR4 signaling on the response to FLT3 inhibitors in FLT3/ITD⁺ cells are regulated by RUNX1.

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Ethical Consideration

Human subjects and experimental animals were not involved.

Conflict of Interest Statement

SF received honoraria from Astellas Pharma Inc. for seminar presentation.

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References

- [1] G. Ghiaur, M. Levis, Mechanisms of resistance to FLT3 inhibitors and the role of the bone marrow microenvironment, *Hematol. Oncol. Clin. N. 31* (4) (2017) 681–692.
- [2] M.A. Hospital, A.S. Green, T.T. Maciel, I.C. Moura, A.Y. Leung, D. Bouscary, et al., FLT3 inhibitors: clinical potential in acute myeloid leukemia, *Onco Targets Ther. 10* (2017) 607–615.
- [3] J. Cortes, A.E. Perl, H. Dohner, H. Kantarjian, G. Martinelli, T. Kovacsovic, et al., Quizartinib, an FLT3 inhibitor, as monotherapy in patients with relapsed or refractory acute myeloid leukaemia: an open-label, multicentre, single-arm, phase 2 trial, *Lancet Oncol. 19* (7) (2018) 889–903.
- [4] J.E. Cortes, S. Khaled, G. Martinelli, A.E. Perl, S. Ganguly, N. Russell, et al., Quizartinib versus salvage chemotherapy in relapsed or refractory FLT3-ITD acute

- myeloid leukaemia (QuANTUM-R): a multicentre, randomised, controlled, open-label, phase 3 trial, *Lancet Oncol.* 20 (7) (2019) 984–997.
- [5] C.C. Smith, The growing landscape of FLT3 inhibition in AML, *Hematol. Am. Soc. Hematol. Educ. Program* 2019 (1) (2019) 539–547.
- [6] C.M. McMahon, T. Ferng, J. Canaani, E.S. Wang, J.J.D. Morrisette, D.J. Eastburn, et al., Clonal selection with RAS pathway activation mediates secondary clinical resistance to selective FLT3 inhibition in acute myeloid leukemia, *Cancer Discov.* 9 (8) (2019) 1050–1063.
- [7] C.C. Smith, Q. Wang, C.S. Chin, S. Salerno, L.E. Damon, M.J. Levis, et al., Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia, *Nature* 485 (7397) (2012) 260–263.
- [8] A. Greenbaum, Y.M.S. Hsu, R.B. Day, L.G. Schuettpelz, M.J. Christopher, J. N. Borgerding, et al., CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance, *Nature* 495 (7440) (2013) 227–230.
- [9] R. Mohle, F. Bautz, S. Raffi, M.A.S. Moore, W. Brugger, L. Kanz, The chemokine receptor CXCR-4 is expressed on CD34(+) hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1, *Blood* 91 (12) (1998) 4523–4530.
- [10] Y. Omatsu, T. Sugiyama, H. Kohara, G. Kondoh, N. Fujii, K. Kohno, et al., The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche, *Immunity* 33 (3) (2010) 387–399.
- [11] A. Peled, I. Petit, O. Kollet, M. Magid, T. Ponomaryov, T. Byk, et al., Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4, *Science* 283 (5403) (1999) 845–848.
- [12] T. Sugiyama, H. Kohara, M. Noda, T. Nagasawa, Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches, *Immunity* 25 (6) (2006) 977–988.
- [13] S. Tavor, I. Petit, S. Porozov, A. Avigdor, A. Dar, L. Leider-Trejo, et al., CXCR4 regulates migration and development of human acute myelogenous leukemia stem cells in transplanted NOD/SCID mice, *Cancer Res.* 64 (8) (2004) 2817–2824.
- [14] C. Voermans, W.P.M. van Heese, I. de Jong, W.R. Gerritsen, C.E. van der Schoot, Migratory behavior of leukemic cells from acute myeloid leukemia patients, *Leukemia* 16 (4) (2002) 650–657.
- [15] S. Fukuda, H.E. Broxmeyer, L.M. Pelus, Flt3 ligand and the Flt3 receptor regulate hematopoietic cell migration by modulating the SDF-1alpha(CXCL12)/CXCR4 axis, *Blood* 105 (8) (2005) 3117–3126.
- [16] C. Onishi, S. Mori-Kimachi, T. Hirade, M. Abe, T. Taketani, J. Suzumiya, et al., Internal tandem duplication mutations in FLT3 gene augment chemotaxis to Cxcl12 protein by blocking the down-regulation of the Rho-associated kinase via the Cxcl12/Cxcr4 signaling axis, *J. Biol. Chem.* 289 (45) (2014) 31053–31065.
- [17] A.C. Spoo, M. Lubbert, W.G. Wierda, J.A. Burger, CXCR4 is a prognostic marker in acute myelogenous leukemia, *Blood* 109 (2) (2007) 786–791.
- [18] W. Du, C. Lu, X.Y. Zhu, D. Hu, X.J. Chen, J. Li, et al., Prognostic significance of CXCR4 expression in acute myeloid leukemia, *Cancer Med.* 8 (15) (2019) 6595–6603.
- [19] M. Abraham, S. Klein, B. Bulvik, H. Wald, I.D. Weiss, D. Olam, et al., The CXCR4 inhibitor BL-8040 induces the apoptosis of AML blasts by downregulating ERK, BCL-2, MCL-1 and cyclin-D1 via altered miR-15a/16-1 expression, *Leukemia* 31 (11) (2017) 2336–2346.
- [20] Z.H. Zeng, Y.X. Shi, I.J. Samudio, R.Y. Wang, X.Y. Ling, O. Frolova, et al., Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML, *Blood* 113 (24) (2009) 6215–6224.
- [21] K. Kojima, T. McQueen, Y. Chen, R. Jacamo, M. Konopleva, N. Shinjima, et al., p53 activation of mesenchymal stromal cells partially abrogates microenvironment-mediated resistance to FLT3 inhibition in AML through HIF-1alpha-mediated down-regulation of CXCL12, *Blood* 118 (16) (2011) 4431–4439.
- [22] A. Jacobi, S. Thieme, R. Lehmann, F. Ugarte, H.L. Malech, S. Koch, et al., Impact of CXCR4 inhibition on FLT3-ITD-positive human AML blasts, *Exp. Hematol.* 38 (3) (2010) 180–190.
- [23] Z. Zeng, I.J. Samudio, M. Munsell, J. An, Z. Huang, E. Estey, et al., Inhibition of CXCR4 with the novel RCP168 peptide overcomes stroma-mediated chemoresistance in chronic and acute leukemias, *Mol. Cancer Ther.* 5 (12) (2006) 3113–3121.
- [24] B.R. Kim, S.H. Jung, A.R. Han, G. Park, H.J. Kim, B. Yuan, et al., CXCR4 inhibition enhances efficacy of FLT3 inhibitors in FLT3-mutated AML augmented by suppressed TGF-beta signaling, *Cancers* 12 (2020) 7.
- [25] K.N. Kremer, A. Dudakovic, M.E. McGee-Lawrence, R.L. Philips, A.D. Hess, B. D. Smith, et al., Osteoblasts protect AML cells from SDF-1-induced apoptosis, *J. Cell Biochem.* 115 (6) (2014) 1128–1137.
- [26] K.N. Kremer, K.L. Peterson, P.A. Schneider, X.W. Meng, H. Dai, A.D. Hess, et al., CXCR4 chemokine receptor signaling induces apoptosis in acute myeloid leukemia cells via regulation of the Bcl-2 family members Bcl-XL, Noxa, and Bak, *J. Biol. Chem.* 288 (32) (2013) 22899–22914.
- [27] S. Goyama, J. Schibler, L. Cunningham, Y. Zhang, Y. Rao, N. Nishimoto, et al., Transcription factor RUNX1 promotes survival of acute myeloid leukemia cells, *J. Clin. Investig.* 123 (9) (2013) 3876–3888.
- [28] K. Morita, S. Maeda, K. Suzuki, H. Kiyose, J. Taniguchi, P.P. Liu, et al., Paradoxical enhancement of leukemogenesis in acute myeloid leukemia with moderately attenuated RUNX1 expressions, *Blood Adv.* 1 (18) (2017) 1440–1451.
- [29] K. Morita, K. Suzuki, S. Maeda, A. Matsuo, Y. Mitsuda, C. Tokushige, et al., Genetic regulation of the RUNX transcription factor family has antitumor effects, *J. Clin. Investig.* 127 (7) (2017) 2815–2828.
- [30] K. Behrens, K. Maul, N. Tekin, N. Kriebitzsch, D. Indenbirken, V. Prassolov, et al., RUNX1 cooperates with FLT3-ITD to induce leukemia, *J. Exp. Med.* 214 (3) (2017) 737–752.
- [31] T. Hirade, M. Abe, C. Onishi, T. Taketani, S. Yamaguchi, S. Fukuda, Internal tandem duplication of FLT3 deregulates proliferation and differentiation and confers resistance to the FLT3 inhibitor AC220 by up-regulating RUNX1 expression in hematopoietic cells, *Int. J. Hematol.* 103 (1) (2016) 95–106.
- [32] L.M. Kelly, Q. Liu, J.L. Kutok, I.R. Williams, C.L. Boulton, D.G. Gilliland, FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model, *Blood* 99 (1) (2002) 310–318.
- [33] H. Quentmeier, J. Reinhardt, M. Zaborski, H.G. Drexler, FLT3 mutations in acute myeloid leukemia cell lines, *Leukemia* 17 (1) (2003) 120–124.
- [34] M. Abe, L.M. Pelus, P. Singh, T. Hirade, C. Onishi, J. Purevsuren, et al., Internal tandem duplication in FLT3 attenuates proliferation and regulates resistance to the FLT3 inhibitor AC220 by modulating p21Cdkn1a and Pbx1 in hematopoietic cells, *PLoS One* 11 (7) (2016), e0158290.
- [35] U. Naumann, E. Cameron, M. Pruenster, H. Mahabaleshwar, E. Raz, H.G. Zerwes, et al., CXCR7 functions as a scavenger for CXCL12 and CXCL11, *PLoS One* 5 (2) (2010), e9175.
- [36] E.J.C. Rombouts, B. Pavic, B. Lowenberg, R.E. Ploemacher, Relation between CXCR-4 expression, Flt3 mutations, and unfavorable prognosis of adult acute myeloid leukemia, *Blood* 104 (2) (2004) 550–557.
- [37] B. Jacob, M. Osato, N. Yamashita, C.Q. Wang, I. Taniuchi, D.R. Littman, et al., Stem cell exhaustion due to Runx1 deficiency is prevented by Evi5 activation in leukemogenesis, *Blood* 115 (8) (2010) 1610–1620.
- [38] A. Giustacchini, S. Thongjuea, N. Barkas, P.S. Woll, B.J. Povinelli, C.A.G. Booth, et al., Single-cell transcriptomics uncovers distinct molecular signatures of stem cells in chronic myeloid leukemia, *Nat. Med.* 23 (6) (2017) 692–702.