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## Molecular Interaction Between the Microenvironment and FLT3/ITD<sup>+</sup> AML Cells Leading to the Refractory Phenotype

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Additional information is available at the end of the chapter

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#### Abstract

Internal tandem duplication mutations in the FLT3 gene (FLT3/ITD) are detected in 10–15% of children and 30% of adult patients with AML and are associated with an extremely poor prognosis. Although several antagonists against FLT3/ITD have been developed, few of them are effective for the treatment of FLT3/ITD<sup>+</sup> AML because of the emergence of drug-resistant cells. The mechanisms responsible for drug resistance include the acquisition of additional mutations in the FLT3 gene and/or activation of other prosurvival pathways such as microenvironment-mediated resistance. Recent studies have strongly suggested that the reciprocal interaction between the microenvironment and AML cells generates specific machinery that leads to chemore-sistance. This chapter describes the molecular mechanism responsible for the refractory phenotype of FLT3/ITD<sup>+</sup> AML cells resulting from the communication between the microenvironment and FLT3/ITD<sup>+</sup> leukemia cells. Understanding this mechanism enables the discovery of novel and innovative therapeutic interventions for resistant FLT3/ITD<sup>+</sup> AML.

Keywords: FLT3/ITD, microenvironment, niche, drug resistance, CXCL12/CXCR4

#### 1. Introduction

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Mutations in the FLT3 gene represent the most common genetic aberrations among patients with acute myeloid leukemia (AML) [1, 2]. Internal tandem duplication mutations in the FLT3 gene (FLT3/ITD), which are expressed in human acute myeloid leukemia (AML)

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stem cells, are found in ~30% of patients with AML [3]. FLT3/ITD<sup>+</sup> AML is one of the most intractable hematological malignancies because of the emergence of resistant clones to FLT3/ITD inhibitors or chemotherapies [3, 4]. FLT3/ITD allows ligand-independent activation and phosphorylation of the FLT3 receptor. Ectopic FLT3/ITD expression in IL-3dependent mouse Ba/F3 or 32D hematopoietic cells results in growth factor-independent proliferation and produces acute leukemia in mice [5, 6]. Studies have indicated that FLT3/ ITD transforms mouse hematopoietic cell lines via the activation of the STAT5, RAS-MAPK, and PI3-kinase/AKT pathways [5, 7, 8] and blocks differentiation by suppressing C/EBP $\alpha$ , PU1, and RUNX1 [9–11]. Other studies have reported that JAK2 and STAT3 are tyrosine phosphorylated by constitutively active FLT3 [12]. ROCK1 [13], CDKN1a [14], SURVIVIN [15, 16], RUNX1 [9, 17], CXCR4 [18, 19], SOCS1 [20], PIM1 kinase [21, 22], FLT3-ligand [23, 24], SHP-2 [25], and micro-RNA-155 [26], and other molecules are reported to be involved in FLT3/ITD signaling. Although FLT3/ITD has been associated with extremely poor patient prognoses, FLT3 inhibitors fail to show significant efficacy in anti-AML therapies. For instance, AC220 (quizartinib), a second-generation class III tyrosine kinase inhibitor (TKI) used in phase II clinical trials, is a very potent and specific inhibitor of FLT3/ITD compared with other TKIs; however, FLT3/ITD<sup>+</sup> cells can become refractory to AC220 [9, 27]. The mechanism responsible for the resistance of FLT3/ITD<sup>+</sup> AML cells against FLT3/ITD inhibitors can be classified into FLT3/ITD-dependent and FLT3/ITDindependent mechanisms [4, 28]. The former is generally acknowledged as the acquisition of mutations in the FLT3 gene in addition to preexisting FLT3/ITD mutations. The emergence of additional mutations in the kinase domain makes FLT3/ITD no longer sensitive to FLT3/ITD inhibitors by altering the three-dimensional structure of FLT3 kinase, making FLT3 inhibitors difficult to physically interact with FLT3 protein. This mechanism is detailed in the excellent reviews [4, 28]. Although the development of further mutations in the FLT3 gene is associated with being refractory to the FLT3 inhibitor, most patients who became refractory to the FLT 3/ITD inhibitors lacked additional mutation in the FLT3 gene. Therefore, the resistant mechanism of these cases was likely to be attributed to alteration of the activity or levels in the molecules or pathways independent of FLT3/ITD [29], which includes microenvironment-mediated resistance.

Human AML stem cells residing in the endosteal niche of the bone marrow are relatively chemoresistant [30, 31]. This resistance results from survival cues in the form of various cytokines and adhesion molecules provided by niche cells [32]. Studies using the FLT3/ITD inhibitors have demonstrated that FLT3/ITD<sup>+</sup> AML blasts circulating in the peripheral circulation were very sensitive to these inhibitors, whereas those residing in the marrow endosteal region remained resistant to the FLT3/ITD inhibitor [33]. Reports have demonstrated that stromal cells protect FLT3/ITD AML cells from apoptosis induced by FLT3/ITD inhibitors [34–36]. These studies suggest that leukemia niches provide survival cues that protect FLT3/ITD<sup>+</sup> AML blasts from being eradicated by the FLT3/ITD inhibitors. In agreement with these observations, early study demonstrated that releasing leukemia cells from the marrow niche into the peripheral circulation by blocking the *CXCL12/CXCR4* interaction is effective in increasing their sensitivity to cytoreductive treatment [37]. These findings indicate that targeting cells via a cell-autonomous mechanism alone may not be sufficient

for treating FLT3/ITD<sup>+</sup> AML and that antagonizing these protective interactions between FLT3/ITD<sup>+</sup> AML blasts and leukemia niches represents a novel therapeutic strategy to eradicate resistant FLT3/ITD<sup>+</sup> AML cells.

### 2. Microenvironmetal factors inducing the resistance of FLT3/ITD<sup>+</sup> AML cells to FLT3 inhibitors

# 2.1. CXCL12/CXCR4 signaling pathways as a mechanism responsible for the resistance of FLT3/ITD AML cells to the FLT3 inhibitor

One of the machineries that holds AML cells in the bone marrow microenvironment is the interaction between *CXCL12* and *CXCR4* (**Figure 1**). *CXCL12*, a chemokine known as stromal cell–derived factor-1 (*SDF1*) that is expressed by the bone marrow microenvironment, is



# FLT3/ITD<sup>+</sup> AML cells in protective microenvironment

**Figure 1.** FLT3/ITD<sup>+</sup> AML cells in protective microenvironment. Suggested model for the resistance mechanism mediated by the environmental factors is shown. Retention of FLT3/ITD<sup>+</sup> cells in the bone marrow microenvironment increases the risk of resistant phenotype of FLT3/ITD<sup>+</sup> AML cells. This is mediated by adhesion molecules as well as the interaction between *CXCL12* that is provided by the microenvironment and the *CXCR4* on the AML cells. FLT3/ITD increases cell migration to *CXCL12*, thereby enhancing the interaction between AML cells and the microenvironment. Hypoxia and adrenergic inputs in the marrow environment that can enhance expression of *CXCL12* and/or *CXCR4* likely increase this interaction even further. FLT3/ITD itself activates or modulates several intracellular molecules, such as *ROCK1*, *RUNX1*, *PIM1*, *ERK*, *STAT3*, *SURVIVN*, *CDKN1A*, miR-155, and *SOCS1*, through which FLT3/ITD increases cell proliferation. In addition to FLT3/ITD, growth factors, such as *FLT3* ligand, stem cell factor (*SCF*), and *GM-CSF*, can also enhance activity and/or expression of these molecules, events providing survival signaling to the cells independent of FLT3/ITD. Therefore, cells will be able to survive even if FLT3/ITD activity is abrogated by the inhibitors.

responsible for retaining hematopoietic stem cells in the marrow niche through its receptor CXCR4 that is expressed on HSCs [38-41]. Similar to normal hematopoietic cells, CXCR4 is expressed in most AML cells that express CXCR4 and migrate in response to CXCL12 [42]. Antagonizing CXCR4 inhibits the engraftment and development of AML in a human xenograft human AML model, suggesting that CXCR4 is required for human AML to home to the marrow niche [43]. High expression of CXCR4 is associated with the poor prognosis of patients with AML [44, 45]. An early study indicated that FLT3/ITD enhanced chemotaxis to CXCL12 that is expressed in the niche [42]. The data suggest that FLT3/ITD facilitates the interaction between AML cells and the microenvironment via the enhancement of CXCL12/ CXCR4 signaling. The expression of CXCR4 is upregulated by various cytokines, including stem cell factor [46], VEGF, bFGF, EGF, IL2, IL4, IL6, IL7, IL10, and IL15 [47]. The induction of CXCR4 expression by the cytokines derived from the niche suggests that these cytokines promote the migration of AML cells to the microenvironment, thereby increasing the interaction between AML cells and the microenvironment. Indeed, stem cell factor enhances the migration of human AML cells to CXCL12 [48] and enhances their homing to the bone marrow [49]. By contrast, FLT3 ligand [50], TNF $\alpha$ , and INF $\gamma$  downregulate CXCR4 expression [47]. Adrenergic inputs downregulate CXCL12 in the marrow environment during the daytime [51] but upregulate CXCR4 on HSCs at night [52]. Hypoxia induces the expression of CXCL12 [53] and CXCR4 [54] by inducing HIF-1 $\alpha$  expression. Hypoxic conditions in the bone marrow niche that induces the expression of CXCL12 and CXCR4 can increase the lodging of AML cells in the bone marrow microenvironment. A recent study suggested that the mobilization of FLT3/ITD<sup>+</sup>AML cells into the peripheral circulation using the CXCR4 antagonist AMD3465 enhanced the antileukemia effect of chemotherapy and FLT3 inhibitor sorafenib, resulting in a reduced burden of AML and prolonged survival of mice [19]. A combination of AMD3100 (Plerixafor), Sorafenib, and G-CSF in FLT3-mutated patients yielded an overall response rate of 77% [55]. These data indicate that disrupting the interaction between FLT3/ITD<sup>+</sup>AML cells and the bone marrow microenvironment by antagonizing CXCR4 is beneficial to overcome the resistance of leukemia cells against the FLT3 inhibitor or chemotherapy.

Although reports have indicated that CXCL12/CXCR4 signaling can induce apoptosis in human AML cells by regulating BCL-X<sub>L</sub>, NOXA, and BAK [56, 57], stromal cells generally protect FLT3/ITD<sup>+</sup> AML cells from apoptosis induced by *FLT3/ITD* inhibitors [34–36], and *CXCL12* increases the number of FLT3/ITD<sup>+</sup> mouse hematopoietic progenitor cells cultured in the absence of hematopoietic growth factors. These data indicate that *CXCL12* can provide a survival effect on the hematopoietic progenitor cells expressing FLT3/ITD [58]. Consistent with *CXCL12* as a survival factor for FLT/ITD<sup>+</sup> cells, targeting the microenvironment by the *CXCR4* antagonist overcomes the resistance of FLT3/ITD<sup>+</sup> AML cells to the *FLT3/ITD* inhibitors [18, 19, 34, 59–61]. Antagonizing *CXCR4* by BL-8040 and FLT3/ITD inhibition demonstrates synergistic effects in inducing the apoptosis of FLT3/ITD<sup>+</sup> AML cells. The mechanism by which *CXCL12* and *CXCR4* provide resistance to FLT3/ITD<sup>+</sup> AML cells includes the expression of *ERK*, *BCL2*, *MCL1*, and *CYCLIN D1* via the downregulation of miR-15a/16-1 expression [18]. Microenvironment-mediated resistance of FLT3/ITD<sup>+</sup> AML cells to *FLT3* inhibitors through *CXCL12* was partially abrogated by activating p53 in the stromal cells using an *HDM2* inhibitor, suggesting that the combination of HDM2 antagonists and the *FLT3* inhibitor may provide therapeutic efficacy [34]. These data demonstrate that, while antagonizing *CXCR4* induces the mobilization of FLT3/ITD<sup>+</sup>AML cells into the peripheral circulation, which, in turn, sensitizes cells to *FLT3* inhibitors, antagonizing *CXCL12/CXCR4* signaling itself can abrogate resistance to FLT3 inhibitors [18, 19, 34, 59–61]. The data clearly indicate that the resistance of FLT3/ITD<sup>+</sup> AML cells to FLT3/ITD inhibitors depends on the stromal cells and is at least partially mediated through *CXCL12/CXCR4*.

#### 2.2. Cytokine signaling in the microenvironment as salvation factors for FLT3/ITD<sup>+</sup> AML

*CXCL12* is not the only cytokine that confers the resistance of FLT3/ITD<sup>+</sup>AML cells to the *FLT3* inhibitor. Stromal cells secrete various cytokines and growth factors, such as angiopoietins, *TNF-* $\alpha$ , *G-CSF*, *GM-CSF*, and *VEGF* [36]. *FLT3* ligand, stem cell factor, *IL-3*, *GM-CSF*, or *G-CSF* existing in the marrow environment can provide a protective effect on the FLT3/ITD<sup>+</sup> AML cells against *FLT3/ITD* inhibitors [23, 24]. For instance, the culture of FLT3/ITD<sup>+</sup> 32D cells with the *FLT3*-inhibitor AC220 in the absence of growth factors induces the rapid decline in the viable cell number, whereas the addition of *IL-3* significantly inhibits the cytotoxic effect of AC220 (Fukuda & Hirade, unpublished observation). Similarly, *FLT3* ligand that is expressed in the marrow microenvironment increases the resistance of FLT3/ITD<sup>+</sup> AML cells to the *FLT3* inhibitor [23]. These cytokines subsequently enhance the expression or activity of *SURVIVIN*, *CDKN1a*, *ERK*, *N-RAS*, and *PIM1*, all of which are known to be involved in the resistant phenotype against FLT3/ITD antagonists. The data indicate that cytokines in the marrow environment provide resistant activity to the FLT3/ITD<sup>+</sup>AML cells against *FLT3* inhibitors [1].

#### 2.3. STAT3/SURVIVIN signaling pathways

*SURVIVIN*, an antiapoptotic protein that is upregulated by FLT3/ITD, regulates the proliferation of FLT3/ITD<sup>+</sup> hematopoietic progenitor cells [16, 62] and mediates the resistance of FLT3/ITD<sup>+</sup> AML cells against the FLT/ITD inhibitor ABT-869 [15]. Zhou et al. reported that *SURVIVIN* expression was upregulated by FLT3/ITD, and its expression was even higher in the resistant FLT3/ITD<sup>+</sup> AML cells compared with cells sensitive to ABT-869. On the other hand, antagonizing *SURVIVIN* recovered the sensitivity of resistant FLT3/ ITD<sup>+</sup> AML cells to ABT-869, indicating that *SURVIVIN* expression is one of the mechanisms responsible for the resistance to ABT-869. *SURVIVIN* expression was mediated by the activation of STAT protein, and antagonizing *STAT3* using SRC-*STAT3* inhibitor IDR E804 abrogated the expression of *SURVIVIN*, coincident with a significant reduction of ABT-869–resistant FLT3/ITD<sup>+</sup>AML cell proliferation *in vivo*. The combination of ABT-869 with IDR E804 further decreased the burden of ABT-869–resistant FLT3/ITD<sup>+</sup>AML in a xenograft model in mice compared with the administration of ABT-869 or IDR E804 alone [15], suggesting that *STAT3* is also involved in the resistance to ABT-869. Consistent with this finding, recent data have demonstrated that the stroma-based activation of STAT3 Y705 confers resistance to AC220 in FLT3/ITD<sup>+</sup>AML [63]. The culture of FLT3/ITD<sup>+</sup> AML cells in direct contact with stromal cells or in the conditioned medium harvested from the stromal cells increased the IC50 of AC220 in FLT3/ITD<sup>+</sup>AML cells, with a concomitant increase in the phosphorylation of STAT3<sup>Y705</sup> in the AML cells, compared with control medium without stromal cells. Pharmacologic inhibition of STAT3 using BP-5-087 [64] decreased the IC<sub>50</sub> of AC220 in the FLT3/ITD<sup>+</sup> AML cells cultured in direct contact with stromal cells or in the conditioned medium derived from stromal cells, indicating that STAT3 confers FLT3/ITD<sup>+</sup> AML resistance to AC220 that is induced by stromal cells. This finding is consistent with SURVIVIN being a direct transcriptional target of STAT3 in FLT3/ITD<sup>+</sup>AML and lymphoma cells [15, 65], suggesting that the STAT3/ SURVIVIN axis protects FLT3/ ITD<sup>+</sup>AML cells from the antileukemia effect by the FLT3 inhibitors. SURVIVIN expression is also upregulated by exogenous factors such as FLT3-ligand [15, 16], which hampers the efficacy of the FLT3 inhibitor and is involved in the resistant phenotype of FLT3/ITD+ AML cells [23]. Likewise, stem cell factor [66] and GM-CSF [67], all of which are provided by the marrow microenvironment, increase the expression of SURVIVIN (Figure 1). These data suggest that the marrow niche protects FLT3/ITD<sup>+</sup> AML cells from FLT3/ITD antagonists through the upregulation of SURVIVIN by the hematopoietic growth factors secreted by the marrow environmental cells (Figure 1). Therefore, antagonizing SURVIVIN and/or STAT3 would overcome the resistance of FLT3/ITD<sup>+</sup> AML to FLT3 inhibitors.

#### 2.4. ERK/MAPK signaling pathways

An additional mechanism responsible for the resistance to the *FLT3* inhibitor by the niche is the activation of *ERK/MAPK* signaling pathways. FLT3 inhibitors induce apoptosis in FLT3/ITD<sup>+</sup> AML cells , whereas direct contact and proximity to stromal cells were protective toward FLT3/ITD<sup>+</sup> AML cells against FLT3 inhibition. Coculture of FLT3/ITD<sup>+</sup> AML cells with bone marrow stroma cells was associated with cell cycle arrest and persistent activation of ERK, even in the presence of the *FLT3* antagonist [36]. On the other hand, inhibition of MEK significantly abrogated the protective effect of stromal cells or *FLT3* ligand in FLT3/ITD<sup>+</sup> AML cells, indicating that *ERK* activation provided by the stromal cells is responsible for the resistance to *FLT3* inhibition in FLT3/ITD<sup>+</sup> AML cells. It was also reported that direct cell contact is more essential for the persistent activation of ERK compared with exposure to soluble factors [36]. Consistently, a recent report demonstrated that the treatment of FLT3/ITD<sup>+</sup> AML cells with *FLT3* inhibitors for over 48 hours induced rebound in *ERK* phosphorylation [68], suggesting an adaptive feedback mechanism capable of reactivating *ERK* signaling in response to upstream target inhibition in the FLT3/ITD<sup>+</sup> AML. These data suggest that antagonizing *ERK/MAPK* signaling pathways can overcome the resistance of FLT3/ITD<sup>+</sup>AML to the FLT3 inhibitors (**Figure 1**).

#### 2.5. Cyclin-dependent kinase inhibitor 1a/Pbx1 signaling pathways

The report by Yang et al. also noted the cell cycle arrest of FLT3/ITD<sup>+</sup> AML cells cocultured by stromal cells [36], indicating that stromal cells provide factors that induce cell cycle quiescence. *CDKN1a* is one of the cyclin-dependent kinase inhibitors that is known to block G<sub>1</sub>/S and G<sub>2</sub>/M transition [69–71]. It is reported that cell cycle quiescence of leukemia stem cells is one of the mechanisms that leads to refractoriness to anticancer drugs that normally eliminate cells in S-phase [30]. In human AML cells, CDKN1a is upregulated by growth factors, such as stem cell factor, FLT3-ligand, and GM-CSF [14, 70, 72], all of which are present in the marrow microenvironment. Consistent with FLT3 ligandinduced upregulation of CDKN1a, FLT3/ITD also upregulates CDKN1a via Stat5 [73]. Abe et al. reported that knocking down CDKN1a significantly decreases proliferation and cell cycle progression in FLT3/ITD<sup>+</sup> cells concomitant with an increase in Pbx1 mRNA expression [14], indicating that CDKN1a that is upregulated by FLT3/ITD negatively regulates proliferation and cell cycle progression of FLT3/ITD<sup>+</sup> cells. Knocking down Pbx1 expression using shRNAs abrogated the enhanced proliferation that was induced by CDKN1a deletion. The data demonstrate that FLT3/ITD not only contains stimulating activity but also harbors inhibitory activity on cell proliferation, which is mediated by upregulating CDKN1a and downregulating PBX1 expression. More importantly, FLT3/ITD confers resistance to the FLT3 inhibitor by inducing the expression of CDKN1a [14]. When FLT3/ ITD was antagonized with AC220, a selective inhibitor of FLT3/ITD, CDKN1a expression was decreased coincident with PBX1 mRNA upregulation and a rapid decline in the number of viable FLT3/ITD + Ba/F3 cells; however, the cells eventually became refractory to AC220. Overexpressing CDKN1a in FLT3/ITD + Ba/F3 cells delayed the emergence of cells that were refractory to AC220, whereas silencing CDKN1a accelerated their development. These data indicate that FLT3/ITD can inhibit FLT3/ITD<sup>+</sup> cell proliferation through the CDKN1a /PBX1 axis and that antagonizing FLT3/ITD contributes to the subsequent development of cells that are refractory to the FLT3/ITD inhibitor by disrupting CDKN1a expression because of FLT3/ITD inhibition. Similarly, the upregulation of CDKN1a may represent one mechanism responsible for the FLT3 ligand-induced resistance of FLT3/ ITD<sup>+</sup> AML cells against the FLT3 inhibitor [23] because CDKN1a expression is induced by FLT3 ligand [14]. The data also suggest that CDKN1a, which is upregulated by hematopoietic growth factors, such as SCF and GM-CSF, which are secreted by stromal cells, is also responsible for the refractory phenotype of FLT3/ITD<sup>+</sup> AML cells (Figure 1).

#### 2.6. RUNX1 in the resistance of FLT3/ITD<sup>+</sup> AML

A recent report demonstrated that FLT3/ITD signaling is associated with a common expression signature as well as a common chromatin signature. The study identified that FLT3/ITD induces the chronic activation of *MAPK*-inducible transcriptional factor *AP-1* and that *AP-1* cooperates with *RUNX1* to shape the epigenome of FLT3/ITD<sup>+</sup> AML [74]. *RUNX1* is a core-binding transcription factor that plays an important role in hematopoietic homeostasis, particularly in differentiation and proliferation [75, 76]. *RUNX1*-deficient cells showed increased susceptibility to AML development in collaboration with MLL-ENL, N-RAS, and EVI5 [77–79], suggesting that *RUNX1* can function as a tumor suppressor in myeloid malignancies. By contrast, *RUNX1* also promotes the survival of AML cells and lymphoma development and can function as an oncogene [80, 81]. These data suggest that the *RUNX1* has a dual function that promotes and attenuates the proliferation of hematological malignant cells. Hirade et al. identified that *RUNX1* 

expression is upregulated by FLT3/ITD and functions as an oncogene in FLT3/ITD<sup>+</sup> cells [9]. Another group demonstrated that RUNX1 cooperates with FLT3/ITD to induce acute leukemia, validating RUNX1 as an oncogene in FLT3/ITD signaling [17]. With respect to the function of RUNX1 in the resistance to the FLT3 inhibitor AC220, antagonizing RUNX1 significantly accentuated the antiproliferative effect of AC220 in FLT3/ITD<sup>+</sup> 32D cells. RUNX1 expression was elevated in the FLT3/ITD<sup>+</sup> 32D cells, which became refractory to AC220, whereas knocking down RUNX1 significantly inhibited the emergence and proliferation of FLT3/ITD<sup>+</sup> cells refractory to AC220, demonstrating that RUNX1 mediates the development of FLT3/ITD<sup>+</sup> AML cells resistant to AC220 in FLT3/ITD<sup>+</sup> cells. RUNX1 upregulation by AC220-resistant cells was not due to the additional mutation in the FLT3 gene because the upregulation of RUNX1 by AC220 was no longer observed when resistant cells were incubated without AC220. The data indicate that the epigenetic mechanism is likely involved in the upregulation of RUNX1 by AC220 refractory cells [9]. Because RUNX1 cooperated with MAPK-inducible transcription factor AP1 [74] and MAPK is regulated by various growth factors existing in the marrow microenvironment, it is highly likely that RUNX1 function is indirectly modulated by the microenvironmental factors. On the other hand, RUNX1 directly binds to the CXCR4 promoter region, and RUNX1 transactivates CXCR4 in a DNA binding-dependent manner, indicating that RUNX1 transcriptionally upregulates CXCR4 expression [78]. These findings strongly suggest that the upregulation of RUNX1 by FLT3/ITD increases the expression of CXCR4, which, in turn, enhances the chemotaxis of FLT3/ITD<sup>+</sup> AML cells to stromal niche cells, thereby increasing the likelihood of the cells being protected from the insult by the FLT3 inhibitor in the niche. On the other hand, RUNX1 downregulates the expression of cell adhesion factors that promote the residency of stem cells and megakaryocytes in their bone marrow niche [82], suggesting that RUNX1 expression that is induced by FLT3/ITD likely alters the interaction between the FLT3/ITD<sup>+</sup> AML cells and niche cells and is involved in the resistance to the *FLT3* inhibitor (Figure 1).

#### 2.7. FLT3/ITD evades external inhibitory cytokine control

While it has been unclear how leukemia cells escape from normal cytokine control that is indispensable to maintain normal hematopoiesis, a recent study demonstrated that FLT3/ITD facilitates the development of myeloproliferative disease by inhibiting the interferon response [20, 26]. Interferon exhibits an anti-proliferative effect on primitive hematopoietic cells [83–86], including FLT3/ITD<sup>+</sup> cells [20]. In FLT3/ITD<sup>+</sup> cells, activated STAT5 up-regulates SOCS1 expression, which inhibits the antiproliferative effect induced by interferon- $\alpha$  or interferon- $\gamma$  [20]. SOCS1 protects FLT3/ITD<sup>+</sup>AML cells from external interferon control, thereby promoting myeloproliferative disease. Another report also uncovered a novel mechanism responsible for the escape of FLT3/ITD<sup>+</sup> AML cells from interferon signaling. Micro-RNA 155 (miR-155) is significantly overexpressed in FLT3/ ITD AML [87–92] and promotes myeloproliferative disease induced by FLT3/ITD. This was coincided with repression of the interferon response compared with that with wildtype FLT3. Inhibition of miR-155 resulted in the elevation of the interferon response and reduction in the proliferation of human FLT3/ITD<sup>+</sup> AML cells. The data indicate that miR-155 promotes FLT3/ITD<sup>+</sup> AML cell proliferation by blocking interferon signaling [26]. Taken together, FLT3/ITD stimulates AML cell proliferation by evading external antiproliferative cytokine control that is normally provided by the microenvironment (**Figure 1**). It remains to be determined if these mechanisms are involved in the resistance against *FLT3* inhibitors.

FLT3/ITD<sup>+</sup> AML is also found in patients with acute promyelocytic leukemia who harbor the PML-RAR $\alpha$  fusion gene resulting from chromosomal translocation. Recent data have demonstrated that the combination of the FLT3/ITD inhibitor and ATRA, which targets PML-RAR $\alpha$ , displays a synergistic effect of reducing the burden of FLT3/ITD<sup>+</sup> AML both *in vitro* and in a xenotransplantation model [93–95]. This is a promising strategy to facilitate the differentiation of FLT3/ITD <sup>+</sup> AML in the patients; however, recent data have also indicated the inactivation of retinoids in the marrow niche, thereby inhibiting the differentiation of AML cells [96–98]. In this regard, the effect of ATRA with the FLT3/ITD inhibitor may be more complicated than anticipated because the marrow niche may impede the long-term effect of ATRA.

# 2.8. Interaction of FLT3/ITD<sup>+</sup> AML cells with the microenvironment via adhesion molecules

The interaction between AML cells and the microenvironment is mediated by various factors, such as CXCL12, and adhesion molecules. CXCL12 can activate adhesion molecules, particularly very late antigen-4 (VLA-4) and lymphocyte function-associated antigen-1 (LFA-1) on hematopoietic stem and progenitor cells, which also regulate the homing process [99]. FLT3/ ITD decreases the expression of VLA4 expression, coincident with a significant reduction in cell adhesion to VCAM1 [58]. While the data indicate that FLT3/ITD negatively regulates the expression of VLA4 and adhesion to its ligand VCAM1, the inhibition of FLT3/ITD by Fl-700 decreases the affinity of VLA4 to soluble VCAM1 [100], indicating that FLT3/ITD modulates the interaction between VLA4 and VCAM1. The interaction of leukemia cells with the microenvironment is also mediated via E-selection [101]. A recent report has demonstrated that a dual inhibitor for E-selectin and CXCR4 (GMI-1359) exerts efficient antileukemia effects in an FLT3/ITD<sup>+</sup> AML xenograft model by mobilizing AML cells into the peripheral circulation from the bone marrow [102, 103]. The data suggest that antagonizing adhesion molecules that retain FLT3/ITD<sup>+</sup> AML cells in the bone marrow microenvironment is beneficial to abate the resistance of AML cells to the FLT3 inhibitor by mobilizing AML cells into the blood circulation.

Taken together, these data provide evidence that stromal cells, or other cells comprising the microenvironment, support FLT3/ITD<sup>+</sup>AML cells via soluble factors and adhesion molecules, which, in turn, activate survival or proliferative signaling in the AML cells (**Figure 1**). However, the machinery provided by the microenvironment is not confined to these factors described above. A recent report has indicated that bone marrow mesenchymal stromal cells transfer their mitochondria to AML cells to support their proliferation [104, 105], possibly representing an additional mechanism that can enhance the resistance to the *FLT3* inhibitor in FLT3/ITD<sup>+</sup> AML. Likewise, it is highly possible that microsomes containing micro-RNAs

secreted from the microenvironment modulate the function of FLT3/ITD<sup>+</sup> AML cells, although this hypothesis remains yet to be proven.

# 3. Functional interaction between FLT3/ITD and CXCR4 in the migration and homing of AML cells that are associated with resistance

Because CXCL12/CXCR4 provides a survival signal to FLT3/ITD<sup>+</sup> AML cells, it suggests that CXCL12/CXCR4 signaling accentuates FLT3/ITD signaling activity. By contrast, FLT3/ ITD regulates cell migration to CXCL12 [50], indicating that FLT3/ITD modulates CXCR4 signaling. Therefore, FLT3/ITD and CXCL12/CXCR4 signaling mutually interacts. While an earlier study demonstrated that patients with FLT3/ITD<sup>+</sup> AML have higher CXCR4 expression than those with FLT3 wild-type AML [45], subsequent studies have demonstrated controversial findings. We and other groups have demonstrated that overexpressing FLT3/ITD in mouse Ba/F3 cells or human CD34<sup>+</sup> cells significantly downregulated CXCR4 expression [50, 59]. Incubating human CD34<sup>+</sup> cells with FLT3 ligand also decreased the expression of CXCR4 [50]. Moreover, the mRNA expression of CXCR4 was significantly lower in patients with FLT3/ITD<sup>+</sup> AML than in those with wild-type FLT3 [9, 106]. These data indicate that FLT3/ITD can reduce the expression of *CXCR4* in contrast to the data of the earlier report. The mechanism responsible for the modulation of CXCR4 expression by FLT3/ITD remains subject to investigation. PIM1, which is activated by FLT3/ITD, upregulates CXCR4 [107]. Similarly, RUNX1, which is elevated in FLT3/ITD<sup>+</sup> AML, upregulates CXCR4 transcription [78]. On the other hand,  $CEBP\alpha$ , a transcriptional factor that increases CXCR4 expression [108], is inactivated by FLT3/ITD [11, 109]. Therefore, the inactivation of CEBP $\alpha$  by FLT3/ ITD can decrease CXCR4 expression. Because FLT3/ITD inhibits CEBPα but enhances PIM1 and/or RUNX1 expression, the balance between the inactivation of CEBP $\alpha$  and activation of *PIM1* and/or *RUNX1* may determine the expression of *CXCR4* in FLT3/ITD<sup>+</sup> AML.

Although the *FLT3* ligand, as well as FLT3/ITD, increases the migration of mouse hematopoietic cells to CXCL12 [19, 50, 106], FLT3 signaling can decrease the migration of CD34<sup>+</sup> cells and mouse Ba/F3 cells toward CXCL12 [50, 59]. Enhancing migration and decreasing migration in response to CXCL12 by FLT3/ITD appear to be controversial, but the reduction of migration toward CXCL12 is most likely a consequence of a decrease in CXCR4 expression, which, in turn, induces the quantitative reduction of CXCR4 signaling. Jacobi et al. reported that the transient expression of FLT3/ITD decreases CXCR4 expression in human CD34<sup>+</sup> cells, coincident with their reduced migration toward CXCL12 [59]. This is consistent with the reduction in CXCR4 expression in CD34<sup>+</sup> cells or Ba/F3 cells incubated with FLT3 ligand that is accompanied by a decrease in CXCL12-mediated migration [50]. These data indicate that FLT3/ITD, as well as normal FLT3 signaling, can inhibit CXCL12/CXCR4 signaling by downregulating CXCR4 expression. By contrast, the sustained expression of FLT3/ITD enhances migration in response to CXCL12, even with a significant downregulation of the CXCR4 level [50]. Augmentation in cell migration toward CXCL12 despite the reduction in CXCR4 expression suggests that the increase in migration was not due to the qualitative increase in CXCR4 signaling. A subsequent study by Onishi et al. confirmed that enhanced migration by FLT3/ITD was mediated through the qualitative change in *CXCR4* signaling [106]. The data indicated that molecules and/or pathways downstream of *CXCR4* that are regulated in the presence of FLT3/ITD were overlapped but distinct from those regulated in the absence of FLT3/ITD, suggesting that FLT3/ITD regulates *CXCR4* signaling pathways functionally distinct from those of normal cells [106]. This implies that FLT3/ITD functionally alters *CXCR4* signaling. These findings strongly suggest that FLT3/ITD can negatively regulate *CXCR4* signaling by qualitatively decreasing *CXCR4* signaling by downregulating *CXCR4* expression, whereas it also increases *CXCR4* signaling activity by changing the global gene expression downstream of CXCR4 (**Figure 2**). One of the molecules responsible for the activation of *CXCR4* signaling by FLT3/ITD is Rho-associated kinase-1 (*ROCK1*). *ROCK1* promotes the migration of *CXCR4*<sup>+</sup> cells to *CXCL12*, whereas antagonizing *ROCK1* displays the opposite effect. *CXCL12* transiently upregulates *ROCK1* expression but subsequently downregulates its expression in the absence of FLT3/ITD. This downregulation is associated with the attenuation in cell migration to CXCL12, suggesting the presence of negative



**Figure 2.** Quantitative and/or qualitative regulation of CXCR4 signaling by FLT3/ITD. *CXCL12/CXCR4* signaling augments FLT3/ITD activity, but in contrast, FLT3/ITD modulates *CXCL12/CXCR4* signaling, indicating that *CXCL12/CXCR4* and FLT3/ITD signaling mutually interacts. Regulation of CXCR4 signaling by FLT3/ITD is classified into two categories: one is quantitative regulation and the other is qualitative mechanism. FLT3/ITD regulates expression of *CXCR4*, depending on the transcriptional mediators or kinases. For instance, inactivation of CEBPa by FLT3/ITD can decrease *CXCR4* expression, whereas activation of *PIM1* and/or *RUNX1* can increase *CXCR4* expression. Downregulation of CXCR4 diminishes cell migration to CXCL12, whereas upregulation of *CXCR4* expression leads to enhancement in cell migration to *CXCL12*. Classification of genes that are regulated by *CXCL12* in FLT3/ITD<sup>-</sup> cells and those in FLT3/ITD<sup>+</sup> cells based on the molecular pathways or biological process demonstrated that they are functionally overlapped but distinct. The data suggest that FLT3/ITD functionally alters *CXCL12/CXCR4* signaling. For instance, downregulation of *ROCK1* expression by *CXCL12* that is normally observed in control cells is abrogated by FLT3/ITD, which is responsible for the enhancement in cell migration to *CXCL12* by FLT3/ITD.

feedback in *CXCL12/CXCR4* signaling mediated by modulating *ROCK1* expression to prevent excessive migration in normal cells. By contrast, FLT3/ITD or *FLT3* ligand enhances the expression and prevents the subsequent downregulation of the *ROCK1* level that is normally induced by CXCL12, thereby abrogating the negative feedback generated by *CXCL12* and *ROCK1*. The loss of negative feedback on *ROCK1* expression induced by *FLT3* signaling resulted in the sustained activation of *CXCL12/CXCR4* signaling, thereby enhancing the migration of FLT3/ITD<sup>+</sup> cells toward *CXCL12*. Enhanced chemotaxis is also mediated through RAS [58].

An additional molecular machinery that specifically mediates the migration of FLT3/ITD<sup>+</sup> cells is PIM1 kinase. The expression and kinase activity of PIM1 are upregulated in FLT3/ITD<sup>+</sup> AML cells [110]. Enhanced PIM1 activity induced by FLT3/ITD is essential for the migration and homing of AML cells [107]. The effect of PIM1 on the migration and homing of FLT3/ITD cells is mediated by the increase in CXCR4 owing to its recycling by the phosphorylation of serine 339 on CXCR4. These data indicate that PIM1 activity is essential for the proper CXCR4 surface expression and migration of FLT3/ITD<sup>+</sup> AML cells toward CXCL12. In addition to regulating migration and homing, PIM1 modulates the resistance of FLT3/ITD<sup>+</sup> AML cells to FLT3 inhibitors [21, 22]. Targeting PIM1 synergizes with FLT3 inhibition [111] and restores the sensitivity of FLT3 inhibitors in FLT3/ITD<sup>+</sup> AML cells [21]. A recent study in abstract form indicated that the microenvironment-induced expression of PIM kinase supports chronic leukemia (CLL) survival and promotes CXCR4-dependent migration [112]. Although this was investigated in CLL, the data suggest that microenvironmental factors increase the expression of *PIM1* kinase, which promotes the resistance of FLT3/ITD<sup>+</sup> AML. The upregulated PIM1 kinase, in turn, would facilitate the migration of FLT3/ITD<sup>+</sup> AML toward CXCL12 by activating CXCR4 signaling, thereby increasing the interaction between FLT3/ITD+ AML cells and microenvironment cells. In this regard, antagonizing PIM1 represents an additional therapeutic strategy to abrogate the interaction between FLT3/ITD<sup>+</sup> AML cells and marrow niches, particularly for those that have become resistant to FLT3/ITD inhibitors. Similarly, ROCK1 enhances not only CXCL12-induced migration [106] but also the proliferation of FLT3/ITD<sup>+</sup> cells [13]. Therefore, antagonizing ROCK1 is likely to be beneficial to interfere with the communication of FLT3/ITD<sup>+</sup> AML cells between the marrow niches and inhibit their proliferation. These data suggest that FLT3/ITD increases the communication with the bone marrow microenvironment by enhancing the chemotaxis toward CXCL12. Together with CXCL12 protecting FLT3/ITD<sup>+</sup> AML cells from the insult of FLT3 inhibitors, the findings strongly indicate that reciprocal interaction between FLT3/ITD and CXCL12/ CXCR4 signaling exists that accentuates the resistance to FLT3 inhibitors.

#### 4. Effect of FLT3 mutation on the microenvironment

Normal hematopoietic stem cells drive hematopoiesis, but this process requires appropriate factors secreted by adjacent cells, adhesion molecules, neighboring cells such as mesenchymal stromal cells, osteolineage cells, and endothelial cells that exist in the microenvironment [113]. In agreement with the microenvironment mediating the tight control necessary for normal hematopoiesis, earlier studies have demonstrated that malfunction of microenvironmental cells can lead to the development of myeloproliferation, which represents one of the outcomes of aberrant hematopoiesis. Walkley et al. demonstrated that the loss of retinoic acid receptor gamma (PAR $\gamma$ ) resulted in myeloproliferation in mice; however, the transplantation of the marrow cells into PAR $\gamma$ -deficient cells did not cause myeloproliferation in wild-type recipients, whereas the transplantation of wild-type marrow cells caused myeloproliferation in PAR $\gamma$ -deficient recipients, indicating that myeloproliferation caused by the loss of PAR $\gamma$  was microenvironmental [114]. The microenvironmental effect on aberrant myeloproliferation is also supported by experiments using Rb-deficient cells. Knocking out Rb resulted in myeloproliferation in mice; however, the genetic defect in both hematopoietic cells and the microenvironment was necessary for the development of myeloproliferation [115]. Furthermore, deletion of DICER1 in primitive osteolineage cells led to myelodysplastic syndrome and AML [116], indicating that malfunction of *DICER1* in the niche component was sufficient to cause myeloid malignancy. These findings indicate that the genetic alteration and/or malfunction of the microenvironment can induce myeloid malignancies.

Reports have demonstrated that HSCs regulate their own niches by instructing neighboring stromal cells to produce supportive factors or alter the overall microenvironment [117–119]. While the marrow niche supports leukemia cell proliferation or protects cells from chemotherapeutic insult by providing various survival signals, recent evidence has demonstrated that leukemia cells modulate the marrow environment to create a supportive niche favoring survival for AML cells, just as healthy HSCs regulate their niche. Zhang et al. demonstrated that chronic myeloid leukemia (CML) cells modulate the microenvironment in favor of CML cells over healthy HCS by modulating CXCL12 expression and alter the localization of HSCs. CML cells modulate cytokine expression in the microenvironment, such that they support CML cells [120]. A study by Schepers et al. identified that myeloproliferative neoplasia (MPN) remodels endosteal bone marrow niches by stimulating mesenchymal stem cells to produce functionally altered osteoblastic lineage cells. This results in the creation of a self-reinforcing leukemic niche that impairs normal hematopoiesis and favors leukemic stem cell function [121]. Several cytokines, such as thrombopoietin and CCL3, that direct cell-cell interaction, alteration of TGF- $\beta$ , and Notch and inflammatory signaling were involved in the expansion and/ or remodeling in osteoblastic lineage cells. The osteoblastic lineage cells remodeled by myeloproliferation compromised normal HSCs but effectively support leukemia stem cells [121]. Similarly, the latest study by Mead et al. demonstrated that FLT3/ITD modulates the marrow microenvironment and impaired the number of HSCs. In the marrow of FLT3<sup>ITD/ITD</sup> mice, FLT3/ ITD-induced myeloproliferation was associated with a progressive decline in the HSC compartment. Notably, when FLT3<sup>ITD/ITD</sup> marrow cells were transplanted with marrow competitor cells from wild-type mice into healthy recipients, the HSCs derived from the competitor cells were significantly reduced, demonstrating the presence of a cell extrinsic mechanism that diminishes the competitor HSC. Loss of competitor cells in the recipient mice that developed FLT3/ITD-induced myeloproliferation was coincided with the disruption of stromal cells in the recipient marrow, an activity that was associated with reduced numbers of endothelial and mesenchymal stromal cells showing increased inflammation-associated gene expression. The study finally discovered that tumor necrosis factor (TNF), a cell-extrinsic negative regulator of HSCs, was overexpressed in the marrow niche cells in FLT3<sup>ITD/ITD</sup> mice, and anti-TNF treatment partially rescued the loss of HSCs. These data clearly demonstrate that FLT3/ITD compromises HSCs through an extrinsically mediated mechanism of disrupting HSCs that support bone marrow stromal cells by generating an inflammatory environment [122]. The same study also demonstrated that the expression of FLT3 mRNA and protein is absent in HSCs, strongly suggesting that FLT3/ITD protein is not expressed in most primitive HSCs, even if FLT3/ITD mutation exists in the FLT3 gene in HSCs. Because these HSCs harboring the FLT3/ITD gene but lacking the expression of *FLT3/ITD* protein would not be targeted by the *FLT3* inhibitors, they may represent a reservoir for the development of resistant clones, in which additional mutations can be accumulated. The lack of mutant *FLT3/ITD* protein in HSCs harboring FLT3/ITD protein or activity would be ineffective. In this regard, disrupting the FLT3 gene, for instance, by using a gene-editing strategy, would represent an additional approach to eliminate HSCs containing FLT3/ITD mutation. Moreover, because FLT3/ITD<sup>+</sup> AML restructures the marrow environment in favor of AML cells over normal HSCs, factors provided by FLT3/ITD<sup>+</sup> AML cells that influence the marrow environment would represent a novel therapeutic target.

#### 5. Summary

FLT3/ITD<sup>+</sup> AML can become refractory to *FLT3* inhibitors. Factors derived from the marrow microenvironment represent one such mechanism responsible for the refractory phenotype to *FLT3/ ITD* inhibitors. Understanding the molecular mechanism involved in microenvironment-mediated resistance will shed light on the development of innovative therapeutic strategies against FLT3/ITD<sup>+</sup> AML, especially for FLT3/ITD<sup>+</sup> AML that has become refractory to *FLT3* inhibitors.

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