

## ORIGINAL ARTICLE

# Focal adhesion kinase is required for CXCL12-induced chemotactic and pro-adhesive responses in hematopoietic precursor cells

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**Hematopoietic stem/progenitor cells (HSC/P) reside in the bone marrow in distinct anatomic locations (niches) to receive growth, survival and differentiation signals. HSC/P localization and migration between niches depend on cell–cell and cell–matrix interactions, which result from the cooperation of cytokines, chemokines and adhesion molecules. The CXCL12–CXCR4 pathway, in particular, is essential for myelopoiesis and B lymphopoiesis but the molecular mechanisms of CXCL12 action remain unclear. We previously noted a strong correlation between prolonged CXCL12-mediated focal adhesion kinase (FAK) phosphorylation and sustained pro-adhesive responses in progenitor B cells, but not in mature B cells. Although FAK has been well studied in adherent fibroblasts, its function in hematopoietic cells is not defined. We used two independent approaches to reduce FAK expression in (human and mouse) progenitor cells. RNA interference (RNAi)-mediated FAK silencing abolished CXCL12-induced responses in human pro-B leukemia, REH cells. FAK-deficient REH cells also demonstrated reduced CXCL12-induced activation of the GTPase Rap1, suggesting the importance of FAK in CXCL12-mediated integrin activation. Moreover, in FAK<sup>flx/flx</sup> hematopoietic precursor cells, Cre-mediated FAK deletion resulted in impaired CXCL12-induced chemotaxis. These studies suggest that FAK may function as a key intermediary in signaling pathways controlling hematopoietic cell lodgment and lineage development.**

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## Introduction

A signature characteristic of hematopoietic stem/progenitor cells (HSC/P) is their ability to self-renew and differentiate along hematopoietic cell lineages. HSC/Ps and lineage-committed progenitor cells are located in specialized bone marrow (BM) microenvironments called stem-cell 'niches' that are believed to control cell growth, survival and differentiation by secretion of soluble factors, cell–cell and cell–matrix interactions. The niche-originating signals that influence HSC/P cell cycle and migratory status depend on the crosstalk between multiple ligand–receptor signaling pathways (for example, chemokines, cytokines and adhesion molecules, among others).<sup>1,2</sup> Studies

using targeted gene disruption in mice have shown that CXCL12 and its corresponding receptor CXCR4 are essential for B-cell and myeloid-cell lineage development during ontogeny.<sup>3,4</sup> Moreover, both CXCL12/CXCR4 and VLA-4/VCAM-1 axes are important for normal HSC/P and B-cell progenitor retention in the BM,<sup>5–8</sup> and it is speculated that homing of leukemic cells is also controlled by both of these pathways.<sup>9–12</sup> *In vivo* disruption of CXCL12/CXCR4 and VLA-4/VCAM-1 axes interferes with the retention of HSC/P and B-cell precursors in BM niches, resulting in their egress to periphery.<sup>7,13,14</sup> *In vitro*, CXCL12 induces chemotaxis and VLA-4-dependent adhesion to VCAM-1 in HSC/Ps and B-cell progenitors,<sup>15–18</sup> and it is speculated that CXCL12-mediated pro-adhesive interactions might explain in part CXCL12-mediated HSC/P and B-cell progenitor BM lodgment observed *in vivo*.<sup>13,19</sup> Despite the significance of CXCL12/CXCR4 and VLA-4/VCAM-1 axes in hematopoiesis, the intracellular signaling pathways underlying hematopoietic progenitor cell lodgment and lineage development are poorly understood. Previously, we and others observed that CXCL12 activates focal adhesion kinase (FAK) in various hematopoietic cell lineages, as measured by tyrosine phosphorylation.<sup>15,20,21</sup> Furthermore, we demonstrated that the duration of FAK phosphorylation correlates with sustained CXCL12-induced pro-adhesive responses in progenitor B cells.<sup>15</sup> Based on these data, we hypothesized that FAK may mediate CXCL12-induced cellular responses in hematopoietic cells.

FAK plays a fundamental regulatory role in the motility and survival of anchorage-dependent cells, for example fibroblasts. FAK is activated following integrin-mediated adhesion to the extracellular matrix by a process called outside-in integrin signaling.<sup>22</sup> Despite the well-established role of FAK in the integrin-controlled motility of anchorage-dependent cells, the functional role of FAK in the biology of hematopoietic cells remains undetermined, due in part to the early embryonic lethality of FAK-deficient embryos, before hematopoiesis begins.<sup>23</sup> In the present study, we sought to assess the potential role of FAK in chemokine-controlled migration and adhesion in human pro-B acute lymphoblastic leukemia (pro-B ALL) cells as well as normal mouse hematopoietic precursor cells. By specific inactivation of the FAK gene in human pro-B ALL REH cells and murine hematopoietic progenitor cells, we show for the first time that FAK is required for CXCL12-induced responses in hematopoietic cells.

## Materials and methods

### Reagents

RPMI-1640 medium, Hank's balanced salt solution (HBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES),

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fetal calf serum (FCS), penicillin, streptomycin, L-glutamine, 2-mercaptoethanol (2-ME) were from Invitrogen (Carlsbad, CA, USA). Manganese chloride ( $MnCl_2$ ), puromycin, bovine serum albumin (BSA) and polybrene were from Sigma (St Louis, MO, USA). CXCL12 was from Peprotech (Rocky Hill, NJ, USA).

#### *Retrovirus-mediated RNA interference of FAK and transient transfection with wild-type chicken-FAK in REH cells*

Human acute lymphoblastic pro-B cell leukemia (pro-B ALL) REH cell line (CRL8286, ATCC, Manassas VA, USA) was maintained as described.<sup>15</sup> Twenty short-hairpin RNA constructs (shRNA) were designed to silence various regions of the human FAK gene. ShRNA-expressing retrovirus-vector delivery system was employed as described previously.<sup>24</sup> Briefly, 63-nt DNA oligomers (IDT, Coralville, IA, USA) containing the sense sequence, a 9-nt loop (TTCAAGAGA) and the antisense sequence were cloned downstream of the U6 promoter of a modified pBabe-puro retroviral vector. The pBabe-puro-U6-hFAK vectors were co-transfected into phoenix-293T cells with the pCMV-VSVg plasmid. The resulting retrovirus-containing supernatant from the transfected cells was used for the spin-infection of REH cells (2500 r.p.m. for 2 h at room temperature), followed by puromycin selection (300 ng/ml) of stably transfected clones. The best silencing efficiency was achieved by shRNA designated as FAK3-RNAi (RNA interference; FAK target sequence: 5-GGAATGCTTCAAGTGTGCT-3), as analyzed by immunoblotting (not shown); therefore, all further studies were conducted using this construct. In some experiments, FAK3-RNAi-bearing REH cells were transiently transfected with hemagglutinin (HA)-tagged chicken FAK (HA-wtFAK) construct (a generous gift from Dr Jun-Lin Guan, University of Michigan Medical School, Ann Arbor, MI, USA), using Lipofectamine 2000 (Invitrogen), according to the manufacturer's specifications.

#### *Western blotting*

Western blotting was performed as described previously.<sup>15,20</sup> Antibodies were anti-FAK (Upstate, Charlottesville, VA, USA), anti-Pyk2, anti-p38 and anti-Rap1 (all three from Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphospecific (Tyr 397) anti-FAK (Invitrogen's BioSource division) and anti-actin (Sigma). Secondary horseradish peroxidase-conjugated antibodies were from Bio-Rad (Hercules, CA, USA). Enhanced chemiluminescence reagent was from Amersham Biosciences (Piscataway, NJ, USA).

#### *Fluorescence-activated cell sorting analysis*

Anti-CD49d (clone P1H4) and anti-CD29 (clone 6S6) antibodies were from Chemicon (Temecula, CA, USA); anti-CXCR4 (clone 12G5) and isotype controls were from BD Biosciences (San Jose, CA, USA). Secondary antibodies were from Jackson Immuno-Research Laboratories (West Grove, PA, USA). Acquisition and analysis were performed on MoFlo Cytometer using Summit Software (DakoCytomation, Fort Collins, CO, USA).

#### *Chemotaxis assay*

Chemotaxis was performed as described,<sup>17</sup> using Transwell inserts (Costar, Cambridge, MA, USA; 6.5 mm diameter, 5  $\mu$ m filter pore size), 5  $\times$  10<sup>5</sup> cells per well and CXCL12 (500 ng/ml final concentration). Cells were allowed to migrate for 2 h at 37°C and after that time, cells that passed through the membrane to the lower well were collected and enumerated

by timed acquisition (60 s each sample at 2 psi sample pressure differential) on a MoFlo cytometer.

#### *Adhesion assay*

The long-term adhesion assay was performed as described.<sup>15</sup> Briefly, 2  $\times$  10<sup>6</sup> cells per 1 ml of adhesion medium (HBSS buffered with HEPES, supplemented with 0.1% BSA) were stimulated with CXCL12 (1  $\mu$ M final concentration) for 30 min in suspension and were then added to the VCAM-1-coated wells (1  $\mu$ g/ml final concentration) and allowed to settle for 30 min at 37°C. As a negative control, adhesion medium without CXCL12 was used. Wells were then washed manually and the number of adhered cells was determined using CyQUANT cell proliferation kit (Molecular Probes, Eugene, OR, USA). Fluorescence of the samples was measured by a Microtiter Plate Fluorometer (DYNEX Technologies, Chantilly, VA, USA). The percentage of adhered cells was calculated in relation to the number of cells in the input control. In some experiments, cells were stimulated with 1 mM of  $MnCl_2$  for 10 min and then transferred to VCAM-1-coated wells for 30 min, followed by removal of non-adhered cells and determination of adhered cell number as described for CXCL12-stimulated adhesion.

#### *Rap1 pull-down assay*

Cells were stimulated with CXCL12 (500 ng/ml) for indicated times and GTP-bound Rap1 was detected using EZ-Detect Rap1 activation kit (Pierce Biotechnology, Rockford, IL, USA). GTP-bound Rap1 was resolved on SDS-PAGE and analyzed by Western blotting.

#### *Isolation of human CD34<sup>+</sup> cells*

CD34<sup>+</sup> hematopoietic progenitor cells were isolated from BM mononuclear cells that were obtained from healthy volunteers as described previously,<sup>15</sup> with the approval of the local Institutional Review Board. Isolation was carried out using the EasySep CD34 enrichment kit (StemCell Technologies, Vancouver, Canada) according to the manufacturer's protocol. The purity of the resulting population was over 96%.

#### *Lentivirally delivered Cre-mediated FAK deletion in murine FAK<sup>flox/flox</sup> progenitor B, Sca-1<sup>+</sup> and c-kit<sup>+</sup>/lin<sup>-</sup> (KL) hematopoietic progenitor cells*

Mice homozygous for floxed-FAK allele (FAK<sup>flox/flox</sup>) were a generous gift from Dr Louis F Reichardt (University of California San Francisco, CA, USA). Femurs and tibias from 8-week-old FAK<sup>flox/flox</sup> mice were homogenized and BM cells were filtered through a 70- $\mu$ m filter. Total BM B cells were isolated by EasySep selection kit (StemCell Technologies) with the purity being over 96%, as assessed by fluorescence-activated cell sorting (FACS) analysis of CD19 expression (data not shown). To obtain a homogenous population of B-cell progenitors, B cells were cultured for 7 days in interleukin (IL)-7-containing complete RPMI medium (RPMI medium supplemented with 20% FCS, 50  $\mu$ M 2-ME, 2 mM L-glutamine, 1  $\times$  penicillin/streptomycin and 10 ng/ml of recombinant mouse IL-7 (R&D Systems, Minneapolis, MN, USA), as described.<sup>25</sup> Over 95% of expanded cells represented pro-B/early pre-B cell stage of development (B220<sup>+</sup>, c-kit<sup>+</sup>, CD43<sup>+</sup>, IgM<sup>-</sup>, not shown). A population enriched in hematopoietic progenitor cells (c-kit<sup>+</sup>/lin<sup>-</sup>, KL), was obtained by FACS. Briefly, FAK<sup>flox/flox</sup> BM cells were stained with the following monoclonal antibodies (all from BD Biosciences): APC-conjugated c-Kit (2B8, CD117) and

PE-conjugated antibodies to lineage markers (Lin) (anti-Mac-1 (M1/70), anti-Gr-1 (RB6-8C5), anti-B220 (RA3-6B2), anti-CD3e (145-2C11), anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-Ter 119). Lineage-negative (Lin<sup>-</sup>) cells were gated for c-Kit<sup>+</sup> and KL cells were sorted using a MoFlo high-speed cell sorter into SF34 medium (StemCell Technologies). FAK<sup>flox/flox</sup> Sca-1<sup>+</sup> progenitor cells were obtained by FACS of BM cells stained with fluorescein isothiocyanate-conjugated Sca-1 antibody (Ly-6A/E, BD Biosciences). Recombination of the floxed-FAK allele in IL-7-expanded progenitor B cells, KL cells and Sca-1<sup>+</sup> cells was carried out by cell infection with a third generation self-inactivating pHAGE lentiviral vector,<sup>26</sup> in which Cre recombinase expression is driven by the CMV promoter and transduction efficiency can be monitored by ZsGreen expression (lenti-Cre vector). Lentiviruses were produced in 293T cells as described.<sup>27</sup> As a control, the mock lentiviral vector (without Cre) was used. The expanded FAK<sup>flox/flox</sup> progenitor B cells were transduced in RPMI complete medium (viral MOI equal to 100) in the presence of IL-7 (10 ng/ml) and polybrene (5 μg/ml). After 12 h incubation, the supernatants were replaced with fresh medium (containing 10 ng/ml of IL-7) and cells were analyzed at 96 h after transduction. KL and Sca-1<sup>+</sup> cells were transduced as described.<sup>27</sup> Briefly, transduction was performed in serum-free SF34 medium containing 5 μg/ml polybrene, 10 ng/ml stem cell factor (SCF) and 100 ng/ml TPO for 12 h (cytokines were from R&D Systems). After 12 h incubation, the supernatants were replaced with fresh medium and cells were analyzed at 48 h post transduction. For cell viability/apoptosis assessment, cells were stained with Annexin-V-APC and 7-amino actinomycin (7-AAD, BD Biosciences) and analyzed within ZsGreen-positive gate by flow cytometry, according to the manufacturer's protocol. For PCR analysis and chemotaxis assay, ZsGreen-positive cells were isolated by FACS. The efficiency of Cre-mediated FAK deletion was determined by PCR, as described previously.<sup>28</sup>

#### In vitro progenitor colony-forming assay<sup>29</sup>

Lenti-Cre- or mock-transduced KL cells (10<sup>4</sup>) were plated in quadruplicates in the methylcellulose medium (MethoCult 3236; StemCell Technologies) supplemented with SCF (100 ng/ml). Cultures were incubated at 37°C in 5% CO<sub>2</sub> and

saturating humidity for 7 days and colonies were then counted under a dissecting microscope, based on morphologic criteria. As reported previously, KL cells cultured with SCF in methylcellulose are enriched for hematopoietic progenitors consisting primarily of granulocyte- and granulocyte/macrophage-colony-forming unit (CFU).<sup>30</sup> This was confirmed by our flow cytometric analysis: 60% of cells plucked from colonies (CFU-SCF cells) were committed myeloid progenitors (c-kit<sup>+</sup>/Gr-1<sup>low</sup> and/or c-kit<sup>+</sup>/Mac-1<sup>low</sup>), whereas 20% of cells retained the initial c-kit<sup>+</sup>/lin<sup>-</sup> phenotype; the remaining 20% of cells represented mature cells (c-kit-negative/Gr-1 and/or Mac-1-positive, data not shown).

#### Chemotaxis of transduced progenitor B cells, CFU-SCF and Sca-1<sup>+</sup> cells

The assay was carried out essentially as described for REH cells, using 10<sup>6</sup> cells per well and 300 ng/ml of CXCL12; the optimal concentration of CXCL12 was established in preliminary studies.

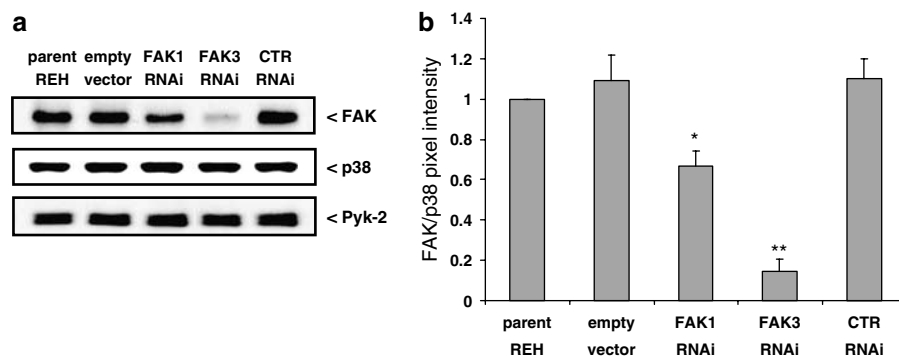
#### Statistical analysis

The values are shown as the mean ± s.d. of at least three experiments or as indicated otherwise. Statistical analysis was carried out using Student's *t*-test.

#### Results

##### Efficient silencing of FAK in REH pro-B cells

REH cells express CXCR4 and respond to CXCL12 by chemotaxis and VLA-4-dependent adhesion to VCAM-1.<sup>15,17,20</sup> Cells were infected with a retroviral vector encoding one of twenty shRNAs targeting different regions of human FAK. As shown in Figure 1, REH cells transduced with shRNA depicted as FAK3-RNAi demonstrated the most significant FAK silencing (above 80%) as compared to parent (untransduced) REH cells, empty vector-transduced cells or cells harboring shRNA against an irrelevant gene (hypoxanthine phosphoribosyltransferase, depicted as CTR-RNAi). Another FAK RNAi (FAK1-RNAi) downregulated about 30% of FAK protein expression, whereas the remaining FAK-RNAi constructs demonstrated very low or no silencing efficiency (data not shown) and therefore only



**Figure 1** Specific silencing of focal adhesion kinase (FAK) by retrovirus-mediated RNA interference (RNAi). (a) Analysis of FAK expression in REH cells infected with either an empty vector, vectors encoding RNAi against various FAK sequences (FAK1 and FAK3 RNAi are shown) or with control RNAi (CTR-RNAi) that targets an irrelevant gene. Cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-FAK or anti-Pyk2 antibodies. Reprobing with anti-p38 antibody was performed to verify protein loading. Blot is representative of three independent infection experiments. (b) Densitometry analysis of western blots. The intensity of protein was calculated using ImageQuant densitometer, Version 1.1 (Molecular Dynamics, Sunnyvale, CA, USA). Mean ± s.d. of three independent infections is shown. Significance was determined by Student's *t*-test (\**P* < 0.05, \*\**P* < 0.01). SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; shRNA, short-hairpin RNA.

FAK3-RNAi was considered for further studies. Importantly, FAK knockdown did not affect Pyk-2 kinase expression (Figure 1a, bottom panel), the only known member of the FAK family that shares a high-sequence homology with FAK.<sup>31</sup>

### FAK silencing impairs pro-B cell chemotaxis to CXCL12

First, we examined whether FAK deficiency affects CXCL12-induced chemotaxis. As shown in Figure 2a, chemotaxis of empty vector-transduced cells and CTR-RNAi-transduced cells was comparable to that of parent REH cells. In contrast, chemotaxis of FAK3-RNAi-transduced cells was completely blocked. The significant abrogation of chemotaxis was observed for a range of CXCL12 concentrations tested (10–1000 ng/ml, not shown). CXCR4 surface expression was unchanged by FAK deficiency (Figure 2b). Moreover, the dynamics and degree of CXCL12-induced internalization of CXCR4 receptor were unaffected by knockdown of FAK (not shown). These results indicate that FAK is required for chemotaxis toward CXCL12, whereas ligand-induced internalization of CXCR4 receptor is FAK-independent.

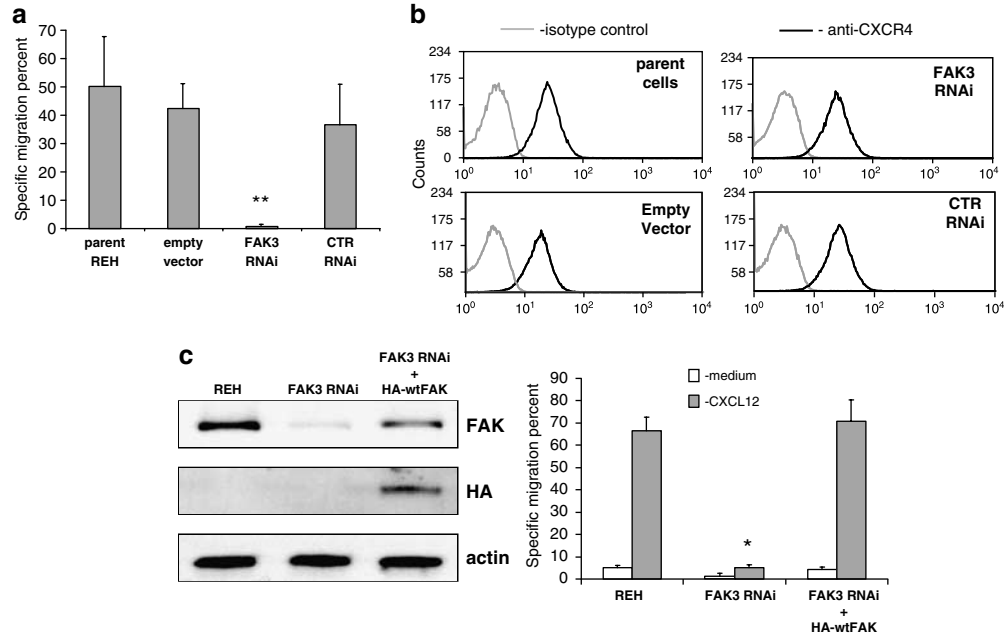
### Rescue of the FAK-RNAi phenotype with wild-type FAK

To confirm that the observed phenotype is the result of sequence-specific silencing of FAK gene, we performed a functional rescue experiment in FAK3-RNAi-infected REH cells that demonstrated 80% of FAK silencing and total inhibition of chemotaxis. HA-tagged wild-type FAK (HA-wtFAK) was transiently transfected into FAK3-RNAi cells. Since the HA-wtFAK

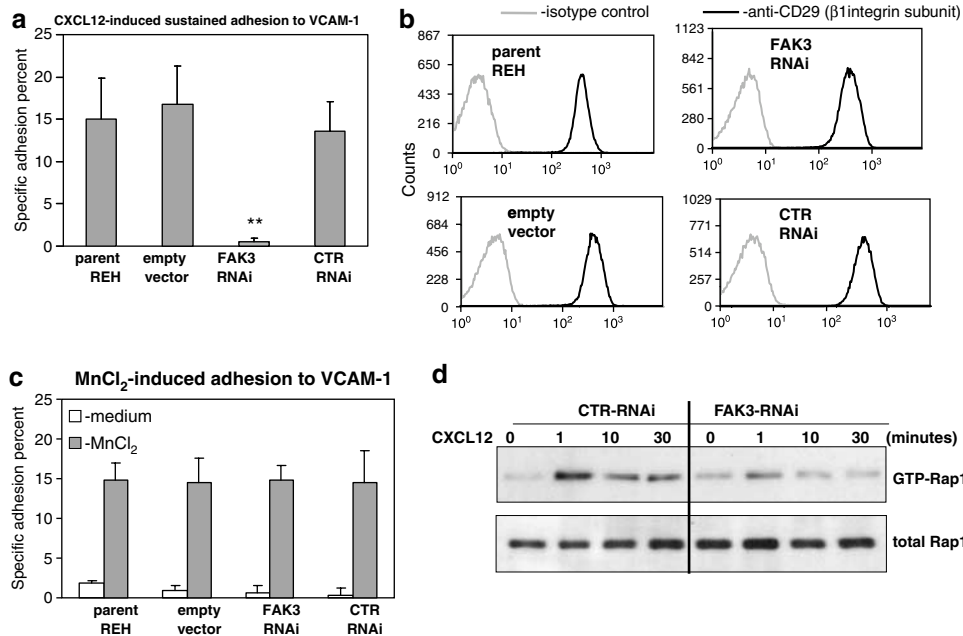
construct is derived from chicken, it is refractory to FAK3-RNAi processing due to a base-pair mismatch.<sup>32</sup> As shown in the left panel of Figure 2c, introduction of HA-wtFAK into FAK3-RNAi cells resulted in restoration of FAK expression. More importantly, wild-type FAK restored the chemotactic responsiveness to CXCL12 in FAK3-RNAi REH cells (Figure 2c, right panel). These results confirm the specificity of RNAi-mediated FAK deletion and further suggest that FAK is required in CXCL12-induced responses of leukemic pro-B cells.

### FAK is required for CXCL12-induced ALL pro-B cell adhesion to VCAM-1

We previously observed that CXCL12 promotes sustained adhesion to VCAM-1 in REH pro-B cells and primary BM B cells.<sup>15</sup> To test whether FAK deficiency affects CXCL12-induced sustained adhesion, FAK3-RNAi and control cells were subjected to the long-term adhesion assay. In this assay, cells are continuously exposed to CXCL12 before and during cell plating on VCAM-1-coated wells, in order to simulate the cell exposure to CXCL12 within the BM microenvironment.<sup>15,33</sup> We found that CXCL12-induced adhesion was completely absent in FAK3-RNAi cells, in contrast to parent REH, empty vector-transduced and CTR-RNAi cells (Figure 3a). The expression of VLA-4 was unaffected by FAK deficiency (Figure 3b). Importantly, FAK downregulation did not impair VLA-4 function since stimulation with MnCl<sub>2</sub>, which activates integrins by direct action on their extracellular domains,<sup>34</sup> resulted in strong upregulation of adhesion to VCAM-1 in FAK-deficient as well as control cells (Figure 3c).



**Figure 2** Silencing of FAK inhibits CXCL12-induced chemotaxis of human ALL pro-B cells. (a) Cells were subjected to Transwell chemotaxis assay toward CXCL12 (500 ng/ml), followed by enumeration of migrated cells by flow cytometry. Data are shown as the percent of specific migration (percent of input cells that migrated in the absence of CXCL12 is subtracted from the percent of cells that migrated toward CXCL12). Mean  $\pm$  s.d. of three independent experiments performed in duplicate is shown,  $**P < 0.01$ . The differences between chemotaxis of REH, empty vector and control RNAi were not statistically significant ( $P > 0.2$ ). (b) FAK knockdown does not affect CXCR4 expression. Gray line represents cells stained with the isotype control antibody, black line represents staining with anti-CXCR4 antibody. (c) Transfection of wild-type FAK into FAK-deficient REH cells restores the FAK-RNAi-mediated defect of migration toward CXCL12. FAK-deficient REH cells (FAK3-RNAi) were transiently transfected with HA-tagged chicken FAK (HA-wtFAK) construct. Left panel shows representative blot, where FAK expression is depicted in parent REH cells (lane 1), FAK3-RNAi cells (lane 2) and FAK3-RNAi cells transfected with chicken FAK (FAK3-RNAi + HA-wtFAK, lane 3). Right panel shows the recovery of chemotaxis to CXCL12 by HA-wtFAK (mean  $\pm$  s.d. of two independent transduction experiments,  $*P < 0.05$ ). ALL pro-B, acute lymphoblastic leukemia pro-B; FAK, focal adhesion kinase; HA, hemagglutinin; RNAi, RNA interference.



**Figure 3** FAK is required for CXCL12-mediated sustained adhesion to VCAM-1. **(a)** Cells were preincubated with CXCL12 for 30 min in solution and were then allowed to settle in VCAM-1-coated wells for another 30 min. Non-adherent cells were subsequently removed by manual washing. Data are presented as specific adhesion percent (percentage of input cells that adhered in the absence of CXCL12 was subtracted from the percentage of input cells that adhered in the presence of CXCL12). Mean  $\pm$  s.d. of three independent experiments performed in triplicate is shown,  $**P < 0.01$ . **(b)** Expression of VLA-4 integrin is unaffected by FAK knockdown. Gray line represents cells stained with the isotype control antibody and black line represents staining with anti-CD29 antibody. Similar results were obtained by staining with anti-CD49d ( $\alpha_4$  integrin subunit) antibody, not shown. **(c)** The effect of FAK knockdown on MnCl<sub>2</sub>-induced adhesion to VCAM-1. Cells were stimulated with 1 mM of MnCl<sub>2</sub> for 10 min and then plated on VCAM-1-coated wells for 30 min. **(d)** Decreased CXCL12-induced Rap1 activation in FAK knockdown cells. FAK3-RNAi and CTR-RNAi cells were stimulated with CXCL12 for indicated time and GTP-bound Rap1 was detected using GTP-Rap1 pull-down assay, followed by western blot with anti-Rap1 antibody. Total cell lysates probed with anti-Rap1 antibody served as a loading control. The results are representative of three independent experiments. FAK, focal adhesion kinase; RNAi, RNA interference.

### Decreased CXCL12-induced Rap1 activation in FAK-deficient cells

The molecular mechanisms involved in chemokine-induced integrin activation are poorly understood, although several signaling molecules have been shown to participate in this process.<sup>35–38</sup> Recently, the Ras-like GTPase Rap1 was described to be crucial in chemokine-induced inside-out integrin activation.<sup>39,40</sup> We thus tested whether silencing of FAK affects CXCL12-induced Rap1 activation. In agreement with previous reports on CXCL12-induced Rap1 activation in hematopoietic cells,<sup>40,41</sup> we show that CXCL12 induced Rap1 activation in CTR-RNAi cells (Figure 3d) and in intact REH cells (not shown). In contrast, Rap1 activation in FAK3-RNAi cells was significantly impaired (Figure 3d). These data provide further evidence of FAK involvement in the CXCL12-induced integrin activation pathway.

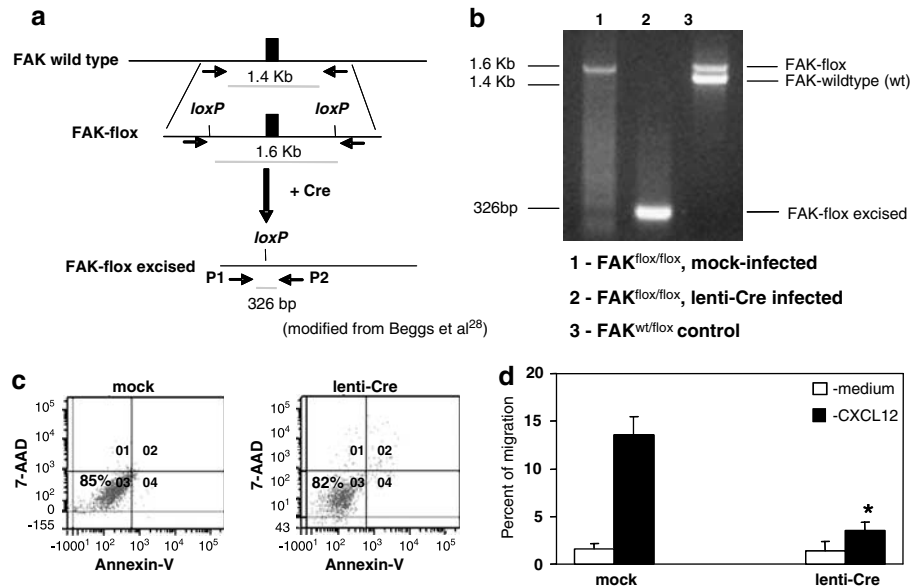
### Cre/lox-mediated FAK deletion in primary murine B cell progenitors inhibits chemotaxis to CXCL12

IL-7-expanded progenitor B cells derived from FAK<sup>lox/lox</sup> mice (Figure 4a)<sup>28</sup> were infected with either the lenti-Cre or mock vector in the presence of IL-7. The efficiency of infection was approximately 90% in both lenti-Cre and mock-transduced cells, as assessed by ZsGreen expression 96 h after infection (not shown). ZsGreen-positive cells were isolated by flow cytometry sorting for further analysis. As shown in Figure 4b, Cre-transduced FAK<sup>lox/lox</sup> B-cell progenitors showed complete deletion of floxed FAK alleles (as demonstrated by the presence

of an excised FAK fragment of 326 bp length), whereas FAK gene in cells infected with mock virus showed no excision (the presence of 1.6 kb band only). The viability of both mock and lenti-Cre-transduced cells was not affected (Figure 4c) and therefore we conclude that neither Cre expression nor FAK gene deletion altered cell viability under these culture conditions. However, similar to what we observed in RNAi-mediated FAK knockdown in human leukemic cell line REH, Cre-mediated FAK deletion significantly decreased progenitor B-cell chemotaxis to CXCL12 (Figure 4d). Thus, our results provide cumulative evidence for the critical role of FAK in CXCL12-mediated responses in normal and leukemic BM B lymphocytes.

### FAK is tyrosine phosphorylated in hematopoietic progenitor cells upon CXCL12 stimulation

To extend our studies in B-lineage cells described above, we also examined FAK function in other hematopoietic cell populations. First, we assessed the effect of CXCL12 stimulation on FAK tyrosine phosphorylation in human (CD34-positive) and murine (Sca-1-positive) hematopoietic progenitors and found that CXCL12 induces FAK tyrosine phosphorylation in both of these subsets (Figure 5a). The weaker signal in murine Sca-1<sup>+</sup> cells may be related in part to their relatively smaller size and/or to the fact that both antibodies used in the study (that is, anti-phospho FAK and anti-FAK), although crossreactive to mouse, were raised against human FAK phosphorylated at tyrosine 397 and total human FAK, respectively, and in our experience, give a weaker signal when used for analyses of murine cells.



**Figure 4** Cre-mediated *FAK* deletion in murine bone marrow (BM) progenitor B cells impairs CXCL12-induced chemotaxis. (a) Schematic diagram of Cre-mediated *FAK* gene recombination in floxed-*FAK* mouse (adapted from Beggs *et al.*<sup>28</sup>). The targeting construct contains the second kinase domain exon of *FAK* (black box) flanked by loxP sites. Primers had following sequences: P1: 5-GACCTTCAACTTCTCATTCTCC-3 and P2: 5-GAATGCTACAGGAACCAATAAC-3. (b) Deletion of floxed-*FAK* allele in *FAK*<sup>flox/flox</sup> BM progenitor B cells. Progenitor B cells expanded for 1 week in IL-7-containing medium were transduced with lentiviral vector encoding Cre and ZsGreen reporter gene (lenti-Cre) or, as a control, the vector with ZsGreen only (mock vector). After 96 h, ZsGreen-positive cells were isolated by flow cytometry sorting and examined for deletion of floxed-*FAK* by PCR. Lenti-Cre expression in BM B cells led to the recombination of floxed-*FAK* gene (326 bp excised PCR fragment, lane 2). Transduction with mock vector showed no excision of floxed-*FAK* (lane 1). Genomic DNA from heterozygous mice (*FAK*<sup>wt/flox</sup>) was used to show wild-type and floxed-*FAK* alleles (lane 3). (c) Analysis of progenitor B-cell viability after Cre transduction. Cells were stained and analyzed by flow cytometry within a ZsGreen gate. The upper right quadrant represents necrotic cells (Annexin-V<sup>+</sup>/AAD<sup>+</sup>); the lower right quadrant represents apoptotic cells (Annexin-V<sup>+</sup>/AAD<sup>-</sup>); and the lower left quadrant represents viable, nonapoptotic cells (Annexin-V<sup>-</sup>/AAD<sup>-</sup>). Numbers represent the relative percentage of viable cells among total cell population. (d) Cre-mediated *FAK* knockdown significantly decreases progenitor B-cell chemotaxis toward CXCL12. ZsGreen-positive cells were sorted and subjected to Transwell chemotaxis assay toward CXCL12 (300 ng/ml) and the number of migrated cells was analyzed by flow cytometry. Experiments were performed in triplicate. Data are shown as percent of chemotaxis (percent of cells that migrated toward chemokine gradient among total cell population). Mean  $\pm$  s.d. of three independent transduction experiments are shown, \* $P < 0.005$ . *FAK*, focal adhesion kinase; IL-7, interleukin.

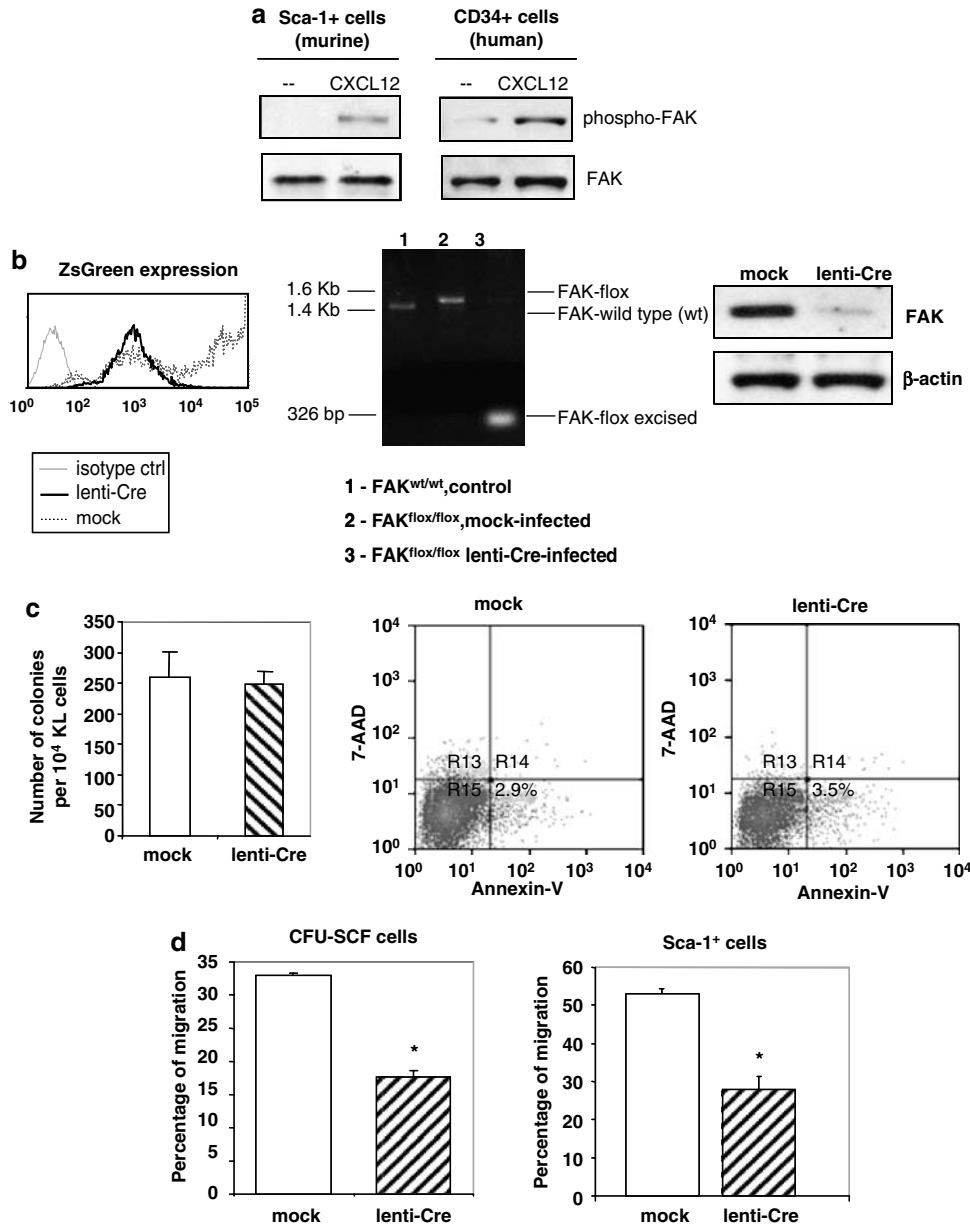
#### Efficient Cre-mediated *FAK* deletion in hematopoietic *c-kit*<sup>+</sup>/*lin*<sup>-</sup> (KL) cells and KL cell-derived hematopoietic precursor cells (CFU-SCF)

To evaluate the effect of *FAK* deletion on hematopoietic progenitor cells, *FAK*<sup>flox/flox</sup> *c-Kit*<sup>+</sup>/*lin*<sup>-</sup> (KL) cells, a population enriched for hematopoietic progenitor cell activity, were isolated by flow cytometry sorting and transduced with the lenti-Cre or mock vector. Genomic DNA analysis confirmed that the infection of *FAK*<sup>flox/flox</sup> KL cells with lenti-Cre vector led to the recombination of floxed-*FAK* allele and the excision of *FAK* gene, whereas *FAK*<sup>flox/flox</sup> KL cells transduced with mock vector showed no *FAK* excision (data not shown). To test whether *FAK* deficiency affects *in vitro* growth potential of hematopoietic progenitor cells, mock-infected and lenti-Cre-infected KL cells were subjected to *in vitro* progenitor colony-forming assay in the presence of SCF.<sup>29,30</sup> After 7 days of culture, both mock and lenti-Cre-transduced *FAK*<sup>flox/flox</sup> KL cell-derived hematopoietic precursor cells (here referred to as CFU-SCF cells) retained high efficiency of transduction; nearly 100% colonies were ZsGreen-positive (not shown and Figure 5b, left panel). Lenti-Cre-mediated *FAK* gene deletion and resulting reduction in *FAK* protein expression was confirmed by PCR and western blotting of cells derived from ZsGreen-positive colonies (Figure 5b, middle and right panels). Lenti-Cre-mediated *FAK* deletion did not cause a significant difference in the number of scored CFU-SCF colonies in comparison to cells transduced with mock vector; KL cells transduced with mock vector formed  $258 \pm 43$

colonies (mean  $\pm$  s.d. per  $10^4$  plated KL cells), whereas KL cells transduced with lenti-Cre formed  $247 \pm 21$  colonies (Figure 5c, left panel). The number of apoptotic cells was low and similar in both mock- and lenti-Cre-transduced KL-derived CFU-SCF cell populations (Figure 5c, middle and right panels).

#### *FAK* deletion significantly reduces CXCL12-mediated migration of hematopoietic precursor cells

To test whether the reduction in *FAK* protein expression affects hematopoietic precursor cell responsiveness to CXCL12, we utilized CFU-SCF cells derived from transduced KL cells. As described in Materials and methods, the resultant population (CFU-SCF cells) retains the characteristics of hematopoietic precursors. Before methylcellulose culture in the presence of SCF, KL cells were transduced with either mock or lenti-Cre vector and *FAK* deletion in KL and in CFU-SCF cells was confirmed as described in the previous paragraph. CFU-SCF cells were then subjected to chemotaxis toward 300 ng/ml of CXCL12. As shown in Figure 5d (left panel), *FAK*-deficient CFU-SCF cells demonstrated a statistically significant ( $P = 0.017$ ), 49% decrease in chemotaxis to CXCL12;  $33 \pm 0.28\%$  (mean  $\pm$  s.d.) of mock vector-transduced cells migrated to CXCL12, whereas only  $17 \pm 0.9\%$  of Cre-transduced colony cells migrated to CXCL12. A similar decrease of chemotaxis to CXCL12 was observed in *FAK*<sup>flox/flox</sup> Sca-1<sup>+</sup> progenitor cells in which *FAK* gene was deleted by lenti-Cre transduction (Figure 5d, right panel).



**Figure 5** Cre-mediated *FAK* deletion in hematopoietic precursor cells. **(a)** CXCL12 induces FAK tyrosine phosphorylation in mouse Sca-1<sup>+</sup> and human CD34<sup>+</sup> hematopoietic progenitor cells. Purified human (CD34<sup>+</sup>) or murine (Sca-1<sup>+</sup>) cells ( $1 \times 10^6$  per stimulation) were incubated with CXCL12 (300 ng/ml) for 3 min at 37°C and lysed. Proteins were separated by SDS-PAGE, followed by transfer and immunoblotting with an anti-human phospho-FAK antibody that recognizes phosphorylated 397 tyrosine residue of FAK. Immunoblotting with total FAK serves as a loading control. **(b)** Efficient Cre/lox-mediated *FAK* deletion is retained in c-Kit<sup>+</sup>/lin<sup>-</sup> (KL) cell-derived hematopoietic precursors (CFU-SCF cells). Lenti-Cre- or mock-transduced KL cells were plated in methylcellulose medium containing 100 ng/ml of SCF for 7 days. Nearly 100% of resulting colonies of lenti-Cre- and mock-transduced KL cells retained ZsGreen expression (left panel shows a representative FACS analysis). Lenti-Cre-mediated deletion of *FAK* gene was confirmed by PCR of DNA isolated from several single colonies (middle panel shows PCR from the representative colony). Right panel shows the decreased FAK protein levels in lenti-Cre-transduced CFU-SCF cells, as analyzed by immunoblotting with anti-FAK antibody and anti-actin as a loading control. **(c)** Effect of *FAK* deletion on hematopoietic progenitor colony formation and colony cell viability. Transduced KL cells were subjected to hematopoietic progenitor colony-forming assay in the presence of SCF. Left panel shows the number of mock (open bar) and lenti-Cre (striped bar) CFU-SCF colonies after 7 days of culture. The number of colonies per 10 000 plated KL cells is shown. For cell viability/apoptosis analysis, mock (middle) or lenti-Cre-transduced (right) CFU-SCF colony cells were analyzed by flow cytometry for Annexin-V and 7-AAD. Numbers in the lower right quadrants of dot-plots show the percentage of apoptotic cells (Annexin-V +/7-AAD-). **(d)** Cre-mediated *FAK* deletion significantly decreases chemotaxis of KL-derived hematopoietic precursors (CFU-SCF) and Sca-1<sup>+</sup> hematopoietic progenitors to CXCL12. Before the chemotaxis experiment, CFU-SCF cells were harvested and kept in serum-free medium overnight. Transwell chemotaxis assay was carried out at 37°C for 2 h (300 ng/ml of CXCL12) and the number of migrated cells was analyzed by flow cytometry. The chemotaxis of *FAK*<sup>lox/lox</sup> mice-derived Sca-1<sup>+</sup> cells infected with lenti-Cre virus was similarly decreased (right panel). Data are shown as percent of specific migration (percent number of cells that migrated toward medium alone is subtracted from percent number of cells that migrated toward CXCL12). Data are presented as mean  $\pm$  s.d. of three independent experiments, \* $P < 0.05$ . CFU-SCF, colony-forming unit-stem cell factor; FACS, fluorescence-activated cell sorting; FAK, focal adhesion kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

## Discussion

Since the cloning of FAK in 1992,<sup>42–44</sup> much knowledge has been acquired regarding its central role in migration, cell cycle and survival in adherent cells, such as fibroblasts. In these cells, adhesion-induced integrin activation promotes the organization of focal adhesions, which are defined as protein complexes consisting of signaling and adaptor proteins linked to cytoskeleton structures.<sup>45</sup> FAK is a key focal adhesion component that coordinates the transmission of integrin signals and thus determines the dynamics and the extent of motility in attachment-dependent cells.<sup>22</sup> Specifically, FAK is implicated in controlling the cycle of focal adhesion assembly/disassembly and cytoskeletal rearrangements in response to external stimuli mediated by integrins, cytokine receptors and G-protein-coupled receptors. Furthermore, FAK is important for cell survival and cell-cycle progression and has been implicated in the biology of multiple neoplasms, including acute myelogenous leukemia in which the increased FAK expression is associated with poor prognosis.<sup>46,47</sup> A number of recent studies employing tissue-specific FAK gene targeting have substantiated the importance of FAK in neuronal and endothelial cell biology.<sup>28,48</sup> In contrast to adherent cells, hematopoietic cells do not form focal adhesion and stress fiber structures<sup>49</sup> and their integrins remain in a non-adhesive state unless activated (for example, by cytokines and/or chemokines).<sup>37</sup>

Based on our previous studies in progenitor B cells, we hypothesized that FAK participates in signaling pathways that determine migratory and adhesive status of hematopoietic cells. Here, we demonstrate that FAK deficiency significantly inhibits CXCL12-induced responses in human and mouse pro-B cells and mouse hematopoietic progenitor cells. First, we found that FAK knockdown by RNAi in human ALL pro-B cell line REH impaired CXCL12-induced chemotactic and pro-adhesive responses. Neither the basal cell adhesion to VCAM-1 nor the adhesion to very high VCAM-1 concentrations in the absence of CXCL12 (as a mean to examine outside-in integrin signaling) were affected by FAK deficiency (not shown), suggesting that the CXCL12-FAK pathway may be involved in inside-out integrin activation. This possibility was further substantiated by the observation that CXCL12-mediated activation of Rap1 is decreased in FAK knockdown pro-B cells. Rap1 is a small GTPase of the Ras superfamily, which acts as a potent trigger for inside-out integrin activation, by modulating integrin affinity and/or membrane clustering.<sup>39</sup> FAK may potentially influence the action of Rap1-specific guanine exchange factors (GEFs) or GTPase-activating proteins, either directly or indirectly, through FAK binding partners (for example, p130Cas) that were shown to activate Rap-1 GEFs.<sup>50</sup>

Next, we generated FAK-deficient primary B cells, by employing Cre-mediated recombination in IL-7-expanded FAK<sup>fllox/fllox</sup> B-cell progenitors. B cells, in which floxed-FAK alleles were efficiently excised, demonstrated a significant decrease in chemotaxis to CXCL12. Thus, the importance of FAK in CXCL12-induced migration observed in the FAK-RNAi-treated pro-B cell ALL line was confirmed by an independent method in primary murine progenitor B cells. Also, similar to progenitor B cells, FAK-deficient Sca-1<sup>+</sup> hematopoietic progenitor cells and hematopoietic precursor cells derived from FAK-deficient KL cells in the presence of SCF (CFU-SCF cells) showed significantly decreased migration toward CXCL12. In FAK-deficient pro-B ALL cells (REH), CXCL12-induced migration was completely inhibited, whereas the effect on migration of normal progenitor B cells as well as hematopoietic precursors

(CFU-SCF cells), albeit significant, was less dramatic. These data point to potential CXCL12/CXCR4 signaling differences between normal hematopoietic cells and leukemic cells. Similar differences in CXCL12-induced migration between normal hematopoietic versus leukemic cells were reported by Spiegel *et al.*<sup>11</sup> In this report, *Clostridium difficile*-derived toxin B, an inhibitor of Rho GTPases, completely attenuated CXCL12-induced chemotaxis of pro-B ALL cells, whereas it had more subtle effect on chemotaxis of normal precursor B cells as well as CD34-positive hematopoietic progenitors. Since FAK is known to regulate the function of Rho GTPases in non-hematopoietic cells,<sup>22</sup> it will be of interest to characterize FAK-Rho interactions in CXCR4-induced signaling in normal and malignant hematopoietic cells.

Because FAK is implicated in cell survival signaling,<sup>22</sup> we examined whether the *in vitro* conditions used to isolate progenitor cells caused enhanced apoptosis and/or cell death of FAK-deficient hematopoietic cells compared to wild-type cells. Although we determined that this was not the case, FAK may still prove to be an important survival factor in hematopoietic cells *in vivo*, where the fate of hematopoietic stem and progenitor cells is determined by a dynamic crosstalk between niche-originating cytokines, chemokines and adhesion molecules.<sup>1</sup> In this regard, FAK deletion did not affect the potential of KL cells to form progenitor colonies, suggesting that FAK signaling may be dispensable for intrinsic hematopoietic progenitor cell proliferation *in vitro*. Similar findings were reported in Rac2-deficient HSC/P cells: the proliferation of Rac2<sup>-/-</sup> HSC/P cells in high proliferative potential colony-forming assays was not different from their wild-type counterparts; however, *in vivo* studies demonstrated that Rac2<sup>-/-</sup> HSC/P ability of long-term engraftment was impaired, suggesting that Rac2 plays an important role in HSC/P microenvironmental localization during the engraftment process.<sup>51</sup>

In summary, the current studies provide evidence for the importance of FAK signaling in chemotactic and pro-adhesive responses of hematopoietic cells. We conclude that *in vivo* studies employing FAK-targeted mouse model systems are needed to provide insight into the function of FAK in various aspects of hematopoiesis, including HSC/P homing, engraftment and lineage development.

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