Title: CCR7 is involved in BCR-ABL/STAP-2-mediated cell growth in hematopoietic Ba/F3 cells

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Abstract

Chronic myeloid leukemia is a clonal disease characterized by the presence of the Philadelphia chromosome and its oncogenic product, BCR-ABL, which activates multiple pathways involved in cell survival, growth promotion, and disease progression. We previously reported that in murine hematopoietic Ba/F3 cells, signal transducing adaptor protein-2 (STAP-2) binds to BCR-ABL and up-regulates BCR-ABL phosphorylation, leading to enhanced activation of its downstream signaling molecules. The binding of STAP-2 to BCR-ABL also influenced the expression levels of chemokine receptors, such as CXCR4 and CCR7. For the induction of CCR7 expression, signals mediated by the MAPK/ERK pathway were critical in Ba/F3 cells expressing BCR-ABL and STAP-2. In addition, STAP-2 cooperated with BCR-ABL to induce the production of CCR7 ligands, CCL19 and CCL21. Our results demonstrate a contribution of CCR7 to STAP-2-dependent enhancement of BCR-ABL-mediated cell growth in Ba/F3 cells.

Keywords: CML; BCR-ABL; STAP-2; CCR7; MAPK/ERK
Introduction

The BCR-ABL fusion oncogene is responsible for the pathogenesis of chronic myeloid leukemia (CML) [1], which is characterized by the premature release of leukemia cells from bone marrow as well as a substantial accumulation of those with a potential of differentiation in the blood, spleen, and bone marrow. Owing to its elevated tyrosine kinase activity, BCR-ABL activates a number of signaling pathways, including the Ras, PI3K/AKT, Janus kinase/signal transducer and activator of transcription, and NF-κB signaling pathways [1, 2]. Moreover, in various human and mouse models, hematopoietic cells expressing BCR-ABL show a growth advantage, resistance to apoptosis, and altered adhesion and homing properties.

Signal transducing adaptor protein-2 (STAP-2) is a novel adaptor molecule, which was isolated as a c-FMS interacting protein [3]. The human homolog of STAP-2 is a known substrate of breast tumor kinase (BRK) [4]. Previous work in our laboratory found that STAP-2 can associate with and influence a variety of signaling or transcriptional molecules [3, 5-9], including STAT3 and STAT5 [3, 5], and FceRI and Toll-like receptor-mediated signals [6, 9]. Further, thymocytes and peripheral T cells from STAP-2-deficient mice show enhanced IL-2-dependent cell growth and integrin-mediated T-cell adhesion and impaired SDF-1α-induced T-cell migration [5, 7, 8]. Because STAP-2 is expressed in a variety of tissues and cells, such as lymphocytes, macrophages, and hepatocytes, STAP-2 is also likely to function in a variety type of cells. Importantly, we identified BCR-ABL as a novel binding partner of STAP-2, and STAP-2 augmented BCR-ABL activity and activation of downstream signaling molecules, including ERK, STAT5, BCL-xL, and BCL2 [10]. We also found that STAP-2 over-expression in murine hematopoietic Ba/F3 cells confers a growth advantage in vitro and is able to induce leukemia in vivo. In addition, STAP-2 controls the migration and homing of BCR-ABL-expressing cells by influencing chemokine receptor expression levels.

In the present study, we found that chemokine receptor CCR7 influences the enhancement of
BCR-ABL-dependent cell growth via STAP-2 and that CCR7 mRNA levels are mediated through the MAPK/ERK pathway in murine hematopoietic Ba/F3 cells.
2. Materials and methods

*Reagents and antibodies.* BCR-ABL tyrosine kinase inhibitor STI571, imatinib mesylate, was kindly gifted by Novartis Pharmaceuticals (Basel, Switzerland). A6730 and U0126 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Expression vectors for human CXCR4 (hCXCR4) was generated by PCR methods and sequenced (primer sequences are available upon request). Anti-ABL, anti-Stat5, anti-BCL-xL, and anti-phospho ERK (pERK) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Myc, anti-pStat5, and anti-actin mAb and were purchased from Sigma-Aldrich. Anti-ERK, anti-Akt, and anti-pAkt antibodies was purchased from Cell signaling Technologies (Beverly, MA).

*Cell culture, establishment of cell lines and siRNA transfection.* An interleukin (IL)-3-dependent murine pro-B cell line, Ba/F3 was maintained in RPMI1640 medium supplemented with 10% FCS with 10% of WEHI-3B conditioned medium as a source of IL-3 (Sekine et al 2005). Stable Ba/F3 transformants expressing pcDNA3 (Ba/F3-pcDNA), pcDNA3-STAP-2 (Ba/F3-STAP-2) or pcDNA3-p210BCR-ABL (Ba/F3-p210) were established as described previously [3, 10]. Stable Ba/F3 transformants expressing human CXCR4 (Ba/F3-p210/STAP-2/hCXCR4#1 and #2) were established by transfection with pcDNA3-hCXCR4. Stable CCR7 knockdown Ba/F3-p210/STAP-2 cell lines (Ba/F3-p210/STAP-2/shCCR7#1 and #2) were established by transfection of with pGPU6/GFP vector (Shanghai GenePharma, Shanghai, China) bearing short hairpin RNA (shRNA) targeting CCR7 (5’-GCATCTTTGGCATCTATAAGTT-3’). Similarly, control shRNA (non-siencing; 5’-TTCTCCGAACTGTCGCTACGT-3’)-transfected Ba/F3-p210/STAP-2 cell lines (Ba/F3-p210/STAP-2/shControl) were also established. Ba/F3-p210/STAP-2 cells transfected with control or Stat5a/b siRNAs using a Nucleofector (Amaxa Biosystems, Cologne, Germany). Cells were transfected with 200 pmol siRNA in Nucleofector solution V. The siRNAs targeting mouse Stat5a and Stat5b used in this study were as
follows: Stat5a, 5'-AGGUCUUUGCAAGUAUUATT-3'; Stat5b, 5'-GCGUGAUGGAAGUAUUGAATT-3'. Control siRNA was obtained from Qiagen (non-silencing; cat. no. 1022076).

**RT-PCR and quantitative real-time PCR**, Cells were harvested, total RNAs from the transfected cells were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in RT-PCR. RT-PCR was performed using RT-PCR high -Plus- Kit (TOYOBO, Tokyo, Japan). Primers used for RT-PCR were: CXCR4: 5'-GGTGGTGCTATGTTGGCGTCT-3' (sense), 5'-TGGAGTGTGACAGCTTGGAG-3' (antisense); Ccl19: 5'-AGACTGCTGCCTGTCTGTGA-3' (sense), 5'-GCCTTTGTTCTTGGCAGAAG-3' (antisense); Ccl21: 5'-GTCCGAGGCTATAGGAAGCA-3' (sense), 5'-GCCCTTTTCCTTTCTTCCAG-3' (antisense); G3pdh: 5'-GAAATCCCATCACCATCTTCCAGG-3' (sense), 5'-CAGTAGAGGCAGGATGATGTTC-3' (antisense). Quantitative real-time PCR analyses of Ccr7 as well as the control G3pdh or Actin mRNA transcripts were carried out using the assay-on-demandTM gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems, Tokyo, Japan).

**Western blot analysis**, Western blot analyses were performed as described previously [3]. Briefly, cells were harvested and lysed in a lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA).

**Cell proliferation assay**, The numbers of viable Ba/F3 cells after the indicated treatments were measured using a WST-8
[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Cell Counting Kit-8; Wako Pure Chemicals)[10]. Briefly, 10 µl of WST-8 solution was added to the cells in each well and incubated for 2 h. The absorbances were measured at a test wavelength of 450 nm and a reference wavelength of 595 nm using a microplate reader (Bio-Rad, Hercules, CA).

FACS Analysis

The following monoclonal antibodies were used: PE-anti-mouse CCR7 and APC-anti-human CXCR4 antibodies (eBioscience, San Diego, CA). All analyses were conducted on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Animal tumorigenesis, Ba/F3-p210/STAP-2/shControl and Ba/F3-p210/STAP-2/shCCR7 #1 cells (1x10⁷) were injected s.c. into BALB/c nude mice aged 4 weeks. After 4 weeks, the animals were sacrificed and the weighs of the tumor, lymph node, and spleen were measured. Mice were housed and bred in the Pharmaceutical Sciences Animal Center of Hokkaido University. All animals were maintained under pathogen-free conditions and in compliance with national and institutional guidelines. All protocols were approved by the Hokkaido University animal ethics committee.

Statistical methods, The significance of differences between group means was determined by Student’s t-test.
3. Results

3.1. STAP-2 cooperates with BCR-ABL to modify the expression of chemokine receptors in BCR-ABL-expressing Ba/F3 cells

We previously reported chemokine receptor expression profiles in Ba/F3 cells and showed that the mRNA levels of CCR7 were higher and those of CXCR4 lower in Ba/F3 cells expressing both BCR-ABL and STAP-2 compared with those expressing BCR-ABL alone. To further investigate these altered levels of CXCR4 and CCR7 expression, we here used four lines of Ba/F3 cells transfected with a control vector (Ba/F3-pcDNA), STAP-2 (Ba/F3-STAP-2), p210 BCR-ABL (Ba/F3-p210), or co-STAP-2/BCR-ABL (Ba/F3-p210/STAP-2) (Fig. 1A). We re-confirmed the expression levels of CCR7 and CXCR4 by quantitative real-time PCR analysis. As shown in Fig. 1B, the mRNA levels of CCR7 were up-regulated in Ba/F3-p210/STAP-2 cells compared with Ba/F3-p210 cells, whereas mRNA levels of CXCR4 were down-regulated in both Ba/F3-p210 and Ba/F3-p210/STAP-2 cells. The enhanced cell surface expression of CCR7 in Ba/F3-p210/STAP-2 cells was confirmed by flow cytometry analysis using an anti-CCR7 mAb (Fig. 1C). Therefore, STAP-2 positively regulates CCR7 expression but down-regulates CXCR4 expression in BCR-ABL-expressing Ba/F3 cells.

3.2. CXCR4 has no significant effect on BCR-ABL-dependent cell growth in Ba/F3 cells

We then tested the effect of CXCR4 expression in Ba/F3 cells on BCR-ABL/STAP-2-dependent cell growth. To this end, we transfected a human CXCR4 (hCXCR4) expression vector into Ba/F3-p210/STAP-2 cells, and established two stable clones (Ba/F3-p210/STAP-2/hCXCR4#1 and #2). Western blot analysis was used to confirm STAP-2 and BCR-ABL protein expression (Fig. 2A). Levels of hCXCR4 expression were also confirmed by PCR (Fig. 2B) and flow cytometry analysis (Fig. 2C) using an anti-hCXCR4 antibody. The reduced level of endogenous CXCR4 expression was completely nullified by the exogenous hCXCR4 expression. As shown in Fig. 4D, both Ba/F3-p210/STAP-2/hCXCR4#1 and #2 cell lines showed similar growth and
resistance to a BCR-ABL inhibitor, STI571, compared with Ba/F3-p210/STAP-2 cells, which had acquired a greater growth advantage and STI571 resistance than Ba/F3-p210 cells. The activation of downstream signaling molecules, including ERK, AKT, STAT5, or BCL-xL, in Ba/F3-p210/STAP-2/hCXCR4#1 and #2 cells was also similar to that in Ba/F3-p210/STAP-2 cells and stronger than the activation seen in Ba/F3-p210 cells (Fig. 4E). Therefore, exogenous hCXCR4 expression did not change the phenotypic of Ba/F3-p210/STAP-2 cells.

3.3. CXCR7 influences BCR-ABL-dependent cell growth in Ba/F3 cells

To examine the role of the elevated endogenous CCR7 expression in BCR-ABL/STAP-2-dependent cell growth, we first established CCR7-knockdown variants of Ba/F3-p210/STAP-2 cells using CCR7 shRNA (Ba/F3-p210/STAP-2/shCCR7#1 and #2), in which CCR7 expression was confirmed to be knocked down by quantitative real-time PCR (Fig. 3A) and flow cytometry analysis (Fig. 3B). Western blot analysis was used to confirm STAP-2 and BCR-ABL protein expression in these clones (Fig. 3C). Ba/F3-p210/STAP-2/shCCR7#1 and #2 cells showed slower proliferation than Ba/F3-p210/STAP-2 cells but faster proliferation than Ba/F3-p210 cells (Fig. 3D; upper panel). Similarly, Ba/F3-p210/STAP-2/shCCR7#1 and #2 cells showed sensitivity to STI571 in between that of Ba/F3-p210/STAP-2 and Ba/F3-p210 cells (Fig. 3D; bottom panel). Further, the activation status of BCR-ABL downstream signaling molecules was also decreased by CCR7 knockdown (Fig. 3E). To investigate the effect of CCR7 on the ability of Ba/F3-p210/STAP-2 cells to induce tumor formation in vivo, we subcutaneously injected Ba/F3-p210/STAP-2/shControl or Ba/F3-p210/STAP-2/shCCR7#1 cells into the flank of BALB/c nude mice. Mice injected with Ba/F3-p210/STAP-2/shControl cells developed small tumors at the injection sites but substantial lymph node swelling. However, such tumor progression was decreased in mice injected with Ba/F3-p210/STAP-2/shCCR7#1 cells (Fig. 3F). Therefore, CCR7 influences cell growth and/or migration of p210/STAP-2-expressing Ba/F3 cells.
3. 4. **Ba/F3-p210/STAP-2 cells express the CCR7 ligands**

CCR7 is a cell surface protein, which transduces signals for cell proliferation and migration in response to its ligands, CCL19 and CCL21. To explain the involvement of CCR7 expression in the observations described above, we speculated that Ba/F3-p210/STAP-2 cells express CCR7 ligands. As shown in Fig. 3G, PCR analysis could not detect CCL19 or CCL21 mRNAs in Ba/F3-p210 cells, but their expression was clearly observed when Ba/F3 cells were transfected with both BCR-ABL and STAP-2 (Fig. 3G). In parallel to the level of CCL19 mRNA expression, the proliferation of Ba/F3-p210/STAP-2 cells, but not of Ba/F3-p210 cells, was enhanced in a dose-dependent manner (Fig. 3H). Therefore, Ba/F3-p210/STAP-2 cells produce and respond to CCL19 and CCL21 in an autocrine manner.

3. 5. **The MAPK/ERK pathway is involved in CCR7 expression in BCR-ABL/STAP-2-expressing Ba/F3 cells**

To confirm which signaling pathway is involved in CCR7 expression and growth in Ba/F3-BCR-ABL/STAP-2 cells, we examined the effect of STAT5 knockdown or of pharmacological inhibition of the AKT or MAPK/ERK pathway. As shown in Fig. 4A and B, neither STAT5 knockdown nor treatment with A6730, an AKT inhibitor, produced a significant effect on CCR7 mRNA levels in Ba/F3-p210/STAP-2 cells. However, treatment of Ba/F3-p210/STAP-2 cells with U0126, a MAPK/ERK inhibitor, produced a significant inhibitory effect on CCR7 mRNA expression (Fig. 4C). Therefore, the MAPK/ERK pathway is involved in CCR7 expression in Ba/F3-p210/STAP-2 cells.

We also examined the effect of STAT5 knockdown or the two pharmacological inhibitors on Ba/F3-p210/STAP-2 cell growth. STAT5 knockdown (Fig. 4A) and pharmacological inhibition of the AKT (Fig. 4B) or MAPK/ERK (Fig. 4C) pathway significantly suppressed growth of Ba/F3-p210/STAP-2 cells. Therefore, Ba/F3-p210/STAP-2 cell proliferation is dependent on STAT5 as well as on AKT and MAPK/ERK pathways.
4. Discussion

STAP-2 enhances BCR-ABL activity and changes the proliferative and/or invasive character of Ba/F3 cells expressing p210 BCR-ABL. STAP-2 cooperates with BCR-ABL to modify the expression of chemokine receptors. In this study, we focused on CCR7 and its ligands, which are critical in the infiltration of tumor cells into lymph nodes and spleen. We found that CCR7 influences BCR-ABL/STAP-2-dependent cell growth in murine hematopoietic Ba/F3 cells and that the MAPK/ERK pathway has an effect on the expression of the CCR7 ligand CCL19 in BCR-ABL/p210-expressing Ba/F3 cells. Further, the expression of the CCR7 ligands, CCL19 and CCL21, was induced only when both BCR-ABL and STAP-2 were expressed simultaneously. Therefore, CCR7 in part mediates STAP-2-dependent enhancement of BCR-ABL-mediated cell growth in Ba/F3 cells in an autocrine manner.

Chemokines and their receptors play essential roles in tumorigenesis, including leukocyte recruitment, tumor cell growth and survival, angiogenesis, and metastasis [11, 12]. One notable finding of our study was that Ba/F3-p210/STAP-2 cells, but not Ba/F3-p210 cells, massively infiltrated lymph nodes. Of importance, ectopic expression of STAP-2 in BCR-ABL-expressing Ba/F3 cells significantly down-regulated CXCR4 but induced CCR7 expression. Lymphatic endothelial cells express CCR7 ligands, CCL19 and CCL21 [13]. In addition, CCR7 has emerged as an important marker in the prediction of axillary lymph node metastasis in breast carcinomas because over-expression of CCR7 correlates with larger primary tumors, deeper lymphatic invasion and poorer survival rates [14, 15, 16]. Thus, the changed expression of CCR7 may well explain the different pattern of cell infiltration. Indeed, the knockdown of CCR7 in Ba/F3-p210/STAP-2 cells completely abolished lymph node infiltration. Further changes included Ba/F3-p210/STA2 cells acquiring more proliferative capacity and resistance to STI571 compared with Ba/F3-p210 cells. Ectopic expression of CXCR4 did not influence Ba/F3-p210/STAP-2 cell growth. However, the
knockdown of CCR7 in Ba/F3-p210/STAP-2 cells partly nullified the enhancement of both cell growth and drug resistance caused by STAP-2 expression, indicating the involvement of CCR7. Because the inhibition of STAT5 or the AKT or MAPK/ERK pathways significantly suppressed Ba/F3-p210/STAP-2 cell growth, an indirect signal mediated by newly induced CCR7 is involved in addition to a direct signal via the enhanced BCR-ABL activities. Therefore, STAP-2 cooperates with BCR-ABL to up-regulate CCR7 expression in Ba/F3-p210/STAP-2 cells, leading to a growth advantage and massive infiltration of lymph nodes.

CCR7 expression is crucial for thymocyte trafficking across the corticomedullary junction in the thymus and for lymph node homing of naive T cells [17, 18]. CCR7 expression in CD4^+CD8^+ T cells and thymocytes requires both ERK- and Ca^{2+}-dependent signaling pathways [19]. In human breast cancer cells, the involvement of AKT-mediated Sp1 activation in prostaglandin E2-induced CCR7 expression has been reported [20]. Here, we showed that STAP-2-mediated CCR7 expression requires the MAPK/ERK signaling pathway in BaF3-p210 cells. AKT phosphorylates Sp1 at S42, T679, and S698 [20]. ERK phosphorylates T453 and T739 of Sp1 to increase its transcriptional activities. C-Jun NH2-terminal kinase 1 phosphorylates SP1 at T278 and T739, which controls its degradation [21]. In STAP-2-mediated CCR7 expression, phosphorylation of Sp1 is likely to determine CCR7 mRNA transcription. The different signals required to induce CCR7 may be dependent on different cell types and stimuli. Further detailed analysis will clarify this issue.

Ba/F3-p210/STAP-2 cells, but not Ba/F3-p210 cells, produced CCR7 ligands, CCL19 and CCL21; therefore, an autocrine loop for the CCR7-CCL19/CCL21 axis is likely to be involved in STAP-2-enhanced cell growth in Ba/F3-p210/STAP-2 cells. The promoter region of the CCL19 gene contains two putative NF-κB binding sites and a potential consensus ISRE element [22]. Thus, the CCL19 promoter is regulated by multiple transcriptional systems, such as NF-κB, IRF, and STATs. The enhancement of BCR-ABL activity by STAP-2 may potentiate CCL19
production. Alternatively, STAP-2 may modulate the BCR-ABL signaling pathway to produce CCL19, because STAP-2 can associate with and influence various signaling and/or transcriptional molecules.

STAP-2 enhanced BCR-ABL activities and cooperated with BCR-ABL to induce the chemokine receptor, CCR7, as well as its ligands, CCL19 and CCL21. The autocrine loop of the CCR7-CCL19/CCL21 axis is likely to be involved in BCR-ABL/STAP-2-mediated growth and resistance to STI571. Because STAP-2 protein is ubiquitously expressed in cells and tissues, CML cells can express both STAP-2 and BCR-ABL. STAP-2 expression, therefore, has the potential to influence splenomegaly and the release of CML cells from bone marrow, as well as to modulate sensitivity to STI571 during CML progression. It would be of interest to determine whether the chemokine receptor expression profile, which depends on STAP-2 expression, is related to splenomegaly observed in CML patients. In addition, STAP-2 and/or CCR7 may represent suitable molecular targets for the treatment of CML.
Conflict of interest

The authors have no conflicting financial interests.

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References


[12] A. Mantovani, B. Savino, M. Locati, et al., The chemokine system in cancer biology and


Figure legends

**Figure 1. STAP-2 cooperates with BCR-ABL to modify the expression of chemokine receptors**

(A) Ba/F3-pcDNA, Ba/F3-STAP-2, Ba/F3-p210 and Ba/F3-p210/STAP-2 cells were lysed, and immunoblotted with anti-ABL, anti-Myc and anti-Actin antibody. (B) Total RNA samples isolated from these cells were subjected to quantitative real-time PCR analyses of CCR7 and CXCR4 as well as the control Actin mRNA transcripts were carried out. (C) These cells were stained with PE-anti-CCR7 antibody and determined the surface expression of CCR7 by FACS analyses.

**Figure 2. CXCR4 has no significant effect on BCR-ABL-dependent cell growth in Ba/F3 cells**

(A) Ba/F3-p210, Ba/F3-p210/STAP-2 and Ba/F3-p210/STAP-2/hCXCR4 (#1 and #2) cells (1x10^6 cells/well) in 12-well plates were cultured without IL-3 for 24 hrs. The cells were lysed, and immunoblotted with anti-ABL, anti-Myc, and anti-Actin antibody. (B) Total RNA samples isolated from these cells were subjected to RT-PCR analyses using CXCR4 and G3PDH primers. (C) These cells were stained with APC-anti-human CXCR4 antibody and determined the surface expression of human CXCR4 by FACS analyses. (D) Ba/F3-p210, Ba/F3-p210/STAP-2 and Ba/F3-p210/STAP-2/hCXCR4 (#1 and #2) cells (3x10^3 cells/well) in 96-well plates were cultured without IL-3 for the indicated periods. The cell numbers were measured using a Cell Counting Kit-8. These cells (2x10^4 cells/well) in 96-well plates were also cultured without IL-3 in the presence of STI571 (0, 0.3, 1, 3 µM) for 24 hrs. The cell numbers were measured using a Cell Counting Kit-8. Shown is a representative experiment, which was repeated at least three times with similar results, and the error bars represent the SD. *p<0.05, **p<0.01, ***p<0.001. (E) These cells (1x10^6 cells/well) in 12-well plates were cultured without IL-3 for 24 hrs. The cells were lysed, and immunoblotted with anti-pERK, anti-ERK, anti-pAkt, anti-Akt, anti-pStat5, anti-Stat5, anti-Bcl-xL, and anti-Actin antibody.
Figure 3. CCR7 influences the BCR-ABL-dependent cell growth in Ba/F3 cells

(A) Ba/F3-p210, Ba/F3-p210/STAP-2/shControl and Ba/F3-p210/STAP-2/shCCR7 (#1 and #2) cells (1x10^6 cells/well) in 12-well plates were cultured without IL-3 for 24 hrs. The cells were lysed, and immunoblotted with anti-ABL, anti-Myc, and anti-Actin antibody. (B) Total RNA samples isolated from these cells were subjected to quantitative real-time PCR analyses of CCR7 as well as the control G3PDH mRNA transcripts were carried out. *p<0.05, **p<0.01. (C) These cells were stained with PE-anti-CCR7 antibody and determined the surface expression of CCR7 by FACS analyses. (D) Ba/F3-p210, Ba/F3-p210/STAP-2/shControl and Ba/F3-p210/STAP-2/shCCR7 (#1 and #2) cells (3x10^3 cells/well) in 96-well plates were cultured without IL-3 for the indicated periods. The cell numbers were measured using a Cell Counting Kit-8. These cells (2x10^4 cells/well) in 96-well plates were also cultured without IL-3 in the presence of STI571 (0, 0.3, 1, 3 μM) for 24 hrs. The cell numbers were measured using a Cell Counting Kit-8. Shown is a representative experiment, which was repeated at least three times with similar results, and the error bars represent the SD. *p<0.05, **p<0.01, ***p<0.001. (E) These cells (1x10^6 cells/well) in 12-well plates were cultured without IL-3 for 24 hrs. The cells were lysed, and immunoblotted with anti-pERK, anti-ERK, anti-pAkt, anti-Akt, anti-pStat5, anti-Stat5, anti-Bcl-xL, and anti-Actin antibody. (F) Ba/F3-p210/STAP-2/shControl and Ba/F3-p210/STAP-2/shCCR7#1 cells were subcutaneously injected into nude mice (n=4). Four weeks postinoculation, tumor, and lymph node were weighed and graphed. *p<0.05. (G) Total RNA samples isolated from Ba/F3-p210 and Ba/F3-p210/STAP-2(#1 and#2) cells were subjected to RT-PCR analyses using CCL19, CCL21 and G3PDH primers. (H) Ba/F3-p210 and Ba/F3-p210/STAP-2 (#1 and #2) cells (3x10^3 cells/well) in 96-well plates were cultured without IL-3 in the presence of CCL19 (0, 1, 10 ng/ml) for 24 hrs. The cell numbers were measured using a Cell Counting Kit-8. Shown is a representative experiment, which was repeated at least three times with similar results, and the error bars represent the SD.
Figure 4. The MAPK/ERK pathway is involved in the CCR7 expression in BCR-ABL/STAP-2-expressing Ba/F3 cells

(A) Ba/F3-p210/STAP-2 cells were nucleofected with control siRNA or STAT5a/b siRNAs. After 24 hrs, cells were lysed, and immunoblotted with anti-pStat5, and anti-Stat5 antibody. Total RNA samples isolated from these cells were subjected to quantitative real-time PCR analyses of CCR7 as well as the control G3PDH mRNA transcripts were carried out. These cells (3x10^3 cells/well) in 96-well plates were cultured without IL-3 for the indicated periods. The cell numbers were measured using a Cell Counting Kit-8. Shown is a representative experiment, which was repeated at least three times with similar results, and the error bars represent the SD. *p<0.05, **p<0.01.

(B)(C) Ba/F3-p210/STAP-2 cells (1x10^6 cells/well) in 12-well plates were cultured without IL-3 in the presence of DMSO, A6730 (10, 50 μM) (B) or U0126 (1, 5μM) (C). After 24 hrs, cells were lysed, and immunoblotted with anti-pAkt, anti-Akt, and anti-Actin antibody. Total RNA samples isolated from these cells were subjected to quantitative real-time PCR analyses of CCR7 as well as the control G3PDH mRNA transcripts were carried out. Ba/F3-p210/STAP-2 cells (3x10^3 cells/well) in 96-well plates were cultured without IL-3 in the presence of DMSO or A6730 (10 μM) for the indicated periods. The cell numbers were measured using a Cell Counting Kit-8. Shown is a representative experiment, which was repeated at least three times with similar results, and the error bars represent the SD. *p<0.05, **p<0.01.
Figure 1

A: Western blot analysis showing the expression levels of CCR7, Actin, p210, and STAP-2 in different cell lines. The bands for ABL, Myc, and Actin are indicated.

B: Graph showing the relative expression levels of Ccr7/Actin in pcDNA3, STAP-2, p210, and p210/STAP-2.

C: Flow cytometry analysis for CCR7 expression in pcDNA3, STAP-2, p210, and p210/STAP-2 cell lines.
Figure 2

**A**

- TCL
- IB: ABL 180
- IB: Myc 75
- IB: Actin 48

**B**

- CXCR4
- G3PDH

**C**

- Cell growth (OD\textsubscript{450})

**D**

- Cell survival (%)
  - STI571 (µM)

**E**

- IB: pErk
- IB: Erk
- IB: pAkt
- IB: Akt
- IB: pStat5
- IB: Stat5
- IB: Bcl-xL
- IB: Actin
**Figure 3**

A. Western blot analysis showing the expression of various proteins such as ABL, Myc, and Actin.

B. Graph showing the expression of Ccr7/G3pdh.

C. Flow cytometry analysis comparing cell growth for different treatments.

D. Graph showing cell survival percentage over days treated with STI571.

E. Western blot analysis for p210, pErk, pAkt, and pStat5.

F. Graph showing tumor and lymph node mass over days treated with STI571.

G. Western blot analysis for Ccl19, Ccl21, and G3pdh.

H. Graph showing cell growth over different concentrations of CCL19.
Figure 4

A. siRNA
- siRNA: A6730
- siControl: TCL
- siStat5a/b

B. A6730
- 0, 10, 50 (µM)
- 0, 10, 50 (µM)
- 0, 10, 50 (µM)

C. U0126
- 0, 1, 5 (µM)
- 0, 1, 5 (µM)
- 0, 1, 5 (µM)

Cell growth (OD₄₅₀)

Days