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REGULATION OF EXPRESSION OF STROMAL-DERIVED FACTOR-1 (SDF-1) RECEPTORS: CXCR4 AND CXCR7 IN HUMAN RHADOMYOSARCOMAS

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Abstract

Rhabdomyosarcomas (RMS) express CXCR4 and CXCR7 receptors that bind prometastatic α -chemokine stromal derived factor-1. In this report we analyzed the activity of both promoters in a model of less metastatic human embryonal-RMS cell line (RD) and more metastatic alveolar-like RMS (RD cells transduced with Paired box gene 3/forkhead homolog - PAX3-FKHR fusion gene). First, CXCR4 is barely detectable on RD and becomes upregulated on RD/PAX3-FKHR cells. In contrast, CXCR7 highly expressed on RD becomes downregulated in RD/PAX3-FKHR cells. Next, promoter deletion and mutation studies revealed that while: i) expression of CXCR4 in RD and RD/PAX3-FKHR cells required nuclear respiratory factor-1 (NRF-1) binding site and ii) was additionally upregulated by direct interaction of NRF-1 with PAX3-FKHR, CXCR7 promoter activity required a proximal nuclear factor-kappa B (NF- κ B) binding motif. The requirement of these factors for CXCR4 and CXCR7 promoter activities was additionally supported after blocking NRF-1 and NF- κ B. Furthermore, CXCR4 expression in PAX3-FKHR⁺ RMS cells seems to be enhanced because of the interaction of PAX3-FKHR and NRF-1 proteins in the proximal part of the promoter that prevents access of the negative regulator of transcription YY1 to its binding site. Finally, while hypoxia enhances CXCR4 and CXCR7 promoter activity and receptor expression on RD cells, it inhibits CXCR7 expression in RD/PAX3-FKHR cells. In conclusion, SDF-1 binding receptors CXCR4 and CXCR7 are differently regulated in RMS cells. The upregulation of CXCR4 and downregulation of CXCR7 expression by PAX3-FKHR or hypoxia may give SDF-1 an advantage to better engage the CXCR4 receptor, thus increasing RMS motility.

Keywords

Rhabdomyosarcoma; SDF-1; CXCR4; CXCR7; PAX3-FKHR

Introduction

Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma of adolescence and childhood and accounts for 5% of all malignant tumors in patients under 15 years of age (1–

10). There are two major histological subtypes of RMS, alveolar (A)RMS and embryonal (E) RMS. Clinical evidence indicates that ARMS is more aggressive and has a significantly worse outcome than ERMS (11,12). Genetic characterization of RMS has identified markers that show excellent correlation with histological subtype. Specifically, ARMS is characterized by the translocation t(2;13)(q35;q14) in 70% of cases or the variant t(1;13)(p36;q14) in a smaller percentage of cases. These translocations disrupt the paired box gene (PAX)3 and PAX7 genes on chromosomes 2 and 1, respectively, and the forkhead homolog in RMS (FKHR) gene on chromosome 13 and generate PAX3-FKHR and PAX7-FKHR fusion genes. These fusion genes encode the fusion proteins PAX3-FKHR and PAX7-FKHR, which increase transcription factor activity of PAX3/7 proteins and enhance metastatic potential of ARMS cells (13).

Chemokines, the small pro-inflammatory chemoattractant cytokines that bind to specific G-protein-coupled, seven-transmembrane receptors present on the plasma membranes of target cells, are the major regulators of trafficking and adhesion of normal and malignant cells (14, 15). More than 50 different chemokines have been cloned so far and some of them were reported to play a pivotal role in cancer metastasis. One of the most important is α -chemokine stromal derived factor-1 (SDF-1), which regulates metastatic behavior of several types of cancer (16–19). SDF-1 binds to G-protein coupled, seven-transmembrane span receptor CXCR4. In our previous work, we demonstrated a pivotal role of the SDF-1 – CXCR4 axis in metastasis of human RMS to various organs including bone marrow (BM) (17).

For many years, it was postulated that CXCR4 was the only receptor for SDF-1. However, the concept of an exclusive interaction of SDF-1 with CXCR4 was questioned recently after a new SDF-1 binding receptor, CXCR7, was identified (20). CXCR7, in contrast to CXCR4, has another ligand, a chemokine called interferon-inducible T-cell chemoattractant (I-TAC) (20). Recently, we noticed that the SDF-1-CXCR7 axis regulates the metastatic potential of human RMS cells similarly to SDF-1-CXCR4 (21). We observed that while SDF-1 signaling through CXCR4 enhances more significant RMS cell motility, CXCR7 is somehow more involved in adhesion of RMS cells. Both these receptors, however, are potential targets for new anti-metastatic strategies (22–24).

To learn more about the SDF-1-CXCR4 and SDF-1-CXCR7 axes in RMS metastasis, we analyzed activity of both promoters in a model of less metastatic embryonal RD cells and more metastatic (alveolar-like) RD cells transduced with PAX3-FKHR fusion gene (RD/PAX3-FKHR). We found by employing promoter deletion and mutation studies that CXCR4 promoter activity depends on a proximal nuclear respiratory factor-1 (NRF-1) binding site and is enhanced after direct interaction of NRF-1 with PAX3-FKHR. In addition, promoter deletion/mutation and chromatin immunoprecipitation (ChIP) assay studies revealed that NRF-1-PAX3-FKHR interaction in proximal part of promoter may prevent access of YY1, that is a negative regulator of transcription to its binding motif located between NRF-1 and proximal PAX3-FKHR binding site. In contrast, CXCR7 promoter activity depends on a proximal nuclear factor-kappa B (NF- κ B) binding site. The requirement of NRF-1 and NF- κ B for CXCR4 and CXCR7 promoter activity, respectively, was additionally supported after blocking expression of these transcription factors by employing NRF-1 shRNA or BAY 11–7082 (small molecular NF- κ B inhibitor). We also noticed that CXCR4 and CXCR7 are differently regulated in human RMS cells in response to hypoxia. We postulate that the hypoxia and PAX3/FKHR-mediated upregulation of CXCR4 and downregulation of CXCR7 may provide an advantage for SDF-1 to better engage the CXCR4 receptor. As a result of this, improved SDF-1 interaction with the CXCR4 receptor increases RMS motility and enhances the metastatic potential of RMS cells.

Material and Methods

Cell Lines and Normoxia culture conditions

Three RMS cell lines were used in the experiments: RH30 (gift of Dr. Peter Houghton, St. Jude Children's Research Hospital, Memphis, TN); RD; and RD transfected cell line. All cell lines were maintained in a humidified atmosphere at 5% CO₂, 37°C at an initial cell density of 2.5×10^4 cells/flask (Corning, Cambridge, MA) and the media were changed every 48 hours. The RMS cell line (RD) was transfected with empty vector or PAX3-Forkhead box O1 (FOXO1) (RD/PAX3-FKHR) (25). Cell cultures were grown in cultured RPMI 1640 (Sigma, St Louis, MO) supplemented with 100 IU/ml penicillin, 10 µg/ml streptomycin, and 50 µg/ml neomycin (Life Technologies, Inc., Grand Island, NY) in the presence of 10% heat-inactivated FBS (Life Technologies) and 300µg/mL G-418.

Cell Surface Expression of CXCR4 and CXCR7

Cell surface expression was measured by flow cytometry. Cells were stained for surface CXCR4 with APC-conjugated antibody (Ab) anti-CXCR4 (BD Biosciences, San Jose, CA; clone #12G5) and for surface CXCR7 with phycoerytherin (PE)-conjugated Ab anti-CXCR7 (R&D Systems, Minneapolis, MN; clone #11G8). Isotype-matched APC and PE-conjugated immunoglobulin (Ig) served as controls (BD Biosciences). All analyses were performed on an LSR cell cytometer (BD Biosciences). Samples were evaluated in triplicate and the data were averaged for statistical analysis.

Cloning of the CXCR4 and CXCR7 promoters

The promoter region of the CXCR4 gene from -2,237 to +62 and the CXCR7 promoter region from -2409 to +89 relative to the transcription start site were amplified. The confirmed sequence was then inserted into a pGL4.10 vector (Promega, Madison, WI). The pGL4.10 vector was digested by SacI and XhoI (or KpnI/EcoRV for CXCR7) (all restriction enzymes New England Biolabs, Ipswich, MA) and the amplified CXCR4 and CXCR7 promoter fragments were inserted through ligation. The cloned pGL4.10-CXCR4/7 constructs were confirmed by sequencing. The sequentially shorter CXCR4/7 promoter fragments were amplified by standard polymerase chain reaction (PCR) methods, sequenced, and cloned to pGL4.10 vector. In both CXCR4 and CXCR7 promoters, three mutated constructs were prepared, i.e., CXCR4 NRF-1mut, CXCR4 hypoxia responsive element (HREmut), and CXCR4 YY1mut as well as CXCR7 NF-κBmut, CXCR7 HREmut, and CXCR7 YY1mut with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Transient transfection assay

RD and RD/PAX3-FKHR cells were transfected with 1.8µg of CXCR4/CXCR7 constructs and pGL4.72 vector (ratio 50:1) using Lipofectamine (Invitrogen, Carlsbad, CA) in 12-well plates according to the manufacturer's protocol. At 24 hours after transfection, cells were lysed and 10µl of each sample was analyzed for firefly/*Renilla* luciferase activity with the dual luciferase assay system (Promega) and measured on a luminometer (Turner Biosystems, Sunnyvale, CA) with provided software. The firefly/*Renilla* luciferase activity ratios were calculated by the software and used to evaluate the changes in promoter activity in hypoxia and normoxia. Fold difference was based on empty pGL4.10 vector activity.

Immunoprecipitation and Western blot

The 5×10^6 cells were lysed in 1 mL of lysis buffer (50mM Tris-HCL pH 8.0, 150mM NaCl, 1% triton $\times$ 100, and protease inhibitors) for 10 minutes on ice, syringed 8 times with a 21 gauge syringe, and spun down for 15 minutes at 10,000g 4°C. Supernatants were transferred to new tubes and pre-cleared with 100µl Preclearing Matrix (Santa Cruz Biotechnology, Santa

Cruz, CA) overnight at 4°C rotating. Samples were spun down for 1 minute at full speed and supernatants were incubated with 0.2µg/mL of a goat anti-human PAX3/7 polyclonal Ab clone N-19 (Santa Cruz Biotechnology) and IP Matrix F (Santa Cruz Biotechnology) overnight at 4°C rotating. Samples were washed 5 times with lysis buffer and employed for western blot analysis using a polyclonal rabbit anti-human NRF-1 Ab clone H-285 (Santa Cruz Biotechnology).

ChIP assay

Either 1×10^6 RD or RD/PAX3-FKHR cells were fixed with 1% formaldehyde (10 minutes at 37°C), quenched with glycine (12mM, 5 minutes at room temperature), lysed, and extracts were employed for ChIP analysis according to the manufacture's protocol (Upstate Biotechnology, Lake Placid, NY). Extracts were sonicated (5 times with 10 second pulses with 1 minute breaks) on ice with a 60 Sonic Dismembrator (Fisher Scientific, Pittsburg, PA). For the CXCR4 promoter, 3µg of a PAX3/7 Ab clone N-19 or NRF-1 Ab clone H-285 or YY1 clone H-414 (Santa Cruz Biotechnology) and 20µl protein G magnetic beads were used to immunoprecipitate protein/DNA complexes. For the CXCR7 promoter, soluble chromatin was incubated with 3µg of NF-κB P50 Ab. Negative controls were incubated with rabbit IgG (Santa Cruz Biotechnology). After incubation, complexes were extensively washed and separated on a magnetic separator. Immunoprecipitates were incubated with proteinase K (62°C for 2 hours and 95°C for 10 minutes) and DNA was cleaned up with spin columns and resuspended in Tris-EDTA buffer. Subsequently, a series of PCR reactions was carried out. Sequences of primers employed are shown in Supplementary Table 1.

Real-time quantitative reverse transcription PCR (RQ-PCR)

Total RNA was isolated from cells treated with hypoxia and controls with RNeasy Kit (Qiagen, Valencia, CA). The RNA was reverse-transcribed with MultiScribe reverse transcriptase and oligo dt primers (Applied Biosystems, Foster City, CA). Quantitative assessment of mRNA levels was performed by RQ-PCR on an ABI 7500 instrument and Power SyBR Green PCR Master Mix reagent. Real-time conditions were as follows: 95°C (15 sec), 40 cycles – 95°C (15 sec), 60°C (1 min). According to a melting point analysis, only one PCR product was amplified under these conditions. The relative quantization value of a target, normalized to the endogenous control β-2 microglobulin gene and relative to a calibrator, is expressed as $2^{-\Delta\Delta Ct}$ (-fold difference), where $\Delta Ct = (Ct \text{ of target genes}) - (Ct \text{ of endogenous control gene, } \beta\text{-2 microglobulin})$, and $\Delta\Delta Ct = (\Delta Ct \text{ of samples for target gene}) - (\Delta Ct \text{ of calibrator for the target gene})$. The following primer pairs were used:

CXCR4 forward 5- GGTTCCTTCATGGAGTCATAGTC -3,

CXCR4 reverse 5- CGGTTACCATGGAGGGGATC -3;

CXCR7 forward 5- GGCTATGACACGCACTGCTACA -3,

CXCR7 reverse 5- TGGTTGTGCTGCACGAGACT -3.

YY1 forward 5- CCCACGGTCCCAGAGTCCA -3,

YY1 reverse 5-GTGTGCGCAAATTGAAGTCCAGT -3

Knockdown of NRF-1 with short hairpin (sh)RNA and inhibition of NF-κb with BAY 11–7082

In RNAi experiments, shRNA-generating plasmid pSuper (Seattle, WA) was used. The oligonucleotide targeting base sequence for human NRF-1 was 5'-CATATGGCTACCATAGAAG-3'. Rhabdomyosarcoma cells were plated at 80% confluency and transfected with shRNA vector using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Commercially available, negative-scrambled control plasmid was

used (Dharmacon, Lafayette, CO). NF- κ b inhibitor BAY 11–7082 was purchased from Sigma (St. Louis, MO). Cells were treated for 16 hours with 1 μ M of inhibitor and then receptor expression was analyzed as described before. In the hypoxia assay, cells were pretreated for 30 minutes with the inhibitor and then subjected to hypoxia.

Hypoxia assay

A hypoxic condition was acquired using a nitrogen-balanced hypoxia chamber providing a gas mixture containing 5% CO₂ and 1% O₂ at 37°C. In the assay, three RMS cell lines were used (RD, RD/PAX3-FKHR and RH30). Cells were treated overnight or for different periods of time, and then subjected to fluorescence-activated cell sorting (FACS) analysis or dual luciferase assay.

Transmembrane chemotaxis

The 8 μ m pore polycarbonate membranes were covered with 50 μ L of 0.5% gelatine. Cells were detached with 0.5mmol/L EDTA, washed, resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium with 0.5% bovine serum albumin (BSA), and seeded at a density of 3 $\times$ 10⁴ in 120 μ L into the upper chambers of Transwell inserts (Corning Costar, Corning, Lowell, MA). The lower chambers were filled with medium alone or medium containing SDF-1 (300ng/mL) or I-TAC (100ng/mL) or conditioned medium or 0.5% BSA RPMI 1640 (control). Plates were put into normoxic and hypoxic conditions. After 24 hours, the inserts were removed from the Transwells. Cells remaining in the upper chambers were scraped off with cotton wool. Cells that had transmigrated were stained by hydroxyethyl methacrylate (HEMA) 3 (Protocol, Fisher Scientific) and counted either on the lower side of the membranes.

Statistical Analysis

All results are presented as mean $\pm$ standard error (SEM). Statistical analysis of the data was performed using the non-parametric Mann-Whitney test and the student's t-test for unpaired samples with $p < 0.05$ considered significant.

Results

CXCR4 and CXCR7 are differently expressed on RD and RD/PAX3-FKHR cells

As previously reported, human RMS cells express both SDF-1 binding receptors (17,26). Figure 1A shows the cell surface expression of CXCR4 and CXCR7 at the protein level on human RD and RD/PAX3-FKHR cell lines employed in the current study. RD cells that belong to the ERMS subtype highly express CXCR7 and very little CXCR4. In contrast, RD cells stably transduced with PAX3-FKHR construct (ARMS-like subtype) highly express CXCR4 and downregulate expression of CXCR7. This reciprocal expression of CXCR4 and CXCR7 was subsequently confirmed by RQ-PCR (Fig. 1B). This pattern of expression of CXCR4 and CXCR7 receptors is consistent with our data obtained on nine other human ERMS and ARMS cell lines (21).

To learn more about the molecular mechanisms governing expression of both receptors, we cloned CXCR4 and CXCR7 promoters and analyzed activity of promoter fragments subcloned in a luciferase reporter gene (pGL4.10 vector) in both RD and RD/PAX3-FKHR cell lines.

Cloning the CXCR4 promoter and generating CXCR4 promoter fragments

The 2299 base pair (bp)-long sequence of the CXCR4 promoter from –2,237 to +62 was generated from genomic DNA by PCR (Supplementary Fig. 1). The cloned promoter region was sequenced and scanned for possible PAX3 binding sites. We identified 10 putative PAX3 binding sites located at –558–572, –596–609, –638–658, –709–721, –1480–1510, –1593–

1597, -1753-1757, -1839-1848, -2097-2101, and -2199-2212 of the cloned CXCR4 promoter sequence (Fig. 2A). We also noticed that the CXCR4 promoter contains 4 HREs (-852-856, -1038-1042, -1282-1286, and -2019-2023), one NF- κ B (-213-223), and one NRF-1 binding site -37-54bps from transcription start (Fig. 2A and Supplementary Fig. 1) in addition to putative PAX3 binding sites. Furthermore, we identified at -303-307 a binding site for negative transcription regulatory factor YY1. Based on this, 9 luciferase reporter gene constructs were generated containing smaller CXCR4 promoter fragments subcloned into a pGL4.10 vector (Fig. 2A). We also generated three promoter constructs where NRF-1, one HRE, and one YY1 binding site were mutated (Fig. 2A). The HRE mutation construct in Figure 2A (lower part) was also subsequently employed in hypoxia and CXCR4 expression studies.

PAX3-FKHR regulation of the CXCR4 promoter

Next, RD and RD/PAX3-FKHR cells were transfected by a luciferase reporter gene that was driven by different CXCR4 promoter fragments. Overall, we found a higher luciferase level in RD cells that were transduced with PAX3-FKHR (Fig. 2B). We found that CXCR4 Frag 7, which contains a NRF-1 binding site but not PAX3 domains, is still able to drive the luciferase gene in both RD and even at a much higher level in RD/PAX3-FKHR cells. This could be explained by the 124 bp fragment of the CXCR4 promoter possibly containing some cryptic PAX3 binding site that is responsible for higher activity of the CXCR4 promoter in the latter cells in the presence of PAX3-FKHR fusion protein. Subsequently, deletion of NRF-1 completely abrogated promoter activity as seen in cells transduced with the shortest CXCR4 Frag 8. To confirm that NRF-1 is crucial for expression of the CXCR4 promoter, RD and RD-PAX3/FKHR cells were transduced with a whole promoter fragment in which a NRF-1 binding site was mutated (NRF-1mut; Fig. 2A). As shown in Fig. 2B, after NRF-1 mutation, CXCR4 promoter activity was abolished in RD and severely reduced in RD-PAX3-FKHR cells. Thus, our data confirm a crucial role of intact NRF-1 in CXCR4 promoter activity (27,28). They also indicate that PAX3-FKHR, which is a strong transcription factor, may additionally increase CXCR4 promoter activity in RMS cells. Moreover, PAX3-FKHR seems to maintain some level of CXCR4 promoter activity even when NRF-1 was mutated (Fig. 2B).

PAX3-FKHR protein interacts with NRF-1

Next, to confirm that a PAX3-FKHR protein can bind directly to the CXCR4 promoter, we performed ChIP analysis using anti-PAX3/7 and anti-NRF Abs. By employing sets of primers (P3, P2, and P1) designed to amplify different promoter regions that contain PAX3 binding sites and primers (N) designed to amplify the NRF-1 binding region (Fig. 2A and Supplementary Table I), we detected the presence of PAX3 and NRF-1-protected regions in the CXCR4 promoter (Fig. 2C). We found that PAX3/7 protein binds to the CXCR4 promoter region flanked by P1 primers in both RD and RD/PAX3-FKHR cells and to the region flanked by P2 primers in RD/PAX3-FKHR cells. Protection of the PAX3 binding site flanked by P2 primers in RD/PAX3-FKHR cells, but not RD cells, is likely explained as being a result of enhanced transcriptional activity of PAX3-FKHR fusion protein.

At the same time, we confirmed NRF-1 protein binding to a proximal fragment of CXCR4 promoter (Fig. 2C). To our surprise, however, NRF-1 protein protected the proximal PAX3 binding region in RD/PAX3-FKHR cells that was flanked by P1 primers and, at the same time, PAX3-FKHR protein protected the NRF-1 binding site flanked by N primers. This suggests a direct interaction of the PAX3-FKHR fusion protein with the NRF-1 transcription factor. This direct interaction between PAX3-FKHR and NRF-1 in RD/PAX3-FKHR was confirmed by Western blot analysis (Fig. 3A and B), where the PAX3-FKHR-NRF-1 complex was immunoprecipitated with anti-PAX3/7 Ab and subsequently probed on the gel with Ab against NRF-1 (Fig. 3C) as well as when reverse immunoprecipitation was performed by employing anti-NRF-1 Abs (Fig. 3D).

Cloning the CXCR7 promoter and generating CXCR7 promoter fragments

CXCR7 promoter was cloned by employing DNA-specific primers. We found that CXCR7 promoter contains three potential HRE- (-100-104, -965-969, -1306-1310), five NF- κ B- (-32-42, -308-318, -1019-1029, -1375-1379, -2145-2155), and four NRF binding sites (-1030-1040, -1468-1478, -1980-1990, -2085-2095) located within 2.5 kilobases upstream of the transcriptional start site (Fig. 4A and Supplementary Fig. 2). Furthermore, we identified at -702-706 a binding site for negative transcription regulatory factor YY1. Subsequently, we generated 8 constructs containing smaller CXCR7 promoter fragments and two constructs containing mutated proximal NF- κ B and HRE as well as YY1 binding sites that were subcloned into a pGL4.10 vector (Fig. 4A). The proximal HRE mutation construct shown in Figure 4A (lower part) was also subsequently employed in hypoxia and CXCR7 expression studies.

NF- κ B is required for CXCR7 promoter activity

In the next step, RD and RD/PAX3-FKHR cells were transfected by luciferase reporter gene constructs driven by those different CXCR7 promoter fragments (Fig. 4B). We noticed that the PAX3-FKHR protein somehow diminished CXCR7 promoter activity as seen for Frag 1, Frag 2, and Frag 5. The highest promoter activity was observed for a 528 bp fragment (Frag 5) that contains two NF- κ B (N1 and N2) and proximal HRE binding sites (Fig. 4B). We noticed that while removal of distal NF- κ B (N2) and HRE binding sites in this construct resulted in a significant decrease of promoter activity, removal of the proximal NF- κ B (N1) binding site completely abolished its activity. Similarly, we observed almost complete inhibition of promoter activity when the proximal NF- κ B binding site was mutated in the longest CXCR7 promoter fragment (NF- κ Bmut; Fig. 4B). Subsequently, we performed ChIP analysis using anti-NF- κ B Ab confirmed so that NF- κ B binds to three proximal NF- κ B binding sites flanked by N1, N2, and N3 primers (Fig. 4C).

Furthermore, analysis of promoter activity of Frag 3 and Frag 4 suggested the presence of a putative negative regulatory site(s) located between NF- κ B (N2) and the distal HRE binding site (Fig. 4A and B). Because this part of the promoter contains a YY1 binding site, we hypothesize that this negative regulator of transcription may affect CXCR7 expression. Our RQ-PCR analysis revealed that in normoxic conditions, the YY1 mRNA level is ~4 times higher in PAX3-FKHR-expressing cells (Fig. 5A, black bars). Consistent with this observation, when the YY1 binding site was mutated in the CXCR4 promoter, we observed upregulation of promoter activity in RD but not RD/PAX3-FKHR cells (Fig. 5B). This further supports our data shown in Figure 2C that the NRF-1-PAX3-FKHR interaction prevents access of YY1 to the CXCR4 promoter. In contrast, mutation of the YY1 binding site in the CXCR7 promoter produced higher promoter activity in RD/PAX3-FKHR cells and at the same time did not affect CXCR7 promoter activity in the RD cell line (Fig. 5C). This could be explained by RD/PAX3-FKHR expressing YY1 at much higher level and that YY1 is negative regulator of CXCR7 expression in these cells. Thus, our data support negative involvement of YY1 in CXCR4 and CXCR7 promoter activities in RD and RD/PAX3-FKHR cells, respectively.

Interestingly, we noticed that YY1 expression at the mRNA level, which is higher in normoxic conditions in RD/PAX3-FKHR cells (Fig. 5A and Supplementary Fig. 4), becomes upregulated in hypoxic conditions in RD cells and downregulated in RD/PAX3-FKHR cells (Fig. 5A, white bars).

Expression of CXCR4 and CXCR7 receptors is differently regulated in RD and RD/PAX3-FKHR cells in response to hypoxia

It has been reported that hypoxia upregulates CXCR4 expression in several cell types (29-31). To address this issue better for RMS cells and to determine whether CXCR7 expression is also modulated by oxygen level, we evaluated expression of both receptors in RD and RD/

PAX3-FKHR cell lines in normoxic and hypoxic conditions (Fig. 6A, right and left). In these experiments, RMS cells were exposed to hypoxia for 0–24 hours and expression of both receptors was evaluated by FACS and shown as mean fluorescence intensity. Figure 6A and Figure 1A show that RD cells in normoxia express very low levels of CXCR4 and highly express CXCR7, but if transferred to hypoxic conditions will highly upregulate CXCR4 and downregulate CXCR7 (Fig. 6A, right and left). In contrast, RD/PAX3-FKHR cells that in normoxia express high level of CXCR4 and low level of CXCR7 upregulate expression of both receptors (Fig. 1 and Fig. 6A, right and left). Overall, these protein expression data correlated with changes at the mRNA level (data not shown). Because our data (Fig. 5B and C) indicated involvement of YY1 in CXCR4 and CXCR7 promoter activity, we analyzed potential binding of YY1 to both promoters by employing the ChIP assay (Fig. 6B). As expected, we noticed that in normoxic conditions in RD cells that YY1 binds to CXCR4 but not the CXCR7 promoter. In contrast, in RD/PAX3-FKHR cells, YY1 binds to CXCR7 but not the CXCR4 promoter. In hypoxic conditions, however, YY1 binds to the CXCR7 promoter in RD cells but does not interact with either promoter in RD/PAX3-FKHR cells (Fig. 6B). These differences in YY1 binding to the CXCR7 promoter in normoxic versus hypoxic conditions may explain downregulation of CXCR7 expression on RD cells during hypoxia.

Next, because NRF-1 and NF- κ B are required in RMS cells for CXCR4 and CXCR7 activity, respectively, we perturbed expression of both transcription factors by employing NRF-1 shRNA or BAY 11–7082 (small molecular NF- κ B inhibitor) in RD and RD/PAX3-FKHR cells (Table 1 and Table 2). In addition, we performed similar studies in the human PAX-3-FKHR⁺ ARMS cell line RH30. As expected, perturbation of NRF-1 expression by shRNA (~80% of basic level) led to inhibition of CXCR4 in RD/PAX3-FKHR cells that highly express CXCR4 (Fig. 1 and Table 1). CXCR4 was also downregulated in PAX3-FKHR⁺ RH30 cells. In addition, as expected, perturbation of NRF-1 expression during hypoxia impaired upregulation of CXCR4 in RMS cells (Table 1).

In the next set of experiments, we found that inhibition of NF- κ B activity downregulated CXCR7 but not CXCR4 expression both in normoxic and hypoxic conditions in RD and RD/PAX3-FKHR cells (Table 2). Of note, because PAX3-FKHR⁺ RH30 cells express CXCR7 at a very low level, no significant changes in CXCR7 expression in this cell line were observed.

Subsequently, to better assess a role of HRE binding sites in expression of both receptors in hypoxic conditions, we performed analysis of promoter activities in hypoxia using different CXCR4 and CXCR7 fragments (Fig. 6C and D) where HRE fragments (indicated in Fig. 2A and Fig. 4A) were deleted or mutated. While analyzing CXCR4 promoter fragment activity, we noticed that upregulation of CXCR4 promoter activity was abolished in response to hypoxia in both RD and RD/PAX3-FKHR cells (Fig. 6C) when three proximal HRE binding sites were deleted (Fig. 2A - Frag 4). We observed a similar response when one distal HRE motif from these three putative binding sites was deleted (Fig. 2A - Frag 3.1) or mutated (Fig. 2A - HREmut) as shown in Fig. 6C. This demonstrates that the HRE binding at the –1282–1286 site is crucial for CXCR4 upregulation under hypoxic conditions.

Similar experiments were performed to address the role of putative HRE fragments (Fig. 4A) in CXCR7 expression (Fig. 6D). We noticed that deletion of both distal HRE binding sites did not affect promoter activity under hypoxia. However, deletion of the proximal HRE binding site (Fig. 4A - Frag 6) or its mutation (Fig. 4A - HREmut) prevented upregulation of CXCR7 promoter activity under hypoxic conditions (Fig. 6D).

Next, the changes in CXCR4 and CXCR7 expression under hypoxic conditions were confirmed by functional chemotactic assays in response to SDF-1 and I-TAC (Fig. 7). It is known that while CXCR4 binds SDF-1 only, CXCR7 is activated by both SDF-1 and I-TAC (20). As

predicted from promoter activity/expression studies during hypoxia (Fig. 6), RD cells that upregulate CXCR4 during hypoxia and downregulate CXCR7 (Fig. 6A, right and left) slightly enhance migration to SDF-1 and decrease migration to I-TAC (Fig. 7A). On the other hand, RD/PAX3-FKHR cells that upregulate expression of both receptors under hypoxia (Fig. 6A, right and left), enhance chemotaxis to both ligands (Fig. 7B). Finally, our chemotaxis results were confirmed in functional chemotactic assays where we perturbed expression of CXCR4 and CXCR7 in RMS cells by employing shRNA (NRF-1) or BAY 11-7082 (NF- κ B), respectively (Supplementary Fig. 3).

Discussion

RMS is the most common soft-tissue sarcoma of adolescence and childhood and clinical evidence suggests that ARMS is more aggressive and metastatic than ERMS (11,12). Because of the highly metastatic ARMS phenotype, which is responsible for poor clinical prognosis, there is an urgent need to better identify the mechanisms that control the metastatic behavior of these cells and to develop effective anti-metastatic treatment strategies to improve survival of RMS patients. We became interested in a role of two G-protein coupled seven transmembrane-span chemokine receptors CXCR4 (17) and CXCR7 (21) in RMS metastasis.

In our previous work, we demonstrated that CXCR4 receptor plays a crucial role in SDF-1-mediated metastasis of RMS cells to SDF-1-expressing organs such as BM and lymph nodes (17). Accordingly, we reported that CXCR4 expression correlates with the more metastatic phenotype of RMS and, although SDF-1 did not affect proliferation or survival of RMS cells, it induced locomotion and directional chemotaxis in several RMS cell lines (17).

With the recent identification of CXCR7, a new receptor for SDF-1 that also binds the I-TAC chemokine, we became interested in the role of the CXCR7-SDF-1 and CXCR7-I-TAC axes in RMS progression (21). We noticed that CXCR7, in contrast to CXCR4, is expressed at a high level on ERMS lines. Although signaling from activated CXCR7 was not associated with increased RMS proliferation or cell survival similarly to CXCR4, it plays an important role in adhesion and migration of RMS cells (21).

The studies described in this work were aimed to better understand how expression of both receptors is regulated at the promoter level in human RMS cells. Promoter analysis was performed in ERMS line RD and ARMS-like PAX3-FKHR-transduced RD cells. We also were interested in addressing how hypoxia influences expression of these genes in PAX3-FKHR-negative and PAX3-FKHR-positive RD cells. We identified minimal promoter regions for CXCR4 and CXCR7, obtained information on molecular regulation of both promoters, and provided evidence that expression of both promoters is differently regulated in RMS cells.

First, we defined a minimal CXCR4 promoter fragment and found that the NRF-1 binding site residing in the proximal promoter sequence plays a crucial role in CXCR4 receptor expression in human RMS cells. Based on the literature there were some transcription factors postulated as positive regulators of CXCR4 promoter transcriptional activity including cAMP responsive element, NF- κ B, and HGF (32-36). However, our work is congruent with a previous report indicating the NRF-1 binding site defines a minimal CXCR4 promoter fragment and that NRF-1 plays a crucial role in CXCR4 expression in human RMS cells (28,37). In addition to promoter deletion and NRF-1 binding site mutagenesis studies, the requirement of NRF-1 for CXCR4 promoter activity was additionally supported in our present study after blocking its expression by employing NRF-1 shRNA.

Next, we identified a minimal CXCR7 promoter fragment and provided evidence that the proximal NF- κ B binding motif is required for its activity. The requirement of NF- κ B for CXCR7 promoter activity was additionally supported in this study after exposing RMS cells

to BAY 11–7082, which is a small molecular NF- κ B inhibitor. Interestingly, the NF- κ B binding site was also identified in the proximal region of the CXCR4 promoter and, as mentioned above, it was even postulated to play a role in CXCR4 expression in human breast cancer cells (32). However, our promoter deletion studies revealed that this transcription factor does not play a significant role in basic CXCR4 expression, at least in RMS cells.

We also found that expression of CXCR4 is positively modulated in RMS cells by PAX3-FKHR protein. It is known that this fusion protein is a much stronger transcription factor as compared to wild type PAX3 (13). Our results support this and show that PAX3-FKHR enhances CXCR4 expression in a PAX3 binding site-dependent manner. Furthermore, some promoter activity was observed in RD/PAX3-FKHR cells but not in PAX3-expressing RD cells, even if the NRF-1 site was mutated. To support this latter observation, we performed ChIP analysis and demonstrated that the PAX3-FKHR protein may in fact directly interact with NRF-1 protein. However, further mutagenesis studies are needed to identify which part of the NRF-1 molecule is involved in this protein-protein interaction.

We also noticed that PAX3-FKHR, while enhancing CXCR4 expression, somehow downregulated expression of CXCR7 in RMS cells. This is in agreement with our previous studies showing that CXCR4 is highly expressed on PAX3/7-FKHR-expressing ARMS cells (17). This is in contrast to CXCR7 expression, which is higher on PAX3/7-FKHR negative ERMS cell lines (21). A possible explanation of this differential regulation of both promoters by PAX3-FKHR could be explained by YY1 activity that as reported is a negative regulator of CXCR4 expression (38,39).

However, YY1 binding motif is present in both promoters, in case of CXCR4 in which YY1 binding site is located between proximal NRF-1 and PAX3 binding motif, direct interaction between PAX3-FKHR-NRF-1 may prevent access of YY1 to its binding sequence in PAX3-FKHR⁺ cells. In contrast, analogous sequence in CXCR7 promoter is accessible for YY1 binding. This explains better why CXCR4 is expressed at higher level than CXCR7 in RD/PAX3-FKHR cells. To support this further, when the YY1 binding site was mutated in the CXCR4 promoter, we observed upregulation of promoter activity in RD but not RD/PAX3-FKHR cells. In contrast, involvement of YY1 in regulating CXCR7 promoter activity is more complex. One of the factors affecting its role is the total level of YY1, which is higher in PAX3-FKHR-expressing cells. This explains why CXCR7 seems to be negatively affected by YY1 in RD/PAX3-FKHR cells that express as we demonstrated YY1 at a much higher level.

Hypoxia has an important impact on expression of several genes that contain HRE motifs. It was reported that expression of CXCR4 is upregulated in several cell types in response to hypoxic conditions (29–31). Here, we provide evidence that it is true for CXCR4⁺ RMS cells as well. To support this, we showed that RD cells expressing very low levels of CXCR4 and even RD/PAX3-FKHR cells that already highly express this receptor both upregulate its expression in response to hypoxia. Of note, we identified one of the HRE elements located at the –1282–1286 site in the CXCR4 promoter sequence as being crucial for hypoxia-induced upregulation of this receptor.

Similar studies on CXCR7 expression in RD cells revealed that it is downregulated during hypoxia. We envision that the downregulation of CXCR7 expression by hypoxia on ERMS cells gives SDF-1 an advantage to more robustly engage the CXCR4 receptor, which becomes upregulated during hypoxic conditions. Therefore, because CXCR4-SDF-1 signaling increases motility of RMS cells (17), ERMS cells may become more metastatic in response to low oxygen levels. In contrast to RD cells and as shown in this report, RD/PAX3-FKHR cells upregulate CXCR7 expression during hypoxia. Because these cells already express high levels of CXCR4 in normoxic conditions, additional upregulation of CXCR7 may play an important role in

affecting some other pro-metastatic SDF-1-mediated properties of ARMS cells such as secretion of metalloproteinases or the tethering of migrating RMS cells in potential metastatic sites.

Of note, our data also support a role of YY1 in regulating CXCR4 and CXCR7 expression during hypoxia. We noticed that YY1 expression, which is lower in normoxia in RD cells as compared to RD/PAX3-FKHR, becomes upregulated in hypoxia in RD cells and at the same time is downregulated in RD/PAX3-FKHR cells. Based on this, while hypoxia-induced upregulation of YY1 expression in RD cells correlates with downregulation of the CXCR7 level, hypoxia-induced downregulation of YY1 expression in RD/PAX3-FKHR cells explains the increase in CXCR7 expression on these cells.

In conclusion, in this report for the first time, we analyzed the regulation of promoter activity at the molecular level of newly identified SDF-1-binding receptor CXCR7 and provide evidence on a pivotal role of a proximal NF- κ B binding site in this process. We also provide novel data on a role for NRF-1 in CXCR4 expression in RMS cells and involvement of the PAX3-FKHR-NRF-1 complex in enhancing CXCR4 expression in more metastatic PAX3-FKHR⁺ ARMS cells. We also postulate that the interaction of PAX3-FKHR and NRF-1 proteins in the proximal part of the CXCR4 promoter in PAX3-FKHR⁺ ARMS cells prevents access of the negative regulator of transcription YY1 to its binding site. Furthermore, we report that both of these pro-metastatic receptors are differently regulated in human RMS cells during hypoxia. Overall changes in the receptor expression pattern during hypoxic conditions suggest that ERMS cells may become more metastatic as a result of enhanced CXCR4 expression that primarily governs SDF-1-dependent RMS motility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Panel A. CXCR4 and CXCR7 membrane expression in RMS cell lines RD and RD/PAX3-FKHR

Flow cytometry analysis of membrane expression was performed on the RD cell line transfected with a PAX3-FKHR hybrid construct named RD/PAX3-FKHR and on the RD cell line transfected with empty vector. Percentage of positive cells was calculated on the basis of adequate isotype control (<1%). MFI – Mean Fluorescence Intensity ± SD. The experiment was repeated three times with similar results. A representative study is shown. **Panel B. RQ-PCR analysis of CXCR4 and CXCR7 mRNA expression in RD and RD/PAX3-FKHR cell lines.** The mRNA expression was measured by Real-Time PCR. Fold of difference was calculated on the basis of $2\Delta\Delta C_t$ values, where CXCR4 expression in RD cells =1 and CXCR7 expression in RD/PAX3-FKHR =1.

Figure 2. CXCR4 promoter deletion studies and transcription binding sites analysis

Panel A. Constructed CXCR4 promoter inserts. The promoter region of the CXCR4 gene from -2,237 to +62 relative to the start of transcription was cloned and is described as Fragment 1. Fragment 1 was sequentially shortened according to the positions of PAX3 binding sites, and NRF-1 binding sites as depicted in the theoretical model of the cloned sequences of the promoter. The position of PAX3-, NRF-1- HRE-, NF- κ B and YY1 binding sites as well as P1-3 primer pairs used in the ChIP experiment are shown. **Panel B. CXCR4 promoter activity studies.** RD and RD/PAX3-FKHR cells were transfected with the appropriate plasmids. Cultured cells were harvested after 24 hours and assayed for the amount of luciferase activity. Activity was measured on the basis of firefly/*Renilla* luciferase activity and then the equimolar fold of difference was counted. Results are expressed relative to a value of 1.0 for cells transfected with pGL4.72 vector and empty pGL4.10. Averages of duplicates from three independent experiments are shown. Values are given as the mean $\pm$ SEM. Frag1-7 $p < 0.05$, as compared with empty vector. Fragments indicated with asterisk (*), $p < 0.05$, as compared with NRF-1mut fragments and Frag8 (Mann-Whitney test). **Panel C. The PAX3-FKHR-NRF-1 complex binds DNA.** The ChIP assay was performed on RD/PAX3-FKHR nuclear extracts pulled down with the anti PAX3/7 Ab and revealed two of the PAX3 binding sites assayed were preserved and that the NRF-1 binding site was preserved as well. Moreover, pulling down with NRF Ab resulted in preservation of both PAX3 binding sites. In the RD cell line, beside the NRF binding site, one PAX3 binding site seems to be occupied by transcription

factors. No complex formation was visible in RD cells. A representative example is shown from three independent experiments.

Figure 3. PAX3-FKHR binds NRF-1

The NRF-1 binding site was shown to be critical for the increase in CXCR4 promoter activity.

Panel A - Western blot analysis of RD and RD/PAX3-FKHR nuclear extracts probed with an anti PAX3/PAX7 Ab (Santa Cruz Biotechnology). We found a band corresponding to wild type PAX3 at 56kD in both cell lines; however, we also found a band at 97kD corresponding to PAX3-FKHR fusion protein. **Panel B** - Western blot analysis of whole RD and RD/PAX3-FKHR extracts probed with an anti-NRF-1 Ab revealed the presence of a band at 68kD corresponding to the predicted size of the NRF-1 protein. **Panel C** - an immunoprecipitation with both RD and RD/PAX3-FKHR whole cell extracts with the PAX3/7 Ab. The blot was subsequently probed with the anti NRF-1 Ab. We noticed a band at 68kD in the blot of RD/PAX3-FKHR but not RD immunoprecipitates corresponding to the size of NRF-1. **Panel D** - reverse experiment, immunoprecipitation with NRF-1 and PAX3 detection shows interaction between NRF-1 and fusion protein PAX3-FKHR. IgG represents the anti-rabbit IgG control for both cell lines.

Figure 4. CXCR7 promoter deletion studies and transcription binding sites analysis

Panel A. CXCR7 promoter inserts. The promoter region of the CXCR7 gene from -2,409 to +89 relative to the start of transcription was cloned and is described as Fragment 1. Fragment 1 shorter derivatives were obtained according to the positions of NRF-1, HRE binding sites, and NF-κB binding sites. The individual constructs were cloned to pGL4.10 vector, transfected into either RD or RD/PAX3-FKHR cells, and used in Dual Luciferase assays. (X) – mutated NF-κB and HRE binding sites. Primer pairs used in the ChIP experiment (N1–N5) flanked 5 different potential binding sites for NF-κB transcription factor. **Panel B. NF-κB as a crucial transcription factor that drives CXCR7 promoter activity.** Activity of particular CXCR7 promoter constructs was assayed by Dual Luciferase showing an important role of NF-κB transcription factors. Activity was measured on the basis of firefly/*Renilla* luciferase activity and then the equimolar fold of difference was counted. Results are expressed relative to a value of 1.0 for cells transfected with pGL4.72 vector and empty pGL4.10. Averages of duplicates from three independent experiments are shown. Values are given as the mean ± SEM. Frag 1–6 p<0.05, as compared with empty vector. Fragments indicated with asterisk (*), p<0.05, as compared with NF-κBmut fragment and Frag7 (Mann-Whitney test). **Panel C. NF-κB to CXCR7 promoter.** ChIP assays of indicated cell lines showed that binding sites 0.03kb, 0.3kb, and 1.0kb are required for full CXCR7 promoter activity. NF-κB-based regulation of the promoter seems to be identical in both cell lines.

Figure 5. YY1 transcription factor negatively regulates CXCR4 and CXCR7 expression
Panel A. PAX3-FKHR translocation upregulates YY1 mRNA expression. RQ-PCR shows ~5-fold upregulation of YY1 mRNA expression after transfection with PAX3-FKHR construct. **Panels B and C. YY1 transcription factor is responsible for downregulation of CXCR4 and CXCR7 expression.** Both cell lines were transiently transfected with wild type CXCR4 (B) and CXCR7 (C) promoter fragments and fragments containing YY1 mutations. After 24 hours, luciferase activity was measured and promoter activity was calculated as in Figure 2B and Figure 4B. (*) = $p < 0.05$ (paired Student's t test).

Figure 6. Panel A. Effects of hypoxia on CXCR4 (left) and CXCR7 receptor (right) membrane expression in RD and RD/PAX3-FKHR cell lines
Hypoxia enhances expression of CXCR4 in both RD and RD/PAX3-FKHR cells. At the same time CXCR7 is upregulated during hypoxia in RD/PAX3-FKHR cells, but downregulated in RD cell. Results are the mean of three experiments. Asterisks (*) indicate $p < 0.05$ versus cells cultured in normoxia (paired Student's *t* test). MFI – Mean Fluorescence Intensity $\pm$ SD. **Panel B. YY1 binding to CXCR4 and CXCR7 promoters is divergently regulated by hypoxia.** RD and RD/PAX3-FKHR cells were subjected to normoxia and hypoxia for 16 hours and subsequent ChIP assays were performed. PCR products were visualized on gels. **Panel C. CXCR4 expression in hypoxia is dependent on HIF-1 α .** Sequential deletions of CXCR4 promoter reveals the vast importance of the HRE binding site located 1.3kb from the transcriptional start. When a promoter fragment devoid of this HRE was used, promoter activity was abolished. Fold of difference was calculated as in Figure 2B. Values are given as the mean $\pm$ SEM, *Activities of Frag3.1, Frag4, and HREmut, $p < 0.05$, as compared with full length promoter fragments (Mann-Whitney test). **Panel D. CXCR7 expression in hypoxia is dependent on HIF-1 α only in the RD/PAX3-FKHR cell line.** Specific constructs lacking HRE binding sites were ligated with pGL4 vector and luciferase activity was measured. Depicted is the fold difference of activity relative to *Renilla* vector and control. Fold of difference was calculated exactly as in Figure 2B. Values are given as the mean $\pm$ SEM. *Activities of Frag6 and HREmut, $p < 0.05$, as compared with full length promoter fragments (Mann-Whitney test).

Figure 7. Effect of hypoxia on chemotactic response of RD (A) and RD/PAX3-FKHR (B) cells
Cells were incubated for 16 hours in hypoxia in the presence of either SDF-1 (300ng/mL) or I-TAC (100ng/mL). Results are mean data of three experiments, * = $p < 0.05$ versus cells cultured in normoxia (paired Student's t test).

Table 1
Inhibition of NRF-1 on CXCR4 and CXCR7 expression in RD, RD/PAX3-FKHR, and RH30 rhabdomyosarcoma cells

NRF-1 was downregulated by shRNA-expressing vector (~80% inhibition as evaluated by RQ-PCR). Cells were evaluated in normoxic condition and hypoxia (16 hours). CXCR4 and CXCR7 membrane expression was measured by FACS and is presented as MFI $\pm$ SD. Mean is shown as result from three independent experiments performed.

NORMOXIA (MFI $\pm$ SD)						
	RD		RD/PAX3-FKHR		RH30	
	Scrambled control	shNRF-1	Scrambled control	shNRF-1	Scrambled control	shNRF-1
Isotype APC	37 $\pm$ 4	35 $\pm$ 2	54 $\pm$ 2	39 $\pm$ 3	39 $\pm$ 2	33 $\pm$ 2
CXCR4	48 $\pm$ 5	39 $\pm$ 6	491 $\pm$ 10	137 $\pm$ 5	735 $\pm$ 30	43 $\pm$ 3
Isotype PE	58 $\pm$ 2	42 $\pm$ 3	37 $\pm$ 2	31 $\pm$ 3	26 $\pm$ 4	38 $\pm$ 3
CXCR7	598 $\pm$ 34	582 $\pm$ 45	99 $\pm$ 12	121 $\pm$ 9	37 $\pm$ 3	69 $\pm$ 4
HYPOXIA (MFI $\pm$ SD)						
	RD		RD/PAX3-FKHR		RH30	
	Scrambled control	shNRF-1	Scrambled control	shNRF-1	Scrambled control	shNRF-1
Isotype APC	54 $\pm$ 4	57 $\pm$ 4	39 $\pm$ 5	30 $\pm$ 3	40 $\pm$ 4	49 $\pm$ 4
CXCR4	112 $\pm$ 20	52 $\pm$ 3	805 $\pm$ 38	196 $\pm$ 15	912 $\pm$ 52	87 $\pm$ 3
Isotype PE	20 $\pm$ 1	36 $\pm$ 3	42 $\pm$ 3	38 $\pm$ 5	38 $\pm$ 2	43 $\pm$ 3
CXCR7	252 $\pm$ 22	269 $\pm$ 36	203 $\pm$ 22	215 $\pm$ 30	37 $\pm$ 3	71 $\pm$ 5

Table 2
Inhibition of NF-κB on CXCR4 and CXCR7 expression in RD, RD/PAX3-FKHR, and RH30 rhabdomyosarcoma cells

NF-κB was downregulated by employing BAY 11–7082. Cells were evaluated in normoxic condition and hypoxia (16 hours). CXCR4 and CXCR7 membrane expression was measured by FACS and is presented as MFI ±SD. Mean is shown as the results from three independent experiments performed. In all BAY 11–7082 experiments, DMSO concentration was <0.01% and DMSO-treated cells served as appropriate controls.

NORMOXIA (MFI±SD)						
	RD		RD/PAX3-FKHR		RH30	
	control	BAY 11–7082	control	BAY 11–7082	control	BAY 11–7082
Isotype APC	36±5	30±2	53±4	63±5	43±3	62±2
CXCR4	61±3	59±10	414±30	435±20	823±40	739±10
Isotype PE	28±2	34±3	67±5	39±5	59±5	53±2
CXCR7	544±20	72±5	119±16	39±10	86±7	62±5
HYPOXIA (MFI±SD)						
	control	BAY 11–7082	control	BAY 11–7082	control	BAY 11–7082
Isotype APC	56±6	40±4	62±3	45±12	36±2	35±3
CXCR4	168±30	105±12	708±38	785±51	968±45	915±68
Isotype PE	61±4	38±5	53±5	26±2	35±2	32±3
CXCR7	188±11	91±5	213±16	45±5	35±3	35±4