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CXCR4 CXCR7 effects in melanoma & melanocytes

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ABSTRACT

Chemokines are small signaling proteins released by cells in response to chemical stimuli in their environment. The chemokine stromal cell-derived factor-1 (SDF1/CXCL12) plays a role in the growth and metastasis of multiple cancers, including melanoma. SDF1/CXCL12 has two known receptors: CXCR4 and CXCR7. CXCR4 receptor signaling influences many cell responses, including the migration of neural crest cells and the amount of receptor expression upregulation in melanoma. CXCR7 receptor signaling influences melanocyte migration and constrains melanoma tumor growth *in vivo,* while CXCR4 receptor signaling does not. It is not known, however, if CXCR7 directly affects melanocyte and melanoma cell migration. Here, CXCR4 and CXCR7 receptor signaling were directly studied *in vitro* in human primary melanocytes and mouse B16-F10 melanoma cell line for migration when both receptors, individually and in combination, were genetically silenced. The silencing of CXCR7 clearly resulted in statistically significant inhibition of migration of both melanocytes and B16-F10 melanoma cells while the effects of CXCR4 silencing of migration inhibition was less clear. These results suggest that the CXCR7 receptor is more relevant than the CXCR4 receptor for the migratory capabilities of melanocytes and B16-F10 melanoma cells. These findings support down regulation or inhibition of the CXCR7 receptor through targeted therapies may be beneficial in melanoma treatment.

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CXCR4 CXCR7 effects in melanoma

2.1 Cell lines

Murine melanoma B16-F0 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Dulbecco's Modified Eagle Medium (DMEM, Genesee Scientific, El Cajon, CA, USA) supplemented with 5% fetal bovine serum (FBS, Genesee Scientific), 1x Penicillin/Streptomycin (Genesee Scientific), and 1x L-Glutamine (Gibco, ThermoScientific, Grand Island, NY, USA). Human Epidermal Melanocyte (HEM) cell line was purchased from Cell Applications (San Diego, CA, USA) and grown in all-in-one HEM Growth Medium (Cell Applications). The NCC line was the Spl201 (Lobsiger et al., 2001).,

2.2 Migration Assay

Cells were plated in small Tissue culture dishes at 8.0 x 10^4 cells/mL the day before the assay. Immediately prior to imaging, 80µL of HEPES Buffer was added to the growth media in order to maintain the desired pH conditions outside of the incubator. These experiments were done using DMEM with FBS because cells did not migrate or even survived in the absence of serum (Pijuan et al., 2019), in addition, there is the difficulty in choosing valid serum alternatives (Piletz et al., 2018; van der Valk, 2022).
When SDF1/CXCL12 addition was required, 200ng SDF1/CXCL12 (R&D Catalog #: 350-NS) was added to the media and allowed to incubate for one hour. When no SDF1/CXCL12 was added, imaging could begin right away. A heat lamp was placed on top of the plated cells with a thermometer inside, in order to maintain a desired temperature of 37^{o}C. After finding a representative field of view under AxioImager microscope, a video was initiated and began taking snapshots every 90 seconds for approximately four hours (approximately 161 frames per video). Cell migration was analyzed by manually tracking at least 15 cells at a time from each experiment using an ImageJ plug-in from Ibidi website (https://ibidi.com/chemotaxis-analysis/171-chemotaxis-and-migration-tool.html). The ImageJ software provided the Accumulated Distance values for each cell pathway that was tracked, as well as the cell's Displacement and Y-Axis Displacement. However, the Velocity, Persistence, and Chemotaxic Index were all calculated in Excel. The formulas used are:
Velocity = Accumulated Distance/Number of Frames.
Persistence = Displacement/Accumulated Distance.
Chemotaxic Index = Y-Axis Displacement/Accumulated Distance

2.3 Wound Assay

Cells were plated in a 6-well plate at 1.2 x 10^5cells/mL one to two days prior to the experiment. Once the cells had reached a ~90-100% confluent monolayer, 200ng SDF1/CXCL12 (R&D Catalog #: 350-NS) was added to the proper wells and allowed to incubate for one hour prior to wounding. At Hour 0, each well was scratched with a 10uL pipette tip, creating a “tic-tac-toe” design (Figure 2). Images were then taken of the four crossed sections in each well at hour 0, 5, 8 and 18. At Hour 18, the cells were then collected for RNA purification, cDNA conversion, and eventually qPCR. The images at each time point were then measured using an area analysis in which the area left unhealed was measured using a wound healing tool in ImageJ software.

2.4 siRNA Transfection

B16-F10 melanoma cells were cultured until ~70-80% confluency. Cells were transfected with either CXCR4 siRNA (OriGene, Rockville, MD, USA),CXCR7 siRNA or scramble siRNA (Eupheria Biotech, Dresden, Germany), as a negative control using Lipofectamine 3000 reagent (Invitrogen; ThermoFisher, Carlsbad, CA, USA). Transfections were conducted according to the manufacturer’s protocol. HEK293T cells were also transfected simultaneously as a positive control.

2.5 RNA Isolation and cDNA synthesis

Melanoma and melanocytes transfected with siRNA were lifted from plates and were purified using the GeneJET RNA Purification Kit (ThermoFisher) according to the manufacturer’s protocol. Total RNA extraction was initiated with cells resuspended in lysis buffer supplemented with β-mercaptoethanol followed by an addition of 100% EtOH. The lysate was passed through the GeneJET RNA Purification Column and centrifuged for 1 min at ≥ 12,000 x g. The flow-through was discarded and the filter underwent several wash steps following the manual’s procedure. Total RNA was eluted from the filter using 100 µL of nuclease-free water. The NanoDrop 2000 (ThermoFisher) was used to ensure Total RNA integrity (280/360 at ~1.8) and concentration (~150-200 ng/µL). Total RNA was stored in -80°C until cDNA conversion.
cDNA conversion was performed using the Eppendorf 5333 MasterCycler. SuperScript VILO cDNA Synthesis Kit (ThermoFisher) was manually mixed in a 20 µL reaction with Total RNA extracted from each cell condition. Each reaction mix underwent an incubation 25°C for 10 minutes followed by 42°C for 60 minutes and was terminated at 85°C for 5 minutes. cDNA was stored at -20°C until used for RT-qPCR.

2.6 PCR and gel electrophoresis

cDNA synthesis was performed as previously described. Each reaction consisted of 8 µL Apex 2x Taq RED Master Mix (Genesee Scientific), 1 µL each offorward and reverse primers at 10 µM concentration (Integrated DNA Technologies), and brought up to 16 µL total with RNase-free water. Thermocycling conditions followed a three-step PCR program as indicated in the protocol (step 1, 95°C for 5 minutes; step 2, 35 cycles of the PCR step, 95°C for 20 seconds, 58°C for 30 seconds, 72°C for 30 seconds; step 3, 72°C for 5 minutes). PCR was performed using the Eppendorf 5333 Mastercyler (Eppendorf, Enfield, CT, USA). Primer sequences used for this reaction are listed in Table 1.
Approximately 12 µL of the final PCR product was loaded into the wells of a 1.5% agarose gel supplemented with 0.5 µg/mL ethidium bromide (EtBr) and submerged in 1x TAE. Gel electrophoresis was performed in the Bio-Rad Mini-Sub cell apparatus supplied with the PowerPac power supply (Bio-Rad, Hercules, CA, USA) for 45 minutes at 75V. For analysis, the gel was imaged under ultraviolet light using the Alpha Innotech FluorChem Q System and the provided program (Alpha Innotech, San Leandro, CA, USA). Gel analysis was performed to measure the band intensity through Fiji software.

2.7 Reverse transcription quantitative real-time PCR

qRT-PCR and analysis was performed in QuantStudio 3 Real-Time PCR System (Applied Biosystems, ThermoFisher). Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher) was manually mixed with forward and reverse primers (Integrated DNA Technologies) amplifying GAPDH, CXCR4 and CXCR7 in a 16 µL reaction containing cDNA at a concentration of ≤ 500ng. Triplicate sample reactions were run in MicroAmp Reaction Tube Strips (Applied Biosystems). Results were normalized to GAPDH using specifically the ΔΔCt comparative method to untransfected melanoma cells. Our thermocycling conditions followed the three-step cycling protocol as stated in the user manual and included a UDG pre-treatment (step 1, 50°C for 2 minutes; step 2, 95°C for 10 minutes; 40 cycles of the PCR step, 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds). Primers used are listed in Table 1.

Table 1 List of primers and their sequences used for qPCR analysis of the SDF1/CXCL12 receptors: CXCR4 and CXCR7. Different primers were needed for both the mouse melanoma and the human melanocyte cell lines.

Primer Name	Primer Sequence

Mouse/Human CXCR4 Forward	5'-GCWGTYCATRTCATYTACACWGTCAACCTCTA-3'
Mouse/Human CXCR4 Reverse	5'-GTSGTCTTSARGGCYTTGCGCTTCTGGTGGCC-3'
Mouse CXCR7 Forward	5'-GGTCAGTCTCGTGCAGCATA-3'
Mouse CXCR7 Reverse	5'-GTGCCGGTGAAGTAGGTGAT-3'
Human CXCR7 Forward	5'-AGCATCAAGGAGTGGCTGAT-3'
Human CXCR7 Reverse	5'-TGTGCTTCTCCTGGTCACTG-3'

All primers were purchased from Integrated DNA Technologies, clear 96-well plates purchased from ThermoFisher Scientific, and Bio-Rad CFX96 Real-Time PCR Thermocycler. Primer sequences were from published papers that had previously been found to be successful (Liang et al., 2001; Maishi et al., 2012; Sierro et al., 2007) (See Table 1 of primers used). GAPDH was the housekeeping gene used to normalize the gene expression.

2.8 Statistical Analysis

For the analysis of the migration and wound assays, each experiment was analyzed separately, and the replicates allowed for a final average to be taken. With the average values calculated, t-tests were performed to determine if there was any significance between two conditions – a control and an experimental. Therefore, t-tests were able to show any significance when comparing the KD condition to either the untreated condition (negative control) or the scramble siRNA condition (positive control). For each of these t-tests, the two sets of data were analyzed as a two-tailed distribution, since the results could be greater or lesser than the control, and with equal variance, since each cell line was compared to itself and not to each other.

For the qPCR analysis, the relative gene expressions were determined using the ΔΔCq Calculation Method as described by Horizon Inspired Cell Solutions (https://horizondiscovery.com/-/media/Files/Horizon/resources/Technical-manuals/delta-cq-solaris-technote.pdf) (Boulter et al., 2016).

2.9 Boyden assays

Sterile and TC-treated Corning® Transwell® polycarbonate membrane cell culture inserts 6.5 mm Transwell with 8.0 µm pore polycarbonate membrane inserts were coated with a solution of 0.1ug/ml fibronectin for one hour. The lower wells

were filled with DMEM supplemented with FBS, and the upper wells were seeded with the indicated cells (1.25 x 10^5) resuspended in DMEM with FBS. The chambers were placed in a 37°C incubator for approximately 24 hours. After incubation, cells remaining on the upper surface of the insert were gently scrubbed off with a cotton swab. The cells on the lower surface were fixed with 4% paraformaldehyde (PFA), then washed with phosphate-buffered saline (PBS) before bring stained withtoluidine blue.. Migrated cells were viewed and quantified using a microscope at 100x magnification. Statistical analysis was done using RStudio software and is available in GitHub: https://github.com/nayousefi/CXCRPaper.

2.10 Cell staining

Transfected cultured cells were fixed for 30 minutes in 4% paraformaldehyde and rinsed twice with PBS, then permeabilized and blocked in 1% triton X100 and 10% goat serum respectively for 30 minutes. Then anti-CXCR4 or anti CXCR7 antibodies (APS305 anti-CXCR4, and **PA3-069 anti-CXCR7;**ThermoFisher) were added in PBS for 18 hours at room temperature. The following day, cells were washed 3 times in PBS for 5 minutes each and incubated with anti-rabbit Alexa-488 secondary antibody for 30 minutes, washed 3 times for at least 5 minutes and mounted with DAPI to visualize nuclei and imaged in a Zeiss Axio Imager using AxioVision software.

2.11 Cell staining measurements

Cell area and fluorescent intensity were both measured using FIJI Is Just ImageJ (FIJI) software. For cell area, the cytoskeleton stained with phalloidin, was traced. The traced image's units were given in pixels. Area averages, standard deviation, and standard area were all performed using the micrometer measurements.

For fluorescent intensity, the image shown in the green channel, stained with either CXCR4 or CXCR7 antibody, was traced. Along with the traced cells, small portions of the background alongside the cell were also measured. The relevant data used for data analysis was "Area", "Mean" and "IntDen" (Integrated Density). To get the "Adjusted Integrated Density", first the average Mean of all the background images was calculated. Averages and standard error were performed using the adjusted integrated density.

The following formula was used to calculate the adjusted integrated density:

Integrated Density of the Cell – (Area of the Cell * Average Mean of Background Images)

Statistical Analysis: Statistical analysis was performed in RStudio. The "tidyverse" and "here" R packages were used to get the ANOVA results from the data.All relevant code and results can be found at https://github.com/nayousefi/CXCRPaper.

2.12 Structural modeling of CXCR4 and CXCR7

A novel dimeric structure of SDF1 bound to CXCR4 was released by Tony Ngo et al. (Ngo et al., 2020). This structure is in the inactive conformation. To obtain the active conformation of this structure, the receptor was aligned to our active conformation of CXCR4 bound to SDF1 that was generated based on homology to rhodopsin's active conformation (PDBid: 4zwj; (Kang et al., 2015). This active conformation of the novel SDF1-CXCR4 dimeric complex was used as a template for building our SDF1-CXCR7, ARRB2-CXCR4, and ARRB2-CXCR7 dimeric complexes through receptor alignment using VMD (Humphrey et al., 1996).

Our four dimeric complexes (SDF1-CXCR4, SDF1-CXCR7, ARRB2-CXCR4, and ARRB2-CXCR7) were embedded in a POPC lipid bilayer-based simulation box using CHARMM-GUI. AMBER was then used to simulate and relax our dimeric

complexes using 1 microsecond molecular dynamics (MD) simulation time under physiological conditions of 310 K and 1 atm. MMPBSA method (Botello-Smith and Luo, 2015) was utilized to quantify the free energy of binding of the ligands, SDF1 and β-arrestin, to each receptor, CXCR4 and CXCR7. This protocol is similar to one followed in our earlier study on the G protein selectivity of the muscarinic receptors M1 and M2 (Santiago and Abrol, 2019a).