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The Complementary Roles of CXCR4 and CXCR7 in Melanoma Migration

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Abstract

The two known receptors, CXCR4 and CXCR7, for the chemokine stromal cell-derived factor-1 (SDF1) play a role in the development and metastasis of multiple cancers, including melanoma. CXCR4 receptor signaling influences melanoma cell responses, including proliferation, migration, and metastasis. CXCR7 receptor signaling can also induce melanoma malignancy by increasing proliferation. However, it is unknown if CXCR7 directly affects melanoma cell migration. Here, we blocked CXCR4 and CXCR7 receptor signaling by inhibitors and by reducing their expression through siRNA treatment. Both methods efficiently reduced melanoma cell migration with two different assays. These results suggest that the CXCR7 receptor is as relevant as the CXCR4 receptor in affecting the migratory capabilities of melanoma cells. These findings support that downregulation or inhibition of the CXCR7 receptor through targeted therapies may benefit melanoma treatment.

Subject Areas

Cell Biology

Keywords

Melanoma, CXCR4, CXCR7, Cell Migration

1. Introduction

Metastatic melanoma is a type of skin cancer that features rapid systemic dissemination [1]. Metastasis typically occurs during the disease's later stages (III and

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IV). In the United States, the five-year survival rate of those afflicted with distant metastatic melanoma is approximately 28.5%, compared to those with localized and regional metastatic melanoma, with five-year survival rates of 99% and 69%, respectively [2].

Melanoma arises from melanocytes transformed by genetic mutations. (Shain and Bastian, 2016). Melanocytes are the pigment cells of the body derived from an embryonic pluripotent stem cell population known as the neural crest. During embryonic development, neural crest cells (NCC) delaminate or split into layers of the dorsal portion of the neural tube and begin migrating quickly and extensively throughout the embryo, giving rise to their derivatives. These NCCs are highly migratory mesenchymal stem cells that will contribute to the formation of many diverse structures: connective tissue, cartilage and bone, neurons, glia, and pigmented melanocytes [3].

Metastasis occurs when cells from the initial tumor travel through the lymphatic system or bloodstream and form new tumors in nearby or distant sites. Chemokines and their associated biological pathways partly aid this migration of malignant cells [4]. Chemokines are a specific type of cytokine that causes chemotactic responses in neighboring cells that harbor reciprocal receptors. These small signaling proteins are secreted by cells to direct the migration of other cells. Chemokines are essential for regulating cell migration during embryonic development and immune responses [5] [6]. Tumor growth and metastatic potential of melanoma and other cancers are affected by stromal cell-derived factor-1 (SDF1, also known as CXCL12), a chemokine with two known receptors, C-X-C Motif Chemokine Receptor 4 and 7 (CXCR4 and CXCR7, also called ACKR3) [7]-[10].

Previous research has investigated the possible influences that SDF1/CXCR4/CXCR7 pasthways have on melanoma growth. In a 2014 study, Liedtke and his colleagues studied melanoma growth and metastasis in transgenic fish lines that overexpressed SDF1 exclusively in pigment cells [11]. They determined that the loss of functional CXCR7 could constrain melanoma growth in vivo. Furthermore, experiments using CXCR4-CXCR7 linked together showed a complete change in the activated signaling pathways and trafficking of these receptors [12]. These studies show the importance of SDF1CXCR4 and SDF1/CXCR7-mediated pathways in melanoma migration and cancer. More recent studies have implicated the role of the SDF1/CXCR4/CXCR7 Axis in a wide range of cancers and other pathologies [13]-[18]. Accordingly, a better understanding of the mechanisms behind SDF1's interactions with its CXCR4 and CXCR7 receptors and its role in the onset of melanoma cancer is necessary to lay the groundwork for developing any clinical treatment.

Here, we focus on how the migration of melanoma cells is affected by SDF1 and its receptors, CXCR4 and CXCR7. We propose that both CXCR4 and CXCR7 are equally involved in the chemotaxis and chemokinesis of melanoma cells through the SDF1/CXCR4/CXCR7 interaction. This study tests these processes using

siRNA knockdown and antagonists on both CXCR receptors. We then conduct *in vitro* experiments, including Boyden chamber assays, wound assays, and immunofluorescence studies. We found that: 1) CXCR7 plays a comparable role to CXCR4 in these migratory processes; 2) Knockdown and inhibition of both CXCRs that resulted in a reduction of melanoma migratory processes.

2. Methods

A more detailed protocol is posted at www.Protocols.io as "CXCR4 CXCR7 effects in melanoma & melanocytes" (dx.doi.org/10.17504/protocols.io.e6nvwjwb9lmk/v1).

Cell lines

Murine melanoma B16-F10 cell line was purchased from American Type Culture Collection (ATCC; CRL-6475) and grown in Dulbecco's Modified Eagle Medium (DMEM, Genesee Scientific; Cat #25-501) supplemented with 5% fetal bovine serum (FBS, Genesee Scientific; Cat #25-514), 1x Penicillin/Streptomycin (Genesee Scientific; Cat #25-512), and 1x L-Glutamine (Gibco, ThermoScientific, Cat #25030081). The Human Epidermal Melanocyte (HEM) cell line was purchased from Cell Applications (Cat # 104-05n) and grown in an all-in-one HEM Growth Medium (Cell Applications; Cat # 135-500). The NCC line was the Spl201 [19].

siRNA Transfection

CXCR4 siRNA (OriGene; Cat #SR426532) and CXCR7 siRNA (Eupheria Biotech; Cat #MU-02817-1) were transfected into cells using Lipofectamine 3000 protocol (ThermoFisher; Cat #L3000008). For the wound assays, cells were plated in a multi-well plate at 120,000 cells/mL one to two days before the experiment began. Cell monolayers were wounded with a 10 uL pipette tip. Images of each well's crossed sections were taken at hours 0, 5, 8, and 18. The images at each time point were then measured using an area analysis via a wound healing tool in ImageJ.

CXCR Inhibition

The CXCR4 inhibitor AMD070 hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) and the CXCR7 inhibitor ACT-1004-1239 (MedChemExpress, Monmouth Junction, NJ, USA) were diluted to 2 mg/mL and 5 mg/mL with dimethyl sulfoxide (DMSO), respectively, according to manufacturer protocols.

B16-F10 melanoma cells were cultured until ~80% - 90% confluency in a 10 cm tissue culture-grade petri dish. The cells were resuspended and plated in 6-well plates at 1.2 × 105 cells/mL along with 30 µML of their respective inhibitor for 24 hours in a 37˚C incubator. Transfected cells were used for future experiments after 24 hours had elapsed.

Migration Assays

Melanoma cells were plated at 80,000 cells/mL for migration assays to obtain a 40% - 50% confluency. Cell migration was analyzed by manually tracking at least 15 cells from each experiment using an ImageJ plug-in from Ibidi (https://ibidi.com/chemotaxis-analysis/171-chemotaxis-and-migration-tool.html).

For inhibitor transfected cells: B16-F10 cells were plated in a 6-well plate at 1.2

$\times 10^5$ cells/mL along with 30 μM of their respective inhibitor, or with 20 μL DMSO in the case of the control, 24 hours before the experiment. On the day of the experiment, pictures of the cells were taken with an AxioImager microscope at 5× before wounding. At Hour 0, each plate was scratched with a 200 μL plastic pipette tip to create a "tic-tac-toe" pattern. Pictures of the top two crossed sections and lateral scratches were taken at hours 0-, 4-, 8-, and 18-hours post-wounding. Images were measured and analyzed in ImageJ using a wound healing tool.

RNA Isolation and cDNA synthesis

Melanoma and melanocyte mRNA were purified using the GeneJET RNA Purification Kit (ThermoFisher; Cat #K0731). cDNA conversion was performed using the SuperScript VILO cDNA Synthesis Kit (ThermoFisher; Cat #11754050).

Boyden assays

Melanoma cells (125,000) were plated on fibronectin-coated Boyden chambers (8.0 μm pore inserts, BD, Corning; Cat #3422) containing DMEM. After 24 h incubation, cells remaining on the filter's top side were removed, and cells on the bottom were fixed, stained with toluidine blue, and counted. Statistical analysis was done using R and is available on GitHub:
https://github.com/nayousefi/CXCRPaper.

Cell staining

Transfected cultured cells were fixed in 4% paraformaldehyde and stained with anti-CXCR4 or anti-CXCR7 antibodies (APS305 anti-CXCR4 and PA3-069 anti-CXCR7 from ThermoFisher) to determine CXCR presence. Cell area and fluorescent intensity were both measured using FIJI software. 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.

Protein Extraction and Bradford Assay Analysis

Protein extraction of adherent cells was performed based on the Cell Lysis and Protein Extraction for Western Blotting from Sigma-Aldrich. B16-F10 melanoma cells were coated in lysis buffer and radioimmunoprecipitation assay (RIPA) buffer, scraped off with a plastic scraper, and put on an Open-Air Rocking Shaker (Fisher Scientific) for 30 minutes at 4˚C. After 30 minutes, the tubes were transferred to an Eppendorf Centrifuge 5415 R mini centrifuge, where they were spun at 13,200 g for 20 minutes at 4˚C. The supernatant was transferred to a clean 1.5 mL mini-centrifuge tube and properly labeled while the resulting pellet was discarded. The final product was stored at −20˚C for further use.

Bradford Assay analysis was performed based on the Quick Start™ Bradford Protein Assay protocol from Bio-Rad. Standards were created from the provided Bovine Serum Albumin (BSA) and measured using a NanoDrop 2000 machine to create a standard curve. Once a standard curve was obtained, proteins were thawed over ice. Approximately 25 μL of each protein was pipetted into a separate 1 mL conical tube, and an equivalent volume of the Bradford dye reagent was added to each tube. After sitting for five minutes, the concentrations of the

proteins were measured using NanoDrop 2000 and against the standard created beforehand. The proteins were diluted to 100 ng/μL using RIPA buffer based on the given concentrations and stored at −20˚C for further use.

Structural modeling of CXCR4 and CXCR7 complexes with SDF1

CXCR4:SDF1 structure: The inactive conformation of SDF1 bound to CXCR4 [20] was aligned to the AlphaFold active conformation of CXCR4 [21]. The structure of SDF1 and the N terminus of CXCR4 were conserved from the cryo-EM structure to maintain the interaction between the ligand and the receptor, while the rest of the receptor structure was obtained from AlphaFold.

CXCR7:SDF1 structure: The N-terminus of CXCR7 in the recently published structure of SDF1 bound to active CXCR7 conformation (PDB ID: 7SK3) [22] was incorporated using Modeller [23], as this region was not elucidated in the cryo-EM structure.

These dimeric complexes (SDF1-CXCR4 and SDF1-CXCR7) were embedded in a POPC lipid bilayer-based simulation box using CHARMM-GUI [24]. The KCl salt at 0.15 nM concentration was added to neutralize the system. AMBER program was used to simulate and relax these complexes using NPT molecular dynamics (MD) for 100 ns simulation time under physiological conditions of 310 K and 1 atm. A time step of 2fs (50,000,000 simulation steps) and non-bond interaction cutoff of 8 Å was utilized along with a Monte Carlo barostat and Berendsen thermostat. Periodic boundary conditions were used, and electrostatic interactions were calculated using the Ewald summation method. MMPBSA method [25] was utilized to quantify the free energy of binding ligand SDF1 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 [26].

3. Results

Inhibiting CXCR4/7 signaling reduces melanoma cell migration.

We asked if blocking CXCR4 and CXCR7 receptors inhibits B16-F10 melanoma cell migration. We tested the hypothesis that B16-F10 melanoma cells will migrate more slowly to fill in the gap in a wound assay after using chemical antagonists to inhibit CXCR4/7. Here, we cultured the B16-F10 melanoma cells into monolayers before scratching a path through the monolayer, "wounding" the cells [27]. B16-F10 melanoma cells were treated with CXCR4 inhibitor AMD070 [28] and CXCR7 inhibitor ACT-1004 [29]. Dimethyl sulfoxide (DMSO) was added to one set of untransfected melanoma to determine if DMSO itself could adversely affect B16-F10 migration (**Figure 1**). We observed that after 18 hours of wounding, control-treated melanoma cells were almost 100% healed, and control with DMSO was about 80% healed. Meanwhile, blocking CXCR4 with AMD070 or CXCR7 with ACT-1004 significantly reduced their healing to ~40% ($p < 0.0001$). Both CXCR4 and CXCR7 antagonists resulted in comparable inhibition of wound healing (**Figure 2**).

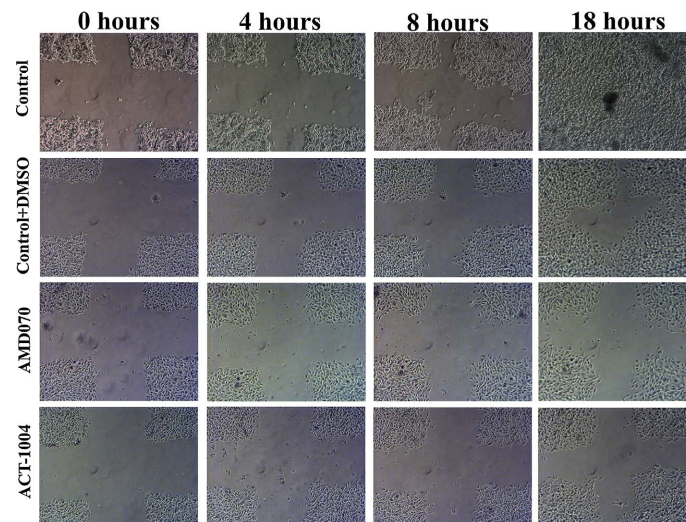


Figure 1. AMD070 and ACT-1004 reduce B16-F10 migration in wound assays. B16-F10 melanoma cells were plated in 6-well plates along with either a 30 μM concentration of their respective inhibitor or 20 μL of DMSO and left in a 37˚C incubator for 24. After 24 hours, the cells were scratch wounded with a 200 μL pipette tip, and images were taken of wounds at 0, 4, 8, and 18 hours after wounding. The photos taken at 18 hours post-wounding show the wound had healed entirely in our control cells, while our control + DMSO cells were about 80% healed. The CXCR4 inhibitor (AMD070) and CXCR7 inhibitor (ACT-1004) still showed a large wound.

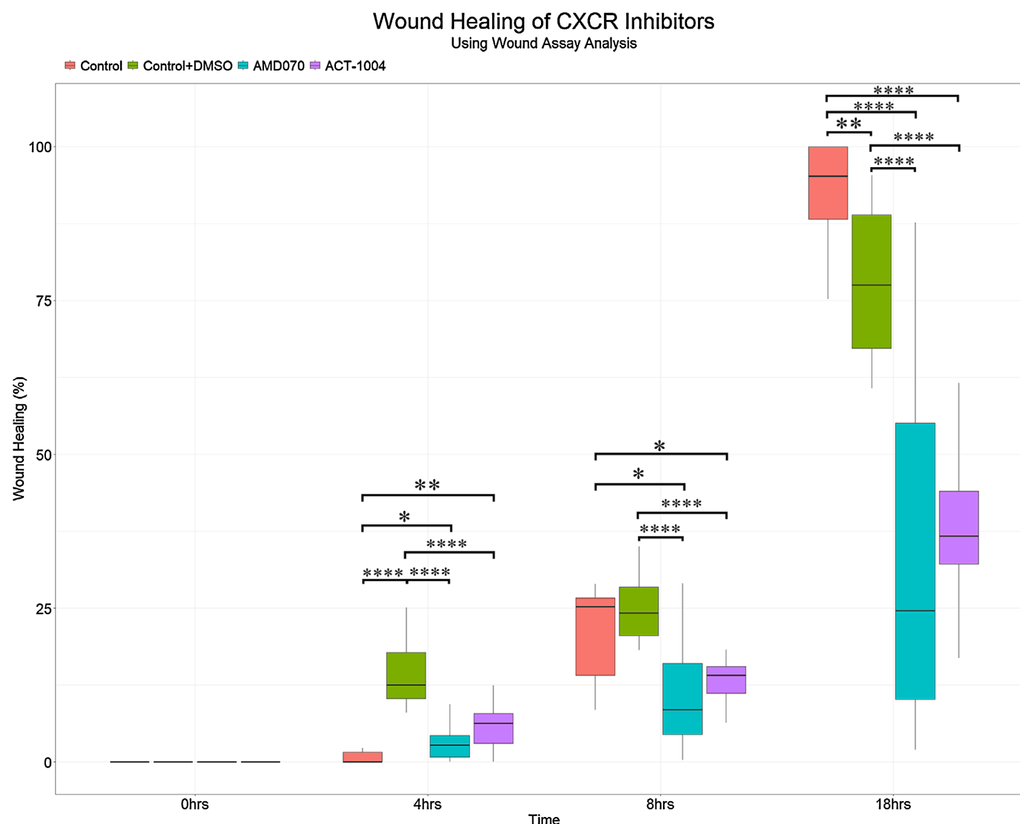


Figure 2. CXCR inhibitors decreased melanoma wound healing by ~60%. Bar graphs showing progressive healing of melanoma wounds at each time point over an 18-hour time frame after CXCR inhibition. The data starts at 0% of the wound area healed at 0 hr, and as time progresses, cells invade the wound and make the area smaller, healing the wound. A difference was observed between the control and the control with DMSO, but an even more significant difference was observed between our controls and our AMD070 and ACT-1004 inhibited cells. *p < 0.05, **p < 0.005, ****p < 0.0001. N = 4.

In tandem with the wound assays, we also utilized Boyden chambers to show that the reduction of B16-F10 melanoma migration was not due to culture conditions. We analyzed chemokines is, random cell movement without a chemical gradient, via these Boyden chambers. The Boyden chamber assay is the standard for cell chemokinesis [30] [31]. Thus, it would support our observations of CXCR roles in melanoma migratory behavior. We observed a 30% decrease in migrated cells in CXCR4-inhibited melanoma and a 40% decrease in CXCR7-inhibited cells (**Figure 3**).

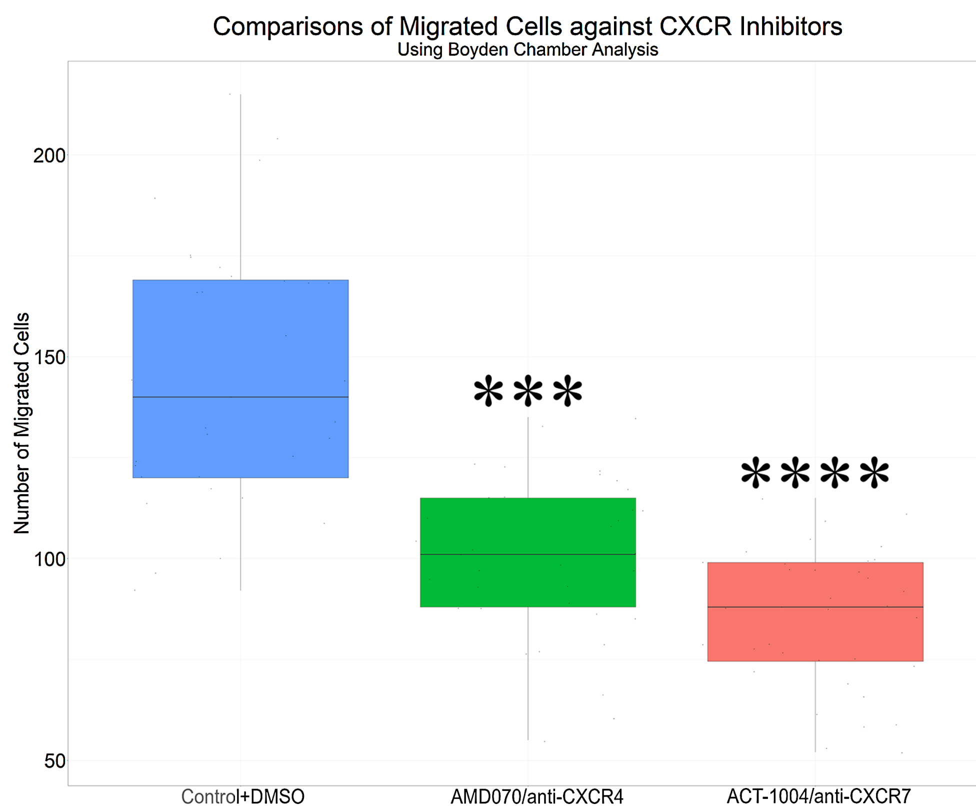


Figure 3. CXCR4 and 7 inhibition decreases melanoma cell chemokinesis. Box plot graphs show how melanoma migration decreased after CXCR4 and CXCR7 inhibition. Data from 4 experiments was normalized to the control values. The number of asterisks present identifies ANOVA significant p-values and are in comparison to the Control + DMSO values. There was no significant difference between the CXCR4 and CXCR7 inhibitors. ***$p < 0.001$, ****$p < 0.0001$. N = 4.

These results with the two inhibitors match what we know about SDF1 binding to CXCR4/7. Experimental studies on the thermodynamics and kinetics of receptor interactions with SDF1 have shown that CXCR7 has a higher affinity for SDF1 than CXCR4 [32]. Our structural modeling of the CXCR4:SDF1 and CXCR7:SDF1 complexes and their relaxation in the lipid bilayer environment; the binding free energy analysis of these interaction partners is shown in **Appendix 2**, along with experimental K_d values. We find that CXCR7 binds with a stronger affinity to SDF1 (more negative DG) than CXCR4, which is consistent with the above experimental observations. The structures provide a biophysical basis for CXCR7,

acting as a sink for SDF1 to downregulate CXCR4 signaling.

Reducing the expression of CXCR4/7 reduces melanoma cell migration.

After corroborating that blocking CXCR4/7 impaired the migration of B16-F10 melanoma cells, we asked if reducing the expression of CXCR4 and/or CXCR7 receptors decreases their migratory behavior. We tested this hypothesis by repeating the wound assays after transfecting B16-F10 melanoma cells with CXCR4/7 siRNA.

Control B16-F10 melanoma cell wounds were ~50% filled (healed) by 8 hours and close to 90% healed after 18 hours (Figure 4). After 18 hours; we did not observe a significant difference between the control cells and those transfected with siRNAs: scramble, CXCR4, or CXCR7.

We repeated the Boyden chamber assays, using siRNA knockdown transfections

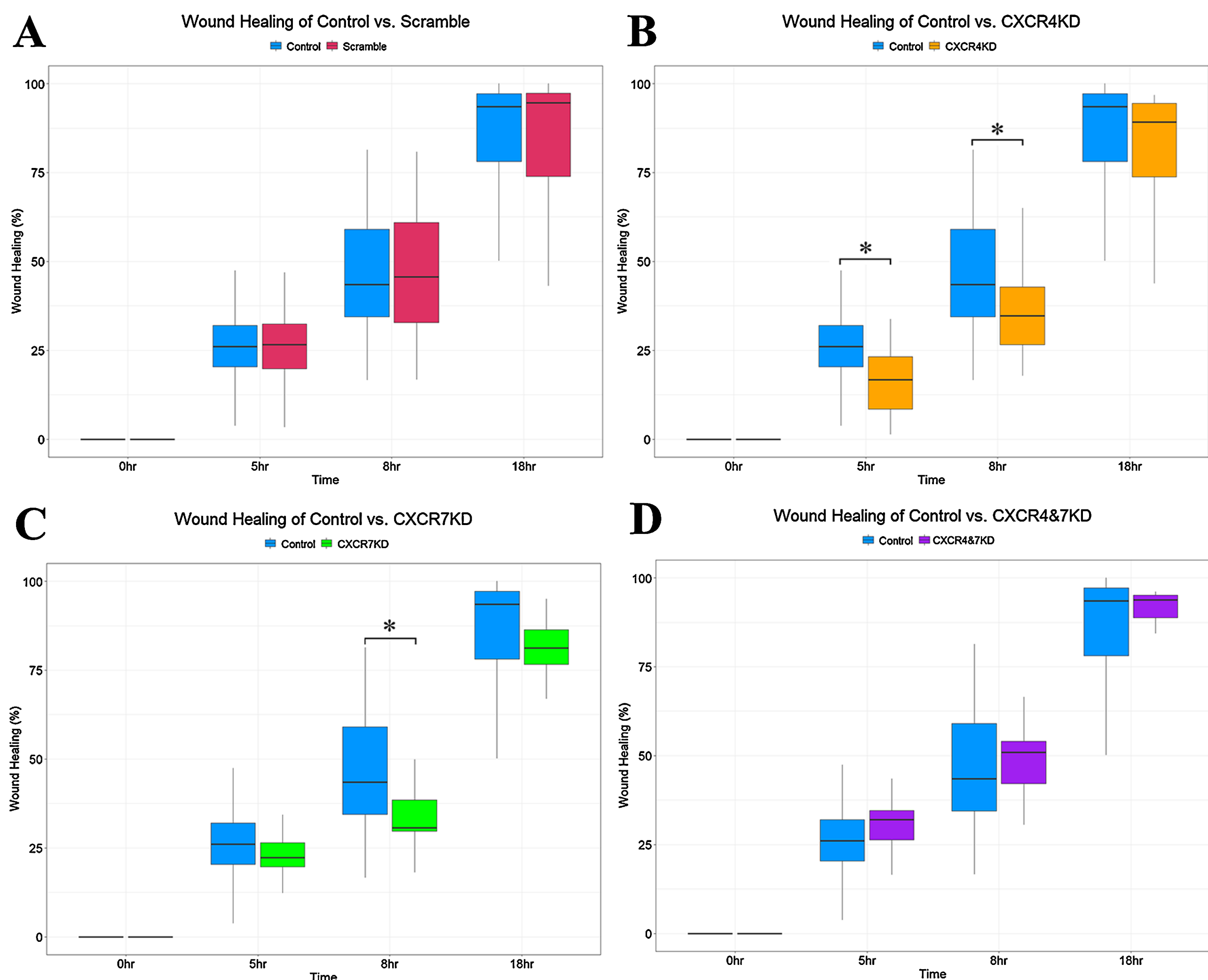


Figure 4. Effect of CXCR4/7 KD in melanoma wound healing. Bar graphs showing progressive healing of melanoma wounds at each time point over an 18-hour time frame for each set of transfections. The data starts at 0% of the wound area healed at 0 hr, and as time progresses, cells invade the wound and make the area smaller, healing the wound. Normal B16-F10 cells were compared to A. Scramble siRNA, B. CXCR4 siRNA, C. CXCR7 siRNA and D. CXCR4 and CXCR7 siRNAs. The only significant difference observed was between our control cells and CXCR4 and CXCR7 siRNA knockdown cells in the presence of SDF1. *$p < 0.05$.

this time. We observed that B16-F10 melanoma cells moved significantly more slowly after CXCR7 KD (55%) and after CXCR4 KD (45%) compared with control or scramble cells (Figure 5).

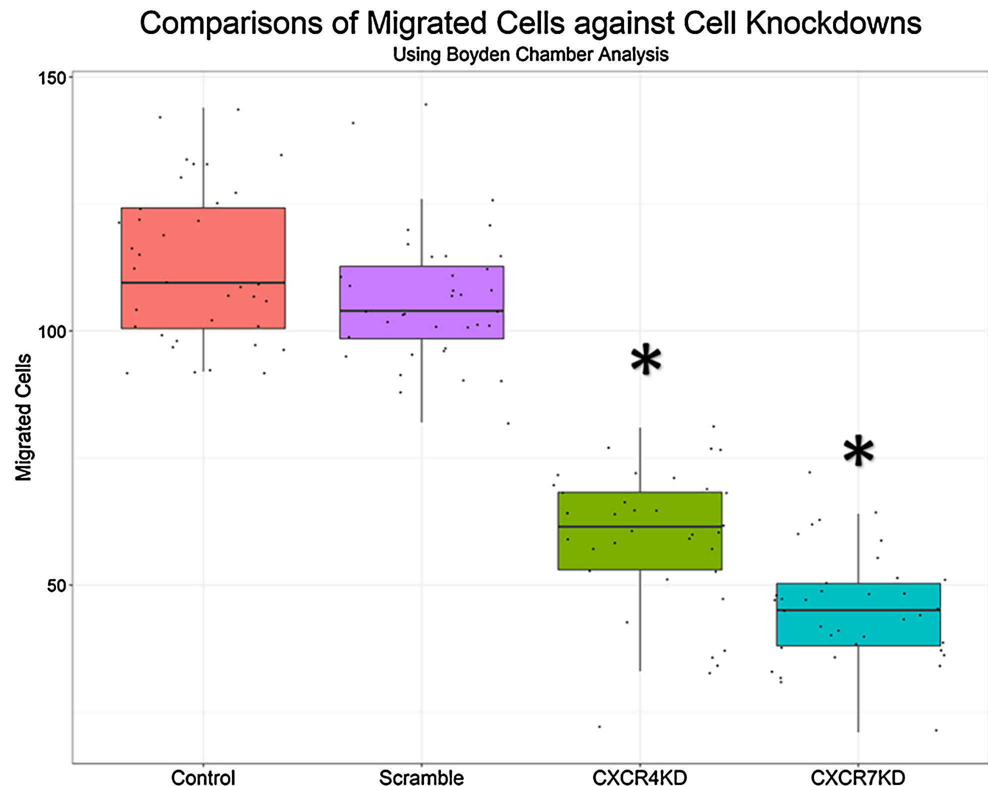
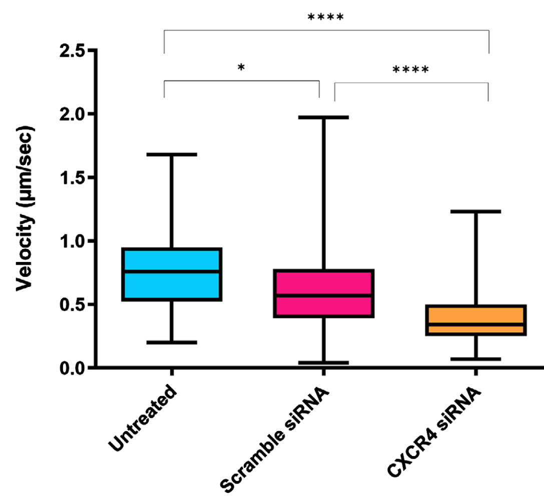


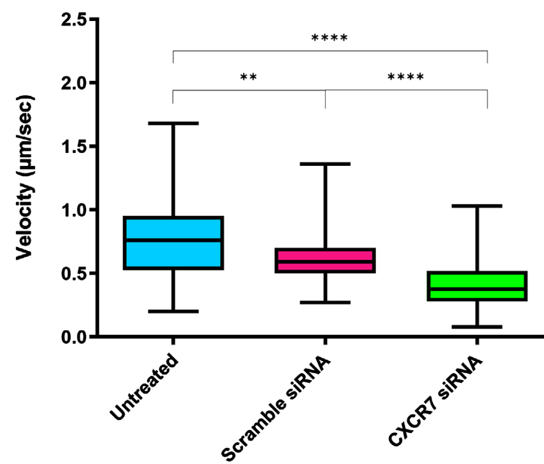
Figure 5. CXCR4/7 KD decreases melanoma cell migration. Box plot graphs show how melanoma cells decrease migration rates after CXCR4 and CXCR7 KD. Data from 5 experiments was normalized to the control values. ANOVA significant p-values are identified by the number of asterisks present: *p < 0.00001.

Cell velocity is one of the critical indicators of increased/decreased cell migration and is necessary for successful metastasis. We wanted to determine if CXCR4/7 influences melanoma migration. B16-F10 cells were transfected with scramble, CXCR4 KD, CXCR KD, or a combination of CXCR4 and 7 KD siRNA to measure a change. B16-F10 cells were then plated either in the presence of or without SDF1. Cell velocity was measured by manually tracking at least 15 cells at a time from each experiment using an ImageJ plug-in. The formula used to measure velocity in ImageJ was the following:

Velocity = (accumulated distance (um)/number of frames)

B16-F10 melanomas without SDF1 showed a 40% reduced velocity after CXCR4 KD compared with a scramble and negative controls (Figure 6A). B16-F10 CXCR4 KD cells in the presence of SDF1 only saw a 10% decrease compared to control melanoma cells with SDF1 (Figure 7A). When we KD CXCR7, we observed reduced velocity after CXCR7 KD without SDF1 compared to scramble and controls (Figure 6B), while their counterparts in the presence of SDF1 saw a





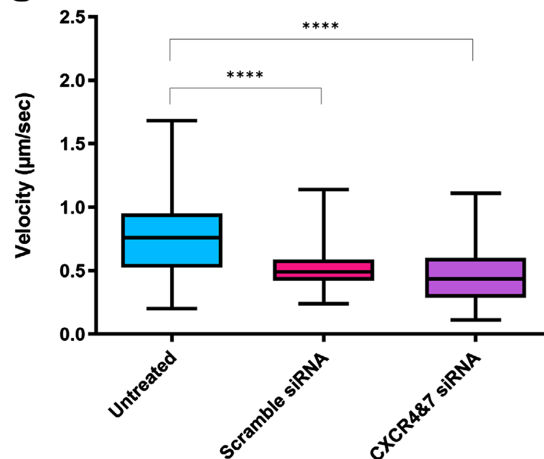


Figure 6. CXCR4/7 KD decreases B16-F10 melanoma cell velocity. Graphs represent the velocity, in µm per second, after melanoma transfection with A. CXCR4 siRNA, B. CXCR7 siRNA, and C. CXCR4 and CXCR7 siRNAs. siRNAs were compared to untreated and scrambled random siRNA. Significant p-values are identified by the number of asterisks present: $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, $^{****}p < 0.0001$.

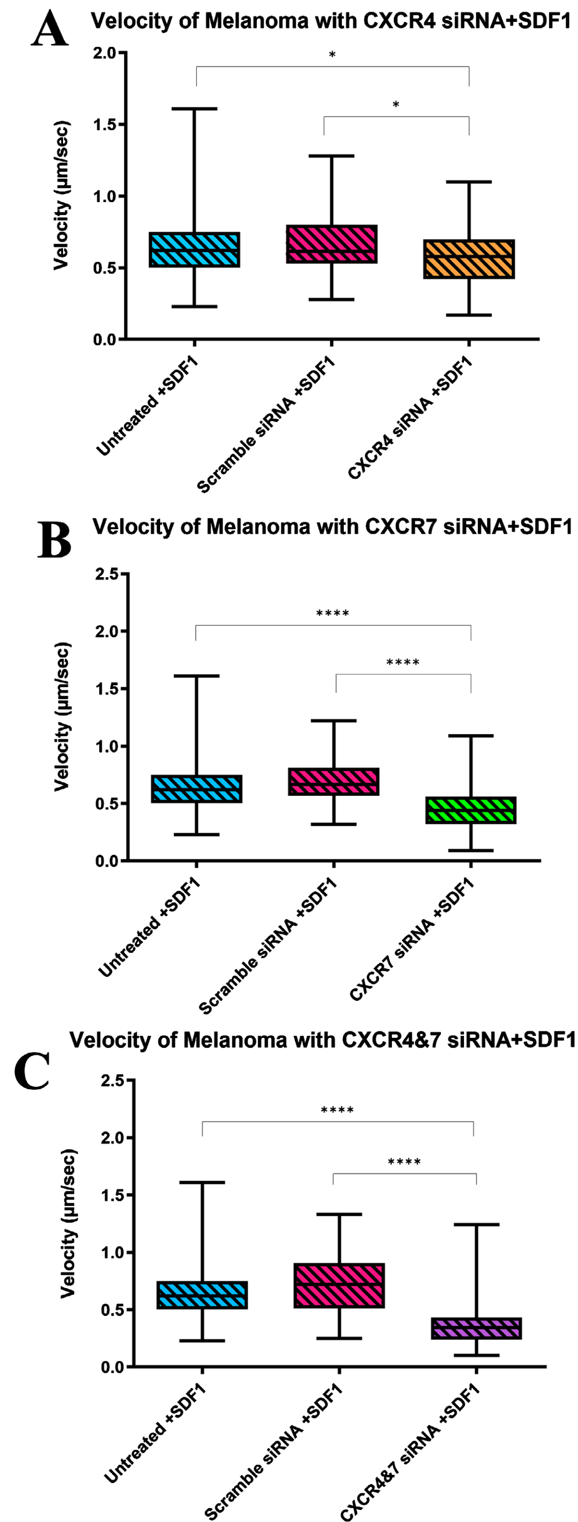


Figure 7. CXCR4/7 KD + SDF1 decreases B16-F10 melanoma cell velocity. Graphs represent the velocity, in μm per second, after melanoma transfection with A. CXCR4 siRNA, B. CXCR7 siRNA, and C. CXCR4 and CXCR7 siRNAs and the addition of SDF1. siRNAs were compared to untreated + SDF1 and scramble random siRNA +SDF1. Significant p-values are identified by the number of asterisks present: *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$.

~25% velocity reduction (Figure 7B). The double CXCR4 and CXCR7 KD without SDF1 also showed a ~33% significant velocity reduction compared with scramble and negative control (Figure 6C); B16-F10 cells with double CXCR4/7 KD and in the presence of SDF1 saw a ~48% reduction in velocity (Figure 7C). CXCR4/7 KD did inhibit an aspect of migratory behavior in B16-F10 melanoma cells, supporting our hypothesis.

Effects of CXCR siRNA Knockdowns on B16-F10 Melanoma Cell Shape

Our observation that CXCR4 and CXCR7 KD reduced B16-F10 melanoma cell migration in a significant and consistent way strongly suggested that CXCR4/7 KD was affecting the actin cytoskeleton of these cells. We looked at B16-F10 cell cytoskeletal morphology and found that B16-F10 melanoma cells had more stress fibers after CXCR7 KD than after CXCR4 KD or untreated cells (Figure 8A). We also measured B16-F10 melanoma cell sizes since they appeared to be smaller. We observed that while CXCR4 KD did not change the size of the cells compared with

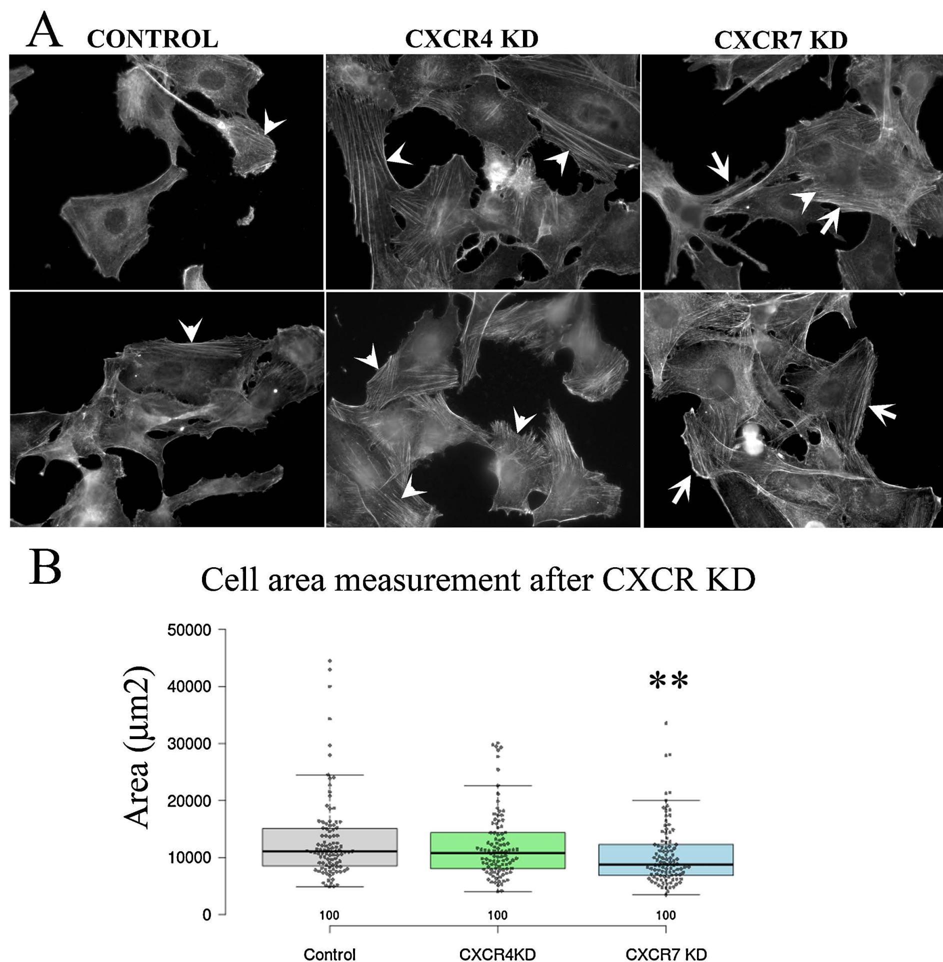


Figure 8. CXCR7 KD decreases melanoma cell size. A) Images of rat B16-F10 melanoma cell line stained with phalloidin-488 after siRNA transfection for CXCR4 or CXCR7. Arrows in CXCR7 KD point to long stress fibers in melanoma cells. B) We plotted the cells' cell size area after transfection. The ** shows the significant reduction of CXCR after siRNA (Anova less than $p < 0.0001$). The number below boxplots corresponds to many cells traced (N = 100).

untreated cells, CXCR7 KD significantly reduced their cell size by 30% (Anova $p < 0.006$) (**Figure 8B**).

4. Discussion

This study showed that the CXCR7 chemokine receptor is as relevant to melanoma cell metastasis as the more studied CXCR4. The data presented here prove that reducing the expression levels of CXCR7 has an equivalent effect to reducing CXCR4 in regulating the migratory capabilities of melanoma cells.

CXCR4 has been thoroughly studied for its links to angiogenesis, cell proliferation, and cell migration in multiple cancers, including melanoma. Typically, increased levels of CXCR4 in certain cancers have been correlated with poor prognoses [33]. However, increasing evidence also suggests that CXCR7 may play a part in cell migration, proliferation, and tumor aggressiveness [10] Fan *et al.*, 2018; [34]. Because CXCR4 has been more widely correlated with aggressive cancers, many more immunotherapy treatments and drugs are in clinical trials targeting CXCR4 versus CXCR7 [35].

In melanoma, CXCR4 expression has often been associated with poor prognoses (Scala *et al.*, 2005). Evidence suggests that high levels of CXCR4 in melanoma correlated with increased melanoma cell migration and tumor growth and survival—this increased migration led to a higher incidence of regional and distal metastases, hence the unfavorable prognoses. In summary, CXCR4 activation leads to enhanced melanoma cell migration [36]-[38].

CXCR7 has not been studied as extensively as CXCR4 in cancers, especially melanoma. Over the years, there have been many studies correlating increased expression of CXCR7 with cancer progression and metastasis in diseases, especially regarding breast, lung, and colon cancers [39]-[41]. Despite this, there is relatively little literature on the relationship of the CXCR7/SDF-1 axis in melanoma, much less experiments directly observing and targeting CXCR7 to understand these effects. One study by McConnell *et al.* found a correlation between CXCR7 and aggressive melanoma [18]. Xu and coworkers found that CXCR7 promotes melanoma tumorigenesis [42].

Previous research has shown that reducing the expression or blocking the signal of CXCR4 in melanoma significantly decreases cell migration [43] [44]. The conclusions from these two CXCR4 studies were based on transwell migration and scratch/wound assays for chemotaxis experiments. Knockdown studies for CXCR7 in melanoma used siRNA [14], CRISPR Cas-9 depletion [42] or using CXCR7 antagonists such as CCX771 [45]. The key findings from these studies were that melanoma migration and metastasis decreased after reduction of CXCR7. Being G-protein coupled receptors, CXCR4 and CXCR7 are constituents in many pathways that aid in cell movement and migration. Suppression of either of these receptors can lead to adverse effects downstream.

Our studies here show for the first time a combination of cell migration assays after receptor inhibition and receptor knockdown. We also show these findings

using two different and relevant cell migration assays for the first time: Boyden and wounds assays. Our results show that both receptors are essential for melanoma migration, but they bring light to CXCR7 as a powerful candidate for therapeutic purposes. An interesting observation was a change in cell shape after knockdowns. We observed increased stress fibers in our knockdown melanoma cells and an overall shrinkage of cell size, specifically the CXCR7 knockdown cells. Past research has shown that these thinner "stress fibers" are more conducive to cell migration [46]. Our results show that reducing the expression of CXCR4 and CXCR7 significantly decreases melanoma migratory capabilities. However, we did not observe any considerable size or cytoskeletal structural changes in our melanoma cells after the chemical inhibition of both receptors.

The crux of this project relied on the ability of both our siRNA knockdowns and the CXCR antagonists to effectively reduce or inhibit the signaling of CXCR in the B16-F10 melanoma cells. It is important to note that despite both mechanisms leading to quite similar results, the methods of delivery and the processes by which these siRNA knockdowns and CXCR antagonists cause this response in melanoma are different. siRNA is taken inside the cell through the cell membrane via a carrier, where it will target specific mRNAs and degrade them, thus inhibiting the levels of the targeted protein [47]. In contrast, the CXCR antagonists work outside the cell by binding directly to the CXCR4 or CXCR7 receptor via AMD070 (CXCR4 antagonist) or ACT-1004-1239 (CXCR7 antagonist), respectively. These molecules occupy the space where SDF-1 would bind, suppressing the GPCR-mediated responses of the CXCR receptors [48].

AMD070, also known as Mavorixafor and AMD11070, is an orally bioavailable inhibitor of CXCR4 that has successfully decreased melanoma cell migration [44] and other cancer metastases such as pancreatic cancer and leukemia [49] [50]. ACT-1004-1239, on the other hand, is a recently discovered CXCR7 antagonist that has shown promise in clinical trials, but its effects on melanoma have yet to be tested [51]. Our findings showed that AMD070 and ACT-1004-1239 significantly decreased B16-F10 melanoma cell migration in the wound and Boyden chamber assays. This corroborated previous experiments regarding the CXCR4 antagonist and explored the effectiveness of the novel CXCR7 antagonist in melanoma.

Based on our Boyden chamber analysis, our siRNA knockdowns also effectively decreased melanoma migration, but they did not elicit a significant change for our wound assays. One likely cause for this result is that transfection levels varied, and in wound assays, melanomas are near each other and secrete SDF1 which would activate the CXCR4 or CXCR7 receptor despite a successful knockdown. Meanwhile, the CXCR antagonists would prevent the binding of SDF1 to the receptors, preventing their activation.

The fact that SDF1/CXCL12 ligand has a higher binding affinity for the CXCR7 receptor compared to the CXCR4 receptor suggests that CXCR7 can also outcompete the CXCR4 receptor for access to the chemokine ligand [32]. Our structural modeling of the CXCR4:SDF1 and CXCR7:SDF1 complexes and their

binding free energy analysis have also confirmed this. Furthermore, a study identified that CXCR4 and CXCR7 work separately in some cells while they work together in others [52]. This information then raises the question of whether these two receptors could form functional heterodimers and if CXCR7 can control the CXCR4 signaling by sequestering the SDF1/CXCL12 ligand. The CXCR4 expression reduction in our CXCR7 KD and no CXCR7 expression reduction in our CXCR4 KD for melanoma suggests the possibility of CXCR4:CXCR7 heterodimers and CXCR7 monomers in melanoma cells. The structural modeling and binding affinity data suggest that CXCR7 could sequester SDF1 if these heterodimers are present, thereby reducing CXCR4's G protein signaling. Another possible result is that targeting CXCR7 might unintentionally enhance CXCR4 activity due to increased SDF1 availability for CXCR4. Therefore, we see the development of a promising therapeutic target in the signaling axis of CXCL12/CXCR4/CXCR7 as separate pathways or in combination in different cancers (including melanoma) [53]. This target is further supported by the fact that β-arrestin has a higher affinity for CXCR7 compared to CXCR4 [32] and cell chemotaxis requires β-arrestin1/2 after CXCL12 stimulation via CXCR4 [54].

5. Conclusion

Melanoma is the deadliest form of skin cancer, and often, prognoses are poor due to late-stage metastasis. GPCRs CXCR4 and CXCR7 have been extensively researched for their roles in cancer progression, including proliferation, cell migration, and eventual metastasis. Our findings show that reducing the expression of either CXCR4 and CXCR7 via siRNA transfection or CXCR antagonism leads to a significant decrease in cell migration. Our data also shows that neither reduction of CXCR4 nor CXCR7 was more effective than the other. We also demonstrated the efficacy of the novel CXCR7 inhibitor ACT-1004-1239, whose antagonistic capabilities in melanoma migration were significant. Altogether, by presenting the effectiveness of reducing CXCR4 or CXCR7 using two different delivery methods, our findings show promising results for melanoma treatment in targeting either of these receptors.

Authors' Contributions

SH, RL, ND, and TT conducted the experiments and data analysis; NK did melanoma siRNA transfection and CXCR Ab staining, wrote the manuscript, and made the figures. RC generated initial structural models of CXCR4 and CXCR7 based complexes. JG refined the structural models and computed free energies of receptor binding to SDF1. RA supervised and designed the receptor structural modeling experiments. RA, CM and MEdB supervised and designed the experiments, and wrote the manuscript.

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Data Availability

This manuscript contains all present study data. Data will be made available on request.

Conflicts of Interest

The authors declare no conflicts of interest.

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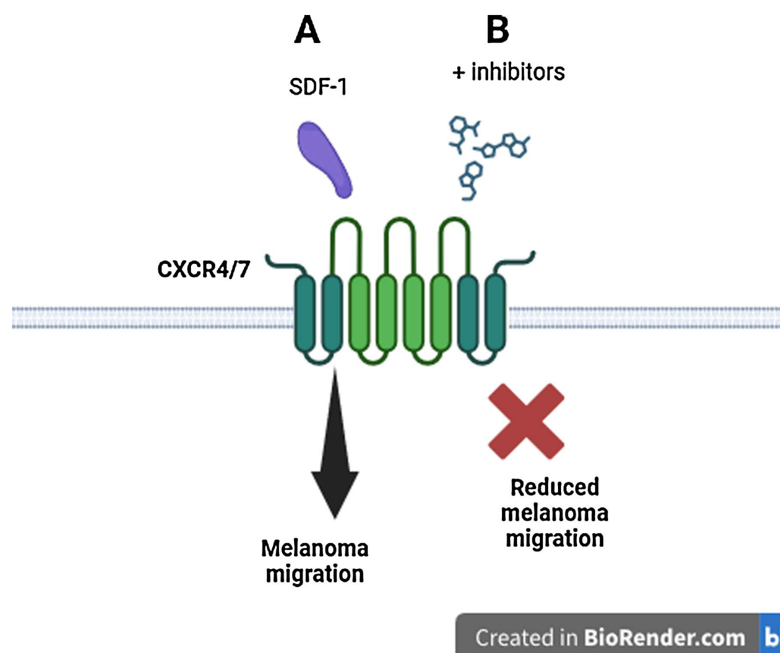
Appendix 1: Graphical Abstract



Appendix 2

Receptor-Ligand System	ΔG	stdev	Experimental K_d
CXCR4:SDF1	-211	23	40 nM
CXCR7:SDF1	-241	14	0.84 nM