CALIFORNIA STATE UNIVERSITY, NORTHRIDGE

The Roles of CXCR4 and CXCR7 in Melanocyte and Melanoma Motility

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for the degree of Master of Science in Biology

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Abstract

The Roles of CXCR4 and CXCR7 in Melanocyte and Melanoma Motility

By

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Master of Science in Biology

Chemokines are signaling proteins released by cells in response to chemical stimuli in their environment. The chemokine stromal derived factor 1 (SDF1) has been regularly studied due to its role in the growth and metastasis of multiple cancers, including melanoma. SDF1 has two known receptors: CXCR4 and CXCR7. Previous research has mainly focused on CXCR4 receptor signaling, which influences many cell responses, among them the migration of neural crest cells and the amount of receptor expression is upregulated in melanoma. CXCR7 receptor signaling is not as well studied but has been shown to influence melanocyte migration and constrain melanoma tumor growth *in vivo* where as CXCR4 could not. It is not known, however, how relevant CXCR7 may be in the capability of melanocyte and melanoma migration. Here, I studied the potential roles of CXCR4 and CXCR7 in melanocyte and melanoma migration *in vitro* by genetically silencing both receptors. My data shows that the CXCR7 receptor is more important for migratory capabilities of melanocytes and melanoma cells than the CXCR4 receptor. These findings suggest that down regulating or blocking the CXCR7 receptor through targeted therapies may show a substantial effect in melanoma treatment.

SECTION 1: INTRODUCTION

Chemokines are a specific type of cytokine that cause chemotactic responses in neighboring cells that harbor reciprocal receptors; these signaling proteins are secreted by cells to direct the movement of other cells. Chemokines are important for their role in regulating cell migration during embryonic development, during immune responses, and even during some cancer cell metastases. Specifically, stromal derived factor 1 (SDF1) is a chemokine that with its two known receptors, CXCR4 and CXCR7, plays a role in tumor growth and metastatic potential of melanoma and other cancers. My project focuses specifically on how the migration of both melanocytes and melanoma cells are affected by SDF1 and its receptors, CXCR4 and CXCR7.

Neural Crest Cell Migration

Melanocytes are the pigment cells of the body that are originally 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, off the dorsal portion of the neural tube and begin migrating quickly and extensively throughout the embryo, giving rise to their derivatives. These NCC are highly migratory mesenchymal stem cells that will contribute to the formation of many diverse structures: connective tissue, cartilage and bone, neurons, glia, as well as the pigment cells known as melanocytes (Baggiolini et al., 2015).

The role that SDF1 has on NCC derivatives via its CXCR4 and CXCR7 receptor pathways, have been well studied. Most of the studies have focused on the SDF1/CXCR4

pathway and its importance in the regulation of NCC migration during embryonic development (Balmadani et al., 2005). One study used Zebrafish embryos that were mutant for the CXCR4 receptor and found that the receptor was vital for the proper migration of NCC, specifically for the proper development of the craniofacial features. However, this study did not find any effect after mutating CXCR7 in Zebrafish cranial NCC (Killian et al., 2009). Experiments in chicken showed SDF1 as a chemoattractant for trunk NCCs and that specifically CXCR4 signaling pathway was critical for guiding the specific migration of sympathetic ganglia precursors (Kasemeier-Kulesa et al., 2005, 2006, 2010; Saito et al., 2012). These and other studies lead scientists to conclude that SDF1/CXCR4 signaling is required for NCC guidance and migration.

Melanocytes

Melanocytes are the cells responsible for making melanin, providing crucial protective pigmentation against ultraviolet radiation that can have damaging effects on DNA throughout the body (D'Orazio et al., 2013). It is well known that too much ultraviolet radiation can damage the melanocytes, resulting in skin cancer known as melanoma (Fears et al., 2002). Healthy skin melanocytes have a way of responding to ultraviolet radiation through signaling mechanisms, resulting in the up regulation of pigmentation as well as the activation of DNA repair pathways (Abdel-Malek et al., 2010).

The migration of melanocyte precursors after NCC delaminate from the neural tube is characterized by their entering what is usually referred to as the dorsolateral pathway (Larue et al., 2003) (see Figure 1). These cells enter the space between the ectoderm and dorsal surface of the somites, while dividing and spreading rapidly throughout the skin of

the developing embryo. Eventually, these precursors will differentiate into mature melanocytes (Luciani et al., 2011).

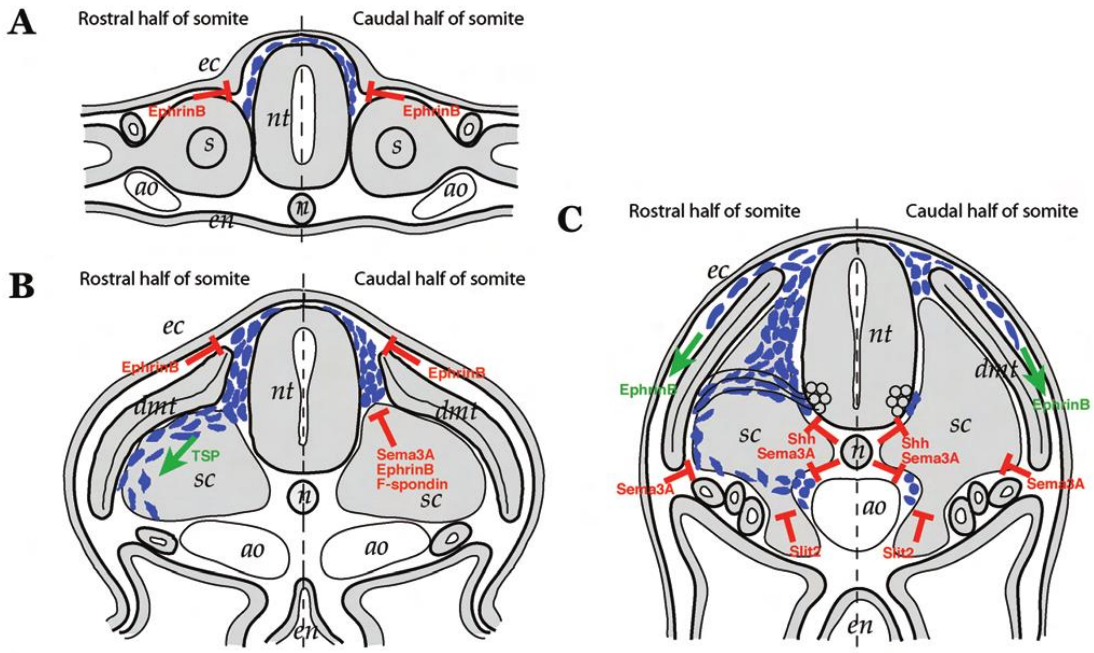

Figure 1: Neural crest cell delamination and migration during embryogenesis. Neural crest cells, shown in blue, migrate through different areas in the embryo. The cells migrating under the ectoderm (ec) will become melanocytes. Source: Neural Crest Delamination and Migration by Jean-Loup Duband, in Neural Crest Induction and Differentiation pp 45-77.

Melanocyte precursors, known as melanoblasts, require specific molecules for proper directed migration and development into mature melanocytes, including growth factors, enzymes and proteins. This includes MGF/KIT, Bace2, Prex1, Cdc42, MITF, and Fasein1 (Wehrle-Haller et al., 2001; Bebber et al., 2013; Lindsay et al., 2011; Woodham et al., 2017; Li et al., 2011; Ma et al., 2013). A loss or mutation of any of these molecules

causes the same consequence: slow and inconsistent migration, as well as hypopigmentation and skin discoloration.

Along with its chemokine role in NCC migration, SDF1 also plays a key chemoattractant role in melanocyte migration. SDF1 is capable of inducing melanocyte directed migration when added at moderate and high concentrations in *in vitro* chemotaxis chambers (10-200ng/ml). In these experiments, they observed that blocking CXCR7 via antibodies showed a significant decrease in melanocyte migration, while blocking CXCR4 had no effect on migration. These findings suggest that SDF1/CXCR7 pathway plays a vital role in melanocyte migration of melanocytes (Lee et al. 2013).

Chemokines and Melanoma

As previously mentioned, chemokines get their name because they are chemotactic cytokines. “Chemo” comes from chemotactic, referring to movement in response to a chemical stimulus. While “kines” from cytokines, signaling proteins secreted by cells. A trademark of chemokine is that they can induce directed chemotaxis from nearby responsive cells. The SDF1 chemokine that my project focuses on happens to be one of the most evolutionarily conserved chemokine-signaling systems, using receptors CXCR4 and CXCR7 (DeVries et al., 2006). SDF1 has been regularly studied due to its role in promoting growth and metastasis in multiple human cancers, and we desire to look further into its mechanistic pathways. One of these SDF1-responsive cancers includes melanoma, a deadly skin cancer.

Melanoma is the most dangerous type of skin cancer due to its low 5 year survival rate. According to the National Cancer Institute, in 2017 there were nearly 10,000 deaths

due to melanoma. The majority of risk factors for melanoma are out of our control, including exposure to ultraviolet light, fair skin, family history, older age, and sex. Although these risk factors make some people at higher risk, melanoma can affect anyone regardless of age, sex, or race. Individuals with fair complexions are diagnosed more frequently than those with darker complexions. Interestingly, individuals with darker complexions are more likely to be diagnosed at more advanced stages (Wang et al. 2016). As melanoma progresses, it changes from a localized blemish to a metastatic tumor that can travel throughout the body. The early stages where the melanoma is localized can be easily treated via excision. However, as the stages progress and the cancer migrates, the treatment is much more difficult and intense. Current therapies include radiating the entire body via chemotherapy. Therefore, identifying the factors involved in the onset of melanocyte transformation into melanoma is important for future clinical applications to catch the cells before they become malignant.

Typical CXCR4 versus Atypical CXCR7 Receptors

SDF1 signals through two specific receptors, CXCR4 and CXCR7, which are very similar in structure and are embedded in the cellular membrane (Bleul et al., 1996; Moser et al, 2004). These receptor structures each consist of seven transmembrane domains, resulting in three extracellular loops and three intracellular loops. The extracellular loops bind to the chemokine SDF1 while the intracellular loop regions bind to signal molecules known as G-proteins, in order to activate the next pathway (Wang et al., 2014). A 2014 study showed that SDF1 binding to CXCR4 initiated signaling pathways through the recruitment of both G-proteins and another regulating protein: β-arrestin. However, in the

same study, CXCR7 was found to be an atypical chemokine receptor as it did not activate G-proteins in response to SDF1, but it could signal through β-arrestin pathways (Coggins et al., 2014). They found that after binding SDF1, the CXCR7 receptor actually had an eight-fold higher binding affinity to β-arrestin compared to the CXCR4 receptor. This meant that SDF1 binds to CXCR7 with a higher affinity compared to CXCR4, even up to approximately fifty-fold greater affinity for the CXCR7 receptor compared to that for the CXCR4 receptor (Balabanian et al., 2005; Burns et al., 2006).

The fact that CXCR7 receptor has a higher binding affinity for both SDF1 and β-arrestin strongly suggests that it also has the ability to out-compete the CXCR4 receptor for access to both the chemokine ligand, as well as the signaling partner. Furthermore, a study was able to identify that CXCR4 and CXCR7 work separately in some cells, while they work together in others (Puchert et al., 2014). This information then raises the question whether these two receptors could form functional heterodimers and if CXCR7 can control the CXCR4 signaling by sequestering the SDF1 ligand and/or arrestin. Therefore, we see the development of a very promising target in the signaling axis of SDF1/CXCR4/CXCR7, as either separate pathways or in combination, in different cancers (including melanoma) (Hattermann et al., 2013).

This project involves a collaboration with Dr. Abrol's laboratory at CSUN, that has modified *in silico* the CXCR receptors in order to create two specific structures for each receptor that will bind to either G-protein or β-arrestin (Figure 2).

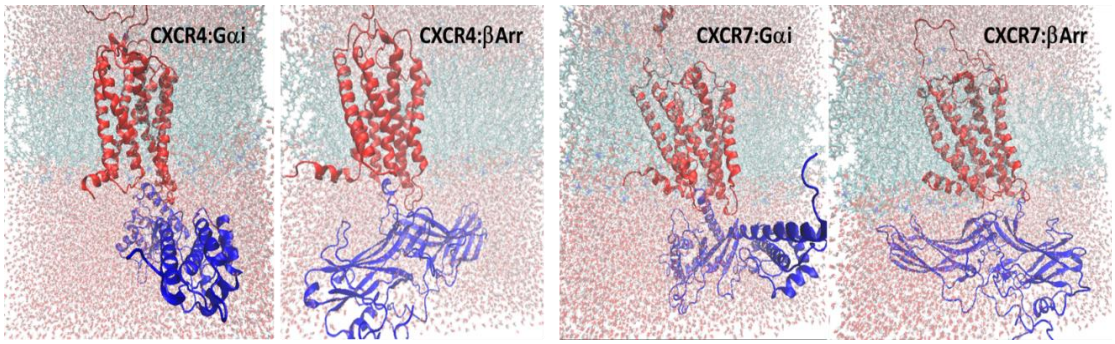

Figure 2: Molecular Dynamics Simulation of CXCR protein complexes (red) embedded in a lipid bilayer with bound regulating protein, either G protein or β-arrestin, in intracellular matrix (blue)

Implications of Studying the Role of CXCR4/7 in the Great Picture of Cancer Metastasis

Previous research has looked at the possible influences that SDF1/CXCR4 and SDF1/CXCR7 pathways 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. They were able to determine that the loss of functional CXCR7 had the ability to constrain the 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 (Décaillot et al., 2011). These recent studies are just some examples that show the importance of both SDF1/CXCR4 and SDF1/CXCR7 mediated pathways in melanocyte migration and cancer. Accordingly, a better understanding of the mechanisms behind the interactions of SDF1 with its CXCR4 and CXCR7 receptors, and its role in the onset of melanoma cancer is necessary to take another step toward developing any type of clinical treatment.

SECTION 2: GOAL/HYPOTHESIS

The main goal of this project was to identify if manipulation of CXCR4 and CXCR7 chemokine receptors would contribute to a malignant transformation of melanocytes or to the amelioration of melanoma phenotype using a melanoma cell line and primary melanocytes. I hypothesize that: A) down regulation and inhibition of the CXCR7 receptor will show a greater change in the phenotype of the melanoma than the down regulation and inhibition of the CXCR4 receptor in causing the cancerous melanoma develop a less aggressive phenotype. B) Down regulation and inhibition of the CXCR7 receptor will show a greater change in the phenotype of the melanocytes than the down regulation and inhibition of the CXCR4 receptor in causing the noncancerous melanocyte develop a more malignant phenotype, one resembling melanoma cells.

SECTION 3: MATERIALS AND METHODS

Two cells lines were used for this project, a mouse melanoma B16-F0 cell line was purchased from ATCC and a Human Epidermal Melanocyte (HEM) cell line was purchased from Cell Applications. Media used to feed the mouse melanoma cells consisted of DMEM, 5% FBS, 1x Pen/Strep, and 1x L-Glutamine, while the media for the human melanocytes cell line was an all-in-one use growth media purchased from the same company as the melanocytes. The assays performed recorded the motility of both cell lines via live cell imaging of (i) simple movement among these cells where their motility could be tracked, and (ii) wound assays to quantify how these cell lines reacted to an outside influence. Then, these experiments were repeated after manipulation of the CXCR4 and CXCR7 proteins, specifically through loss of function assays where I transfected the cells with siRNA to silence either one or both receptors. Each experimental condition was assessed by qPCR to examine at the gene expression of both CXCR4 and CXCR7.

Movement Assay: Cells were plated in small dishes at 80,000 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. When SDF1 addition was required, 200ng SDF1 was added to the media and allowed to incubate for one hour. When no SDF1 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 the microscope, a video was initiated and began taking snapshots every 90 seconds for approximately four hours (approximately 161 frames per video). The cell movement could then be tracked

using ImageJ software, where the manual tracking tool was used to track approximately 20 cells per video. 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 are as follows:

- Velocity = Accumulated Distance/Number of Frames
- Persistence = Displacement/Accumulated Distance
- Chemotaxic Index = Y-Axis Displacement/Accumulated Distance

Wound Assay: Cells were plated in a multi-well plate at 120,000 cells/mL one to two days before the experiment began. Once the cells had reach ~90-100% confluency, 200ng SDF1 was added to the proper wells and allowed to incubate for one hour prior to wounding. At Hour 0, each well was wounded 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 – where the area left unhealed was measured using a wound healing tool in ImageJ.

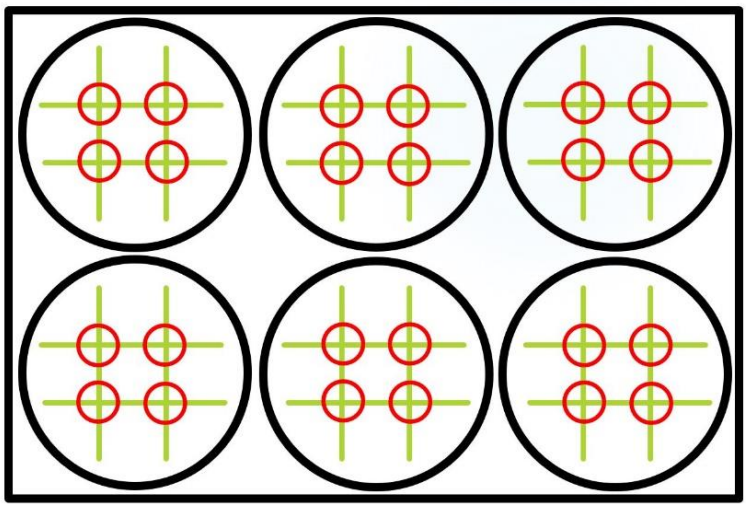

Figure 3: Outline of a wound assay performed on a 6-well plate. The green lines indicate the wound itself. The red circles indicate the crossed sections of the wound where images were taken at different timepoints throughout the 18 hour experiment.

siRNA Transfection: CXCR4 siRNA was purchased from OriGene while CXCR7 siRNA was purchased from Eupheria Biotech. Lipofectamine 3000 was used to transfect the siRNA complexes into the cells that would then fluoresce green with GFP if successfully transfected. Transfections included both a siRNA that would inhibit the gene, as well as a scramble siRNA control that would transfect into the cell but not affect the target gene. HEK293T cells were also transfected simultaneously as a positive control. The transfection incubated for 48-72 hours but was only usable for experimentation for approximately 3-4 days post incubation. For each transfection, the three days of experimentation included 2 wound assays and 8 movement assays: 2 knockdowns, 2 scrambles, 2 knockdowns +SDF1 expression construct, and 2 scrambles +SDF1 expression construct.

Preparation for Wound and Movement Assays: After the siRNA transfection had incubated for 48-72 hours, the cells were split and prepared for the three days' worth of movement and wound assays. For the wound assays, cells were plated at 120,000 cells/mL

and assay was performed two days later in order to obtain a single monolayer of cells to wound. For the movement assays, for day one of experiments, the melanocytes and melanoma cells were plated at 80,000 cells/mL in order to obtain a confluency of approximately 40-50%. For day two and day three movements, the two cell lines were plated at different concentrations since the melanoma cell line grew much faster than the primary cell line. Therefore, day two movements of melanocytes were plated at 50,000 cells/mL and day three movements plated at 40,000 cells/mL. On the other hand, the melanoma proliferated so quickly that day two movements were plated at 30,000 cells/mL and day three movements plated 20,000 cells/mL.

RNA Purification: After each 18-hour wound assay, the cells from each well were collected and labeled according to each condition. GeneJET RNA Purification Kits were purchased from ThermoFisher Scientific. The protocol used was from the kit's user guide: Mammalian Cultured Cells Total RNA Purification Protocol for adherent cells. If purification could not be performed immediately, the cells were frozen after step 2 in the lysis buffer supplemented with β-mercaptoethanol until the rest of the protocol could be completed. Upon completion of RNA purification, the concentration of each RNA sample was calculated using a NanoDrop 2000, also a product of ThermoFisher Scientific. The samples were then stored at -20^{o}C until cDNA conversion could be performed.

cDNA Conversion: Purchased from ThermoFisher Scientific, SuperScript VILO cDNA Synthesis Kit was used for cDNA conversion of the RNA samples. The protocol was

followed as described in the product info sheet, using a total reaction volume of 20μL. Using the concentration given by the NanoDrop 2000, the total amount for each RNA sample (in μL) was calculated by allowing the total concentrations in the reaction volume to be the same (usually 500ng/μL). Since the NanoDrop 2000 cannot calculate cDNA concentrations, this was the best way to ensure the total concentration of each individual tube would be similar, if not the same, in order to proceed to qPCR analysis.

qPCR Analysis: All primers were purchased from Integrated DNA Technologies, Maxima SYBR Green/ROX qPCR Master Mix and clear 96-well plates purchased from ThermoFisher Scientific, and Bio-Rad CFX96 Real-Time PCR Thermocycler. I used primer sequences from published papers that had previously been found to be successful (Maishi et al., 2012; Sierro et al., 2007; Liang et al., 2001) (See Table 1). GAPDH was the housekeeping gene used to normalize the gene expression. As for the protocol, I was able to optimize the reaction volume to 15μL. In each well, the reaction included the following components: 5μL of SYBR Green, 0.5μL of 10μM forward primer, 0.5μL of 10μM reverse primer, 5μL of template cDNA, and 4μL of qPCR grade water. A master mix of all components, minus template cDNA, was made for each set of primers, and 15μL of the master mix was added to each well. The template cDNA was added separately as a dilution was required prior to its addition into the well. The template cDNA for each condition was diluted to 50ng/μL and 5μL was added to each well for a total concentration of 250 ng/μL per reaction. The plate was then sealed, centrifuged and placed into the thermocycler. The thermocycler was programmed based off a three-step cycling protocol and melting curve

analysis, both of which could be found in the user guide for the Maxima SYBR Green/ROX qPCR Master Mix (Figure 4).

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'

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

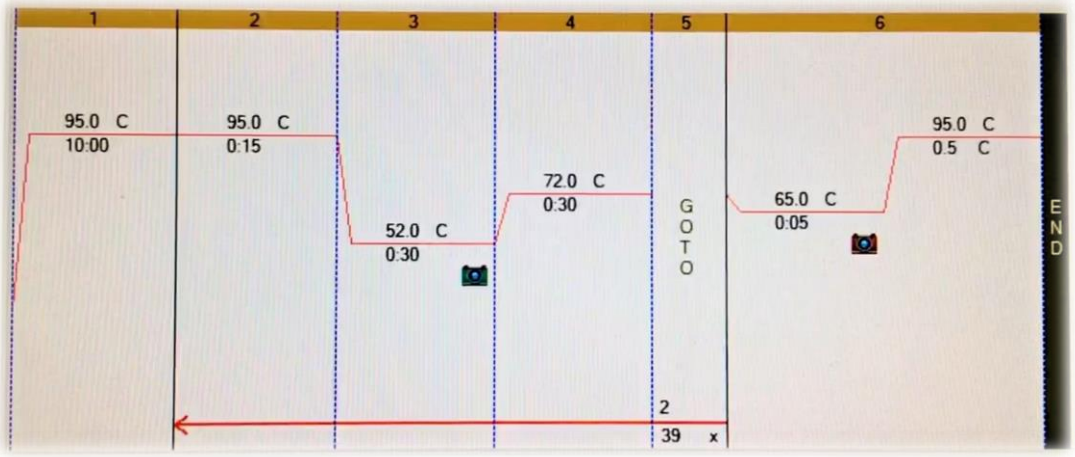

Figure 4: The thermocycler program of qPCR including the three-step cycling protocol as well as melting curve analysis

Human CXCR4

AACTTCAGTTTGTTGGCTGCGGCAGCAGGTAGCAAAGTGACGCCGAG
GGCCTGATGCTCCAGTAGCCACCGCATCTGGAGAACCAGCGGTTACC
ATGGAGGGGATCAGTGTAAGTCCAGTTTCAACCTGCTTTGTCATAAAT
GTACAAACGTTTGAACTTAGAGCGCAGCCCCCTCTCCGAGCGGGCAGA
AGCGGCCAGGACATTGGAGGTACCCGTACTCCAAAAAAGGGTCACCG
AAAGGAGTTTTCTTGACCATGCCTATATAGTGCGGGTGGGTGGGGGG
GGAGCAGGATTGGAATCTTTTTCTCTGTGAGTCGAGGAGAAACGACTG
GAAAGAGCGTTCCAGTGGCTGCATGTGTCTCCCCCTTGAGTCCCGCC
GCGCGCGGCGGCTTGCACGCTGTTTGCAAACGTAAGAACATTCTGTG
CACAAGTGCAGAGAAGGCGTGCGCGCTGCCTCGGGACTCAGACCAC
CGGTCTCTTCCTTGGGGAAGCGGGGATGTCTTGGAGCGAGTTACATT
GTCTGAATTTAGAGGCGGAGGGCGGCGTGCCTGGGCTGAGTTCCCAG
GAGGAGATTGCGCCCGCTTTAACTTCGGGGTTAAGCGCCTGGTGACT
GTTCTTGACACTGGGTGCGTGTTTGTTAAACTCTGTGCGGCCGACGGA
GCTGTGCCAGTCTCCCAGCACAGTAGGCAGAGGGCGGGAGAGGCGG
GTGGACCCACCGCGCCGATCCTCTGAGGGGATCGAGTGGTGGCAGC
AGCTAGGAGTTGATCCGCCCCGCGCGCTTTGGGTTTGAGGGGAAAAC
CTTCCCGCCGTCCGAAGCGCGCCTCTTCCCCACGGCCGCGAGTGGG
TCCTGCAGTTCGAGAGTTTGGGGTCGTGCAGAGGTCAGCGGAGTGGT
TTGACCTCCCCTTTGACACCGCGCAGCTGCCAGCCCTGAGATTTGCG
CTCCGGGGATAGGAGCGGGTACGGGGTGAGGGGCGGGGGCGGTTA
AGACCGCACCTGGGCTGCCAGGTCGCCGCCGCGAAGACTGGCAGGT
GCAAGTGGGGAAACCGTTTGGCTCTCTCCGAGTCCAGTTGTGATGTTT
AACCGTCGGTGGTTTCCAGAAACCTTTTGAAACCCTCTTGCTAGGGAG
TTTTTGGTTTCCTGCAGCGGCGCGCAATTCAAAGACGCTCGCGGCGG
AGCCGCCCAGTCGCTCCCCAGCACCCTGTGGGACAGAGCCTGGCGT
GTCGCCCAGCGGAGCCCCTGCAGCGCTGCTTGCGGGCGGTTGGCGT
GGGTGTAGTGGGCAGCCGCGGCGGCCCGGGGCTGGACGACCCGGC
CCCCCGCGTGCCCACCGCCTGGAGGCTTCCAGCTGCCCACCTCCGG
CCGGGTTAACTGGATCAGTGGCGGGGTAATGGGAAGCCACCCGGGA
GAGTGAGGAAATGAAACTTGGGGCGAGGACCACGGGTGCAGACCCC
GTTACCTTCTCCACCCAGGAAAATGCCCCGCTCCCTAACGTCCCAAAC
GCGCCAAGTGATAAACACGAGGATGGCAAGAGACCCACACACCGGAG
GAGCGCCCGCTTGGGGGAGGAGGTGCCGTTTGTTCATTTTCTGACAC
TCCCGCCCAATATACCCCAAGCACCGAAGGGCCTTCGTTTTAAGACCG
CATTCTCTTTACCCACTACAAGTTGCTTGAAGCCCAGAATGGTTTGTAT
TTAGGCAGGCGTGGGAAAATTAAGTTTTTGCGCTTTAGGAGAATGAGT
CTTTGCAACGCCCCCGCCCTCCCCCCGTGATCCTCCCTTCTCCCCTCT
TCCCTCCCTGGGCGAAAAACTTCTTACAAAAAGTTAATCACTGCCCCT
CCTAGCAGCACCCACCCCACCCCCCACGCCGCCTGGGAGTGGCCTCT
TTGTGTGTATTTTTTTTTTCCTCCTAAGGAAGGTTTTTTTTCTTCCCTCT
AGTGGGCGGGGCAGAGGAGTTAGCCAAGATGTGACTTTGAAACCCTC
AGCGTCTCAGTGCCCTTTTGTTCTAAACAAAGAATTTTGTAATTGGTTC
TACCAAAGAAGGATATAATGAAGTCACTATGGGAAAAGATGGGGAGGA
GAGTTGTAGGATTCTACATTAATTCTCTTGTGCCCTTAGCCCACTACTT
CAGAATTTCCTGAAGAAAGCAAGCCTGAATTGGTTTTTTAAATTGCTTT
AAAAATTTTTTTTAACTGGGTTAATGCTTGCTGAATTGGAAGTGAATGT
CCATTCCTTTGCCTCTTTTGCAGATATACACTTCAGATAACTACACCGA
GGAAATGGGCTCAGGGGACTATGACTCCATGAAGGAACCCTGTTTCC
GTGAAGAAAATGCTAATTTCAATAAAATCTTCCTGCCCACCATCTACTC
CATCATCTTCTTAACTGGCATTGTGGGCAATGGATTGGTCATCCTGGT
CATGGGTTACCAGAAGAAACTGAGAAGCATGACGGACAAGTACAGGC
TGCACCTGTCAGTGGCCGACCTCCTCTTTGTCATCACGCTTCCCTTCT
GGGCAGTTGATGCCGTGGCAAACTGGTACTTTGGGAACTTCCTATGCA
AGGCAGTCCATGTCATCTACACAGTCAACCTCTACAGCAGTGTCCTC
ATCCTGGCCTTCATCAGTCTGGACCGCTACCTGGCCATCGTCCACGC
CACCAACAGTCAGAGGCCAAGGAAGCTGTTGGCTGAAAAGGTGGTC
TATGTTGGCGTCTGGATCCCTGCCCTCCTGCTGACTATTCCCGACTTC
ATCTTTGCCAACGTCAGTGAGGCAGATGACAGATATATCTGTGACCG
CTTCTACCCCAATGACTTGTGGGTGGTTGTGTTCCAGTTTCAGCACAT
CATGGTTGGCCTTATCCTGCCTGGTATTGTCATCCTGTCCTGCTATTG
CATTATCATCTCCAAGCTGTCACACTCCAAGGGCCACCAGAAGCGCA
AGGCCCTCAAGACCACAGTCATCCTCATCCTGGCTTTCTTCGCCTGTT
GGCTGCCTTACTACATTGGGATCAGCATCGACTCCTTCATCCTCCTGG
AAATCATCAAGCAAGGGTGTGAGTTTGAGAACACTGTGCACAAGTGGA
TTTCCATCACCGAGGCCCTAGCTTTCTTCCACTGTTGTCTGAACCCCAT
CCTCTATGCTTTCCTTGGAGCCAAATTTAAAACCTCTGCCCAGCACGC
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Mus musculus CXCR4

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Figure 5: Human and Mouse gene sequences for CXCR4 with qPCR primers listed in Table 1 highlighted for reference.

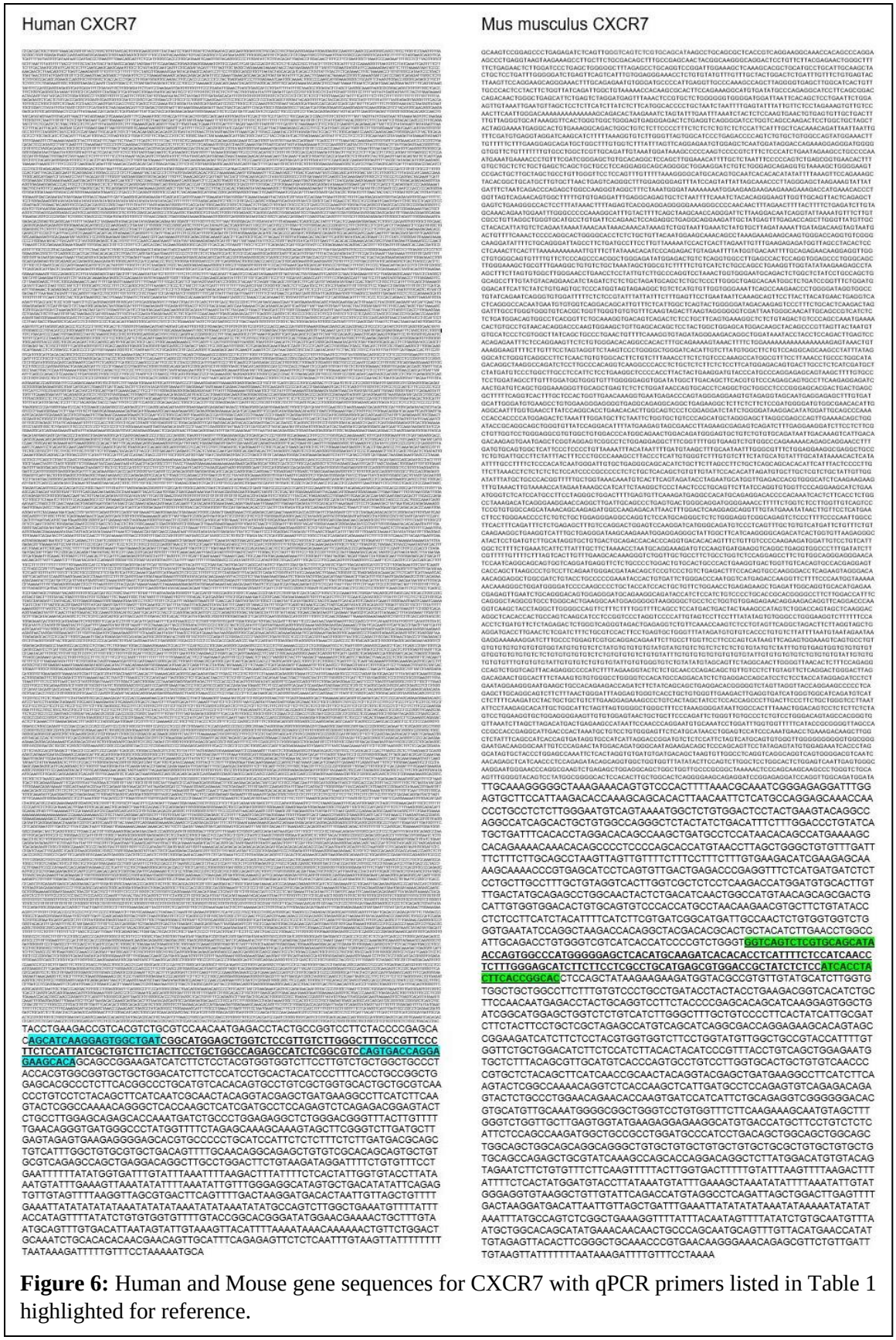

Figure 6: Human and Mouse gene sequences for CXCR7 with qPCR primers listed in Table 1 highlighted for reference.

Statistical Analysis: For the analysis of the movement 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 I only compared each cell line 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 (Haimes & Kelley).

SECTION 4: RESULTS

The first question in my thesis was do CXCR4 and CXCR7 play the same roles in melanocytic cells? In order to answer this question, the first step was to look at the level of expression of the two receptors in both cells: melanocytes and melanoma, as well as the "parental" cell, the NCC. In order to do so, I performed qPCR on these three related cells by calculating the amount of expression of the mRNA after normalizing qPCR results to GAPDH ΔΔCt values (Figure 4).

The results from qPCR for both receptors showed that NCC have higher levels of expression than melanocytes or melanoma (56.8% of CXCR4 mRNA and 78.6% of CXCR7 mRNA compared to GADPH). Melanocytes showed the least expression of both mRNAs, having 46.5% of CXCR4 and 45.2% of CXCR7. While melanoma cells showed a similar expression of the CXCR4 receptor as the NCC (56.3%) and 54.1% of CXCR7. This indicated that the three types of cells I worked with all have good levels of expression for both receptors.

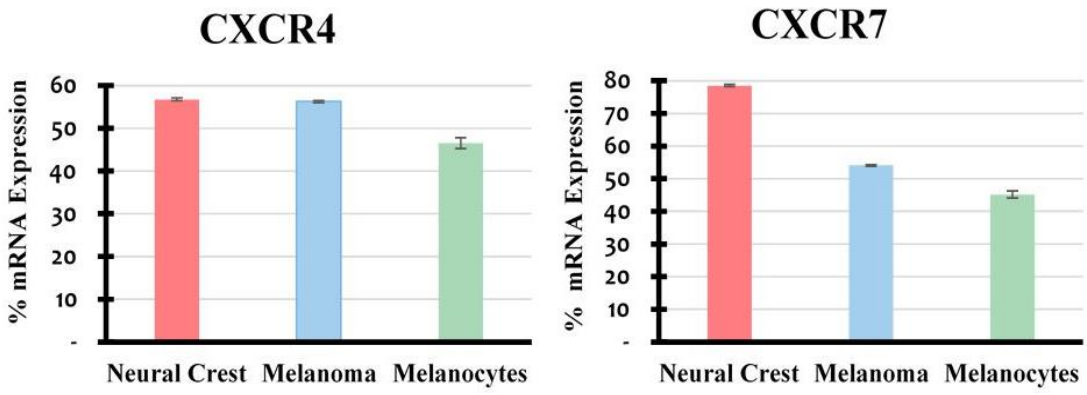

Figure 7: qPCR analysis comparing the gene expression of SDF1 protein receptors CXCR4 and CXCR7 in Neural Crest Cells: SpL201 (red), Melanoma: B16-F0 (blue), and Melanocytes: HEM (green).

Effects of CXCR siRNA Knockdowns on Melanocyte and Melanoma Velocity

The next steps in looking at the role of these two receptors in melanocytic cell behavior was to determine if CXCR receptors affected melanocyte and melanoma motility. If CXCRs have a role in cell migratory behavior reducing or deleting them will change their migratory capabilities, which can be quantified by measuring cell velocity. These experiments consisted of transfecting cells with siRNA and live imaging them to gather cells motility parameters.

Here I live cell imaged these cells after transfecting them with siRNA for CXCR4 and/or CXCR7. From now on I will refer to cells transfected with siRNA as knockdown (KD) for each receptor. Cell movement was analyzed by manually tracking at least 15 cells at a time from each experiment using ImageJ plug-in from Ibidi website (Asano and Horn, 2013). This plug-in generated standard cell migration parameters: velocity, distance, persistence and chemotaxis index.

In analyzing each of these parameters, I found that within each CXCR KD condition there was a significant change when comparing the untreated cells and the scramble siRNA condition, as well as the untreated +SDF1 and the scramble siRNA +SDF1 condition (Figures 6-8). This observation of a difference between control scramble siRNA and untreated cells is likely a result of the cells being exposed to the transfection conditions for 24-48 hours. Therefore, I focused on the CXCR siRNA KD conditions compared strictly to the scramble siRNA condition. However, all significant change is noted in the figures for reference. Additionally, KD of CXCR4 resulted in some off target KD of CXCR7 in both melanocytes and melanoma, and both values will be denoted in each figure. In the same way, KD of CXCR7 resulted in extreme off target KD of CXCR4 in

both melanocytes and melanoma, and thus the KD of CXCR7 in actuality is a KD of both CXCR4 and CXCR7.

Velocity, the first migratory parameter I looked at examines the speed and direction travelled by the cells, these are considered indicators of metastatic capabilities of cells. The results of velocity changes in melanocytes showed that CXCR4 and CXCR7 KD siRNA (76% KD CXCR4 and 39% KD CXCR7; 70% KD CXCR7 and 97% KD CXCR4) have a strong effect compared with the scramble siRNA (Figure 6A and 6C). As expected, adding SDF1 to melanocytes showed a small significant increase in both the scramble siRNA transfections as well as the CXCR siRNA KDs velocity. Melanocyte cell velocities nearly doubled compared to the scramble siRNA condition when CXCR4 or CXCR7 were knocked down (Figure 6A and 6C, Table 2. The addition of SDF1 on top of the CXCR7 KD stimulated the cells even more, causing the melanocyte velocity to more than double compared to both the scramble siRNA +SDF1 condition. Finally, the double KD for CXCR4 and CXCR7 (74% KD CXCR4 and 73% KD CXCR7) showed similar results as to the CXCR7 single KD condition. The only difference noted between the single CXCR7 KD condition and the double KD, is that the double KD showed the effects to a lesser degree when comparing the CXCR4&7 KD conditions to the scramble conditions with or without SDF1.

Next, I looked at the velocity of melanoma cells after transfection of CXCR4 or CXCR7 siRNA (35% KD CXCR4 and 49% KD CXCR7; 56% KD CXCR7 and 90% KD CXCR4). The first observation was that adding SDF1 to melanoma cells caused small but significant decrease in their velocity of untreated versus scramble CXCR KD (Figure 6, Table 3). Second, it was noticeable that KD of CXCR4 or CXCR7 caused a reduction in

melanoma overall velocity. In contrast with melanocytes, the double CXCR4 and CXCR7 KD in melanoma (69% KD CXCR4 and 75% KD CXCR7) caused a significant reduction of their velocity.

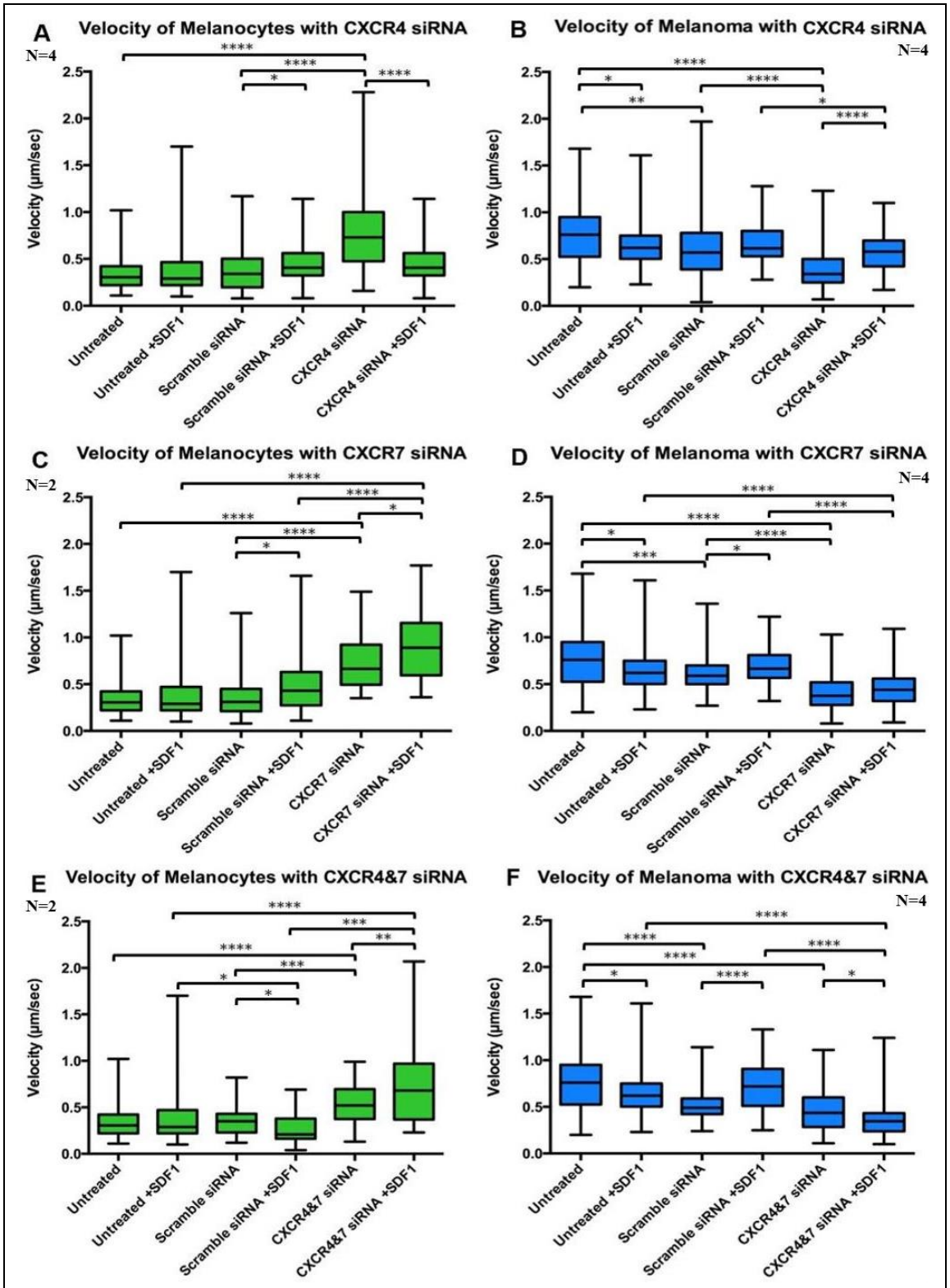

Figure 8: Graphs representing the velocity of cells, in µm per second, after melanocyte transfection with A) CXCR4 siRNA C) CXCR7 siRNA and E) CXCR4 and CXCR7 siRNAs. As well as melanoma transfections with B) CXCR4 siRNA D) CXCR7 siRNA and F) CXCR4 and CXCR7 siRNAs. Significant p-values are identified by the number of asterisks present: * <0.05, ** <0.005, *** <0.0005, **** <0.0001

Effects of CXCR siRNA Knockdowns on Melanocyte and Melanoma Accumulated Distance

The second migratory parameter assessed was the accumulated distance traveled by each cell line after KD of CXCR4/7. The findings from these measurements showed that CXCR4/7 KDs in melanocytes (76% KD CXCR4 and 39% KD CXCR7; 70% KD CXCR7 and 97% KD CXCR4) caused, as expected from velocity results, a strong increase in migrated distance (Figure 7A, Tables 2). The simple addition of SDF1 to the melanocytes cells did show small but significant increases in their accumulated distance traveled of each condition. However, what was very interesting was that transfecting melanocytes with CXCR4 or CXCR7 siRNA caused a doubling of their total distance traveled with CXCR4 KD, and a little less than double with the CXCR7 KD. Both CXCR KDs were significant compared to the scramble siRNA condition as well. Furthermore, an even larger increase was seen in the accumulated distance of the melanocytes upon the addition of SDF1. The CXCR7 siRNA +SDF1 condition showed the distance traveled by cells to nearly triple compared to scramble siRNA +SDF1 condition. The results for melanocytes from double KD for CXCR4 and CXCR7 (74% KD CXCR4 and 73% KD CXCR7) showed very similar results to the CXCR7 single KD condition. The distance travelled by the cells nearly doubled upon CXCR4/7 KD and more than doubled when SDF1 was added.

The results for melanoma, were also found to be similar to those of velocity change after CXCR KD. The simple addition of SDF1 to the melanoma cells caused a small but significant increase in their accumulated distance traveled (Figure 7, Table 3). The distance traveled by melanomas greatly decreased upon the transfection of CXCR4/7 siRNAs (35%

KD CXCR4 and 49% KD CXCR7; 56% KD CXCR7 and 90% KD CXCR4). The total distance traveled by melanoma cells showed a greater than fifty percent decrease compared to the scramble condition upon the transfection of either CXCR4 or CXCR7 siRNA. Furthermore, I also found significant reduction in the total distance of melanoma after addition of SDF1 with the transfection with both CXCR4 and CXCR7 siRNA. This reduction in distance, with and without the addition of SDF1, proved to be more significant upon the CXCR7 KD then compared to the CXCR4 KD. However, in the double KD condition (69% KD CXCR4 and 75% KD CXCR7), I found the decrease in distance not significant compared to the scramble condition.

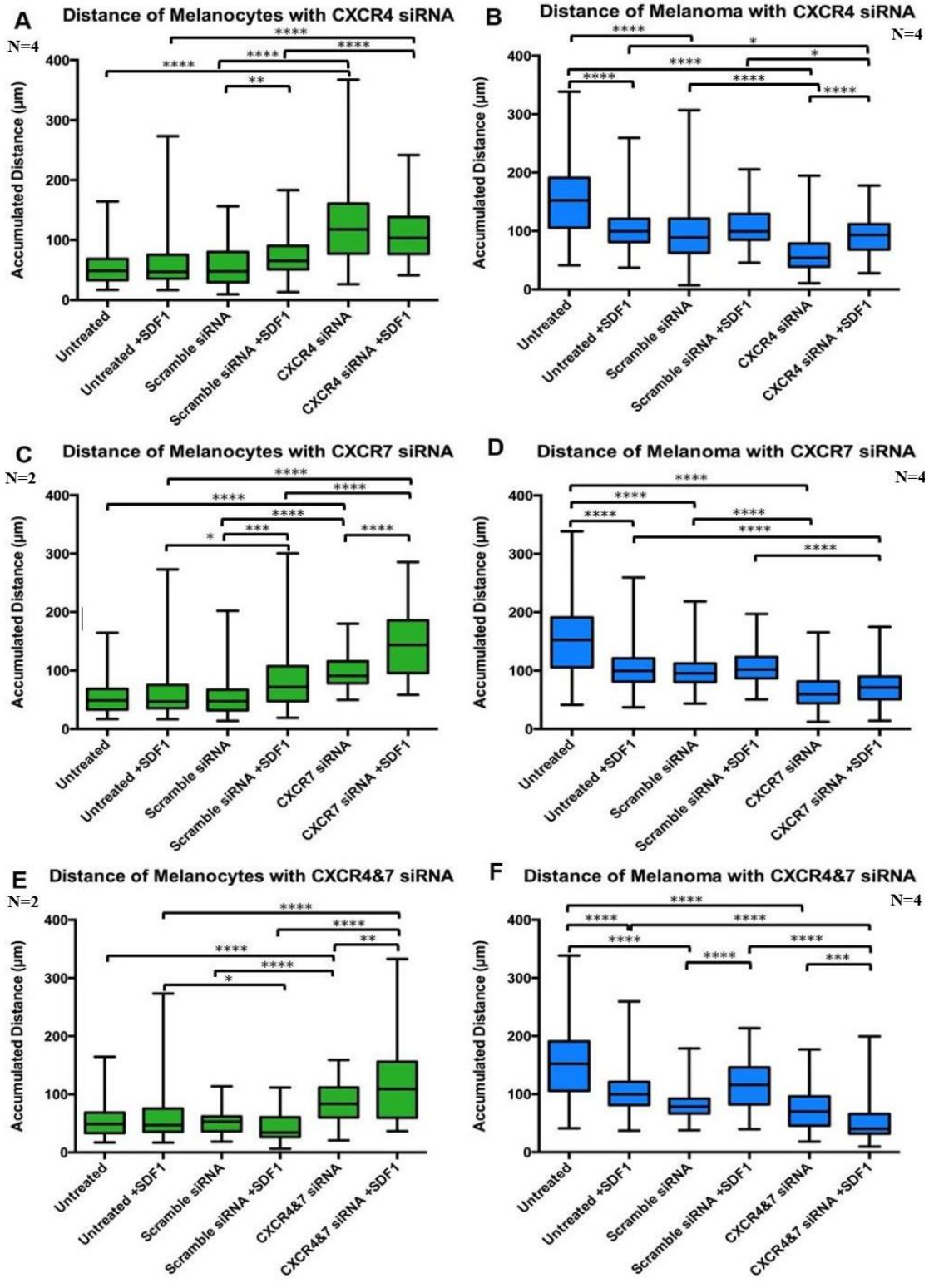

Figure 9: Graphs representing the accumulated distance traveled by cells in µm after melanocyte transfection with A) CXCR4 siRNA C) CXCR7 siRNA and E) CXCR4&7 siRNAs. As well as melanoma transfections with B) CXCR4 siRNA D) CXCR7 siRNA and F) CXCR4&7 siRNAs. Significant p-values are identified by the number of asterisks present: * <0.05, ** <0.005, *** <0.0005, **** <0.0001

Effects of CXCR siRNA Knockdowns on Melanocyte and Melanoma Persistence and Chemotaxis Index

Persistence, the third parameter of migratory capabilities, measures random versus directional movement of cells (Petrie), simply the higher the persistence the more the cell is moving in a single direction. Figure 8, along with Tables 2 and 3, depicts the effects each siRNA transfection had on the persistence of each cell line. Like the other analyses, the simple addition of SDF1 showed slight increases but only in certain transfection conditions, but not consistently. What I also found was that there was no consistent significant change in the KD conditions. In all three sets of CXCR KDs, no significant change was found in the persistence of either cell line in comparison to the scramble siRNA condition.

The final parameter, chemotaxis index, measures the behavioral response of the cells in regard to their movement toward or away from a chemical stimulus. The effects each CXCR KD had on this parameter can be seen in Figure 9, as well as Tables 2 and 3. The chemotaxic index showed no significant change in any cell line for any of the transfections, compared to the scramble conditions, despite the observation that distance and velocity were significantly changed in these experiments.

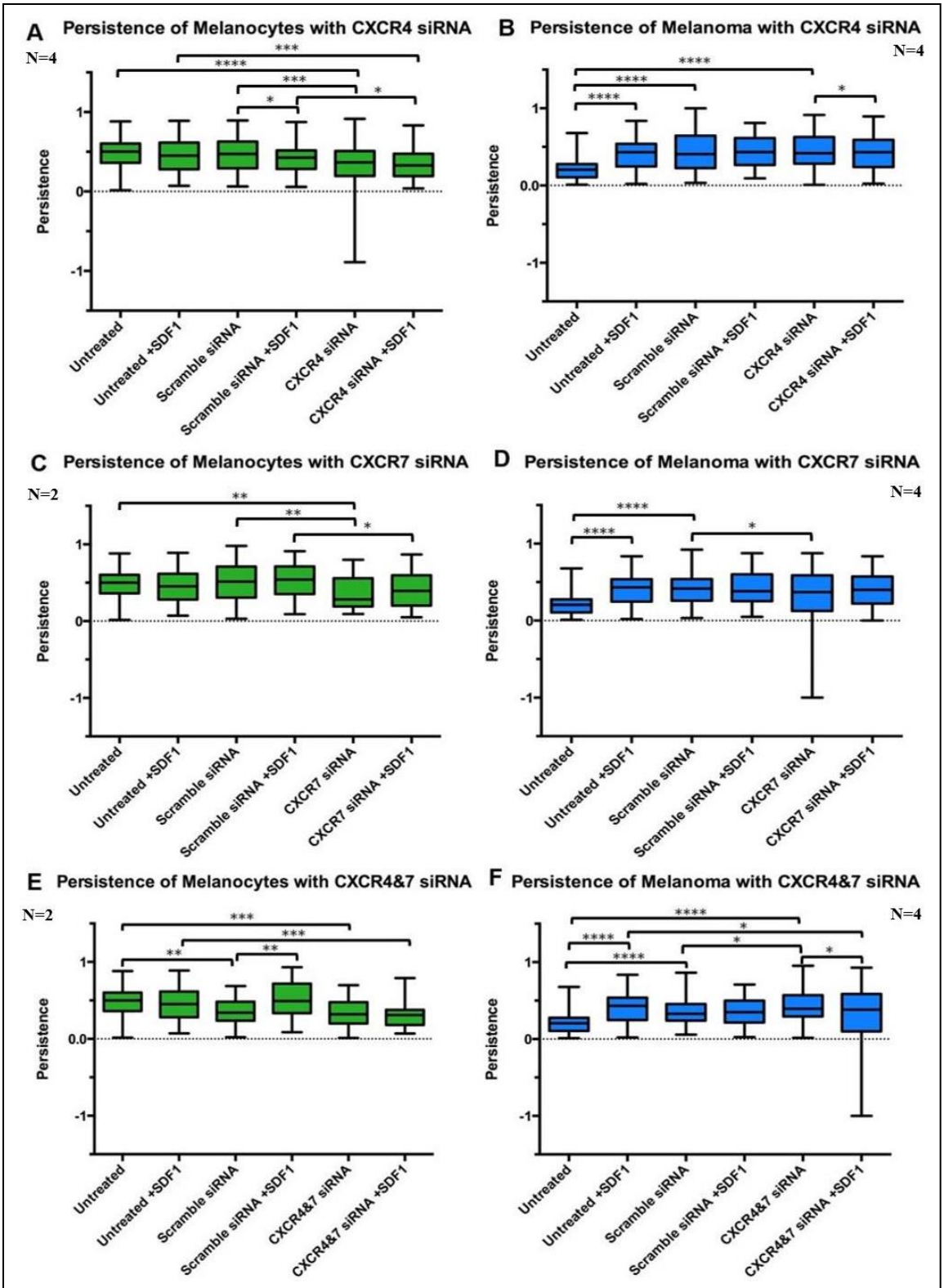

Figure 10: Graphs representing the persistence of cells after melanocyte transfection with A) CXCR4 siRNA C) CXCR7 siRNA and E) CXCR4 and CXCR7 siRNAs. As well as melanoma transfections with B) CXCR4 siRNA D) CXCR7 siRNA and F) CXCR4 and CXCR7 siRNAs. Significant p-values are identified by the number of asterisks present: * <0.05, ** <0.005, *** <0.0005, **** <0.0001

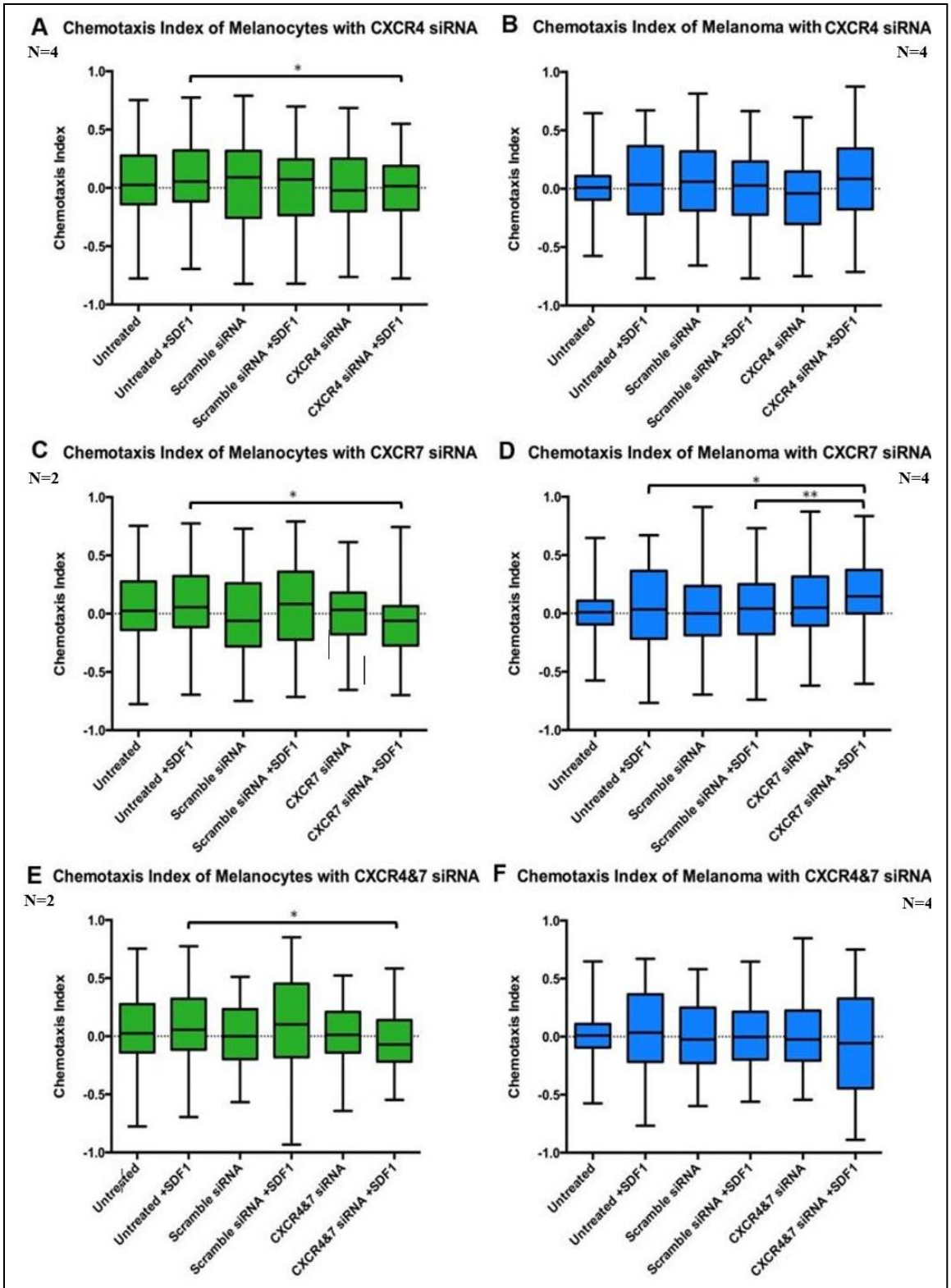

Figure 11: Graphs representing the chemotaxis index of cells after melanocyte transfection with A) CXCR4 siRNA C) CXCR7 siRNA and E) CXCR4 and CXCR7 siRNAs. As well as melanoma transfections with B) CXCR4 siRNA D) CXCR7 siRNA and F) CXCR4 and CXCR7 siRNAs. Significant p-values are identified by the number of asterisks present: * <0.05, ** <0.005, *** <0.0005, **** <0.0001

Effects of CXCR siRNA Knockdowns on Melanocyte Wound Assays

The second set of experiments performed were 18-hour wound assays in order to identify how the KDs can influence motility under a very different paradigm: collective migration. Wound assays for each set of transfections were compared to the wound assay of the untreated cell lines (Figure 10).

The first observation with melanocytes was a significant change in wound healing capabilities in two of the three conditions: CXCR7 (-16%, $p<0.02$) and the double KD (-10%, $p<0.03$) transfections (Figure 11, Table 2). However, the CXCR4 siRNA condition did not show significance in the healing capabilities of melanocytes compared with the changes observed after CXCR7 KD (76% KD CXCR4 and 39% KD CXCR7; 70% KD CXCR7 and 97% KD CXCR4). Importantly in all these experiments, I did not observe a significant change in the healing capabilities of any of the CXCR KDs when comparing the scramble siRNA conditions to the untreated conditions. Similarly, there was also no significance in any condition solely upon the addition of +SDF1.

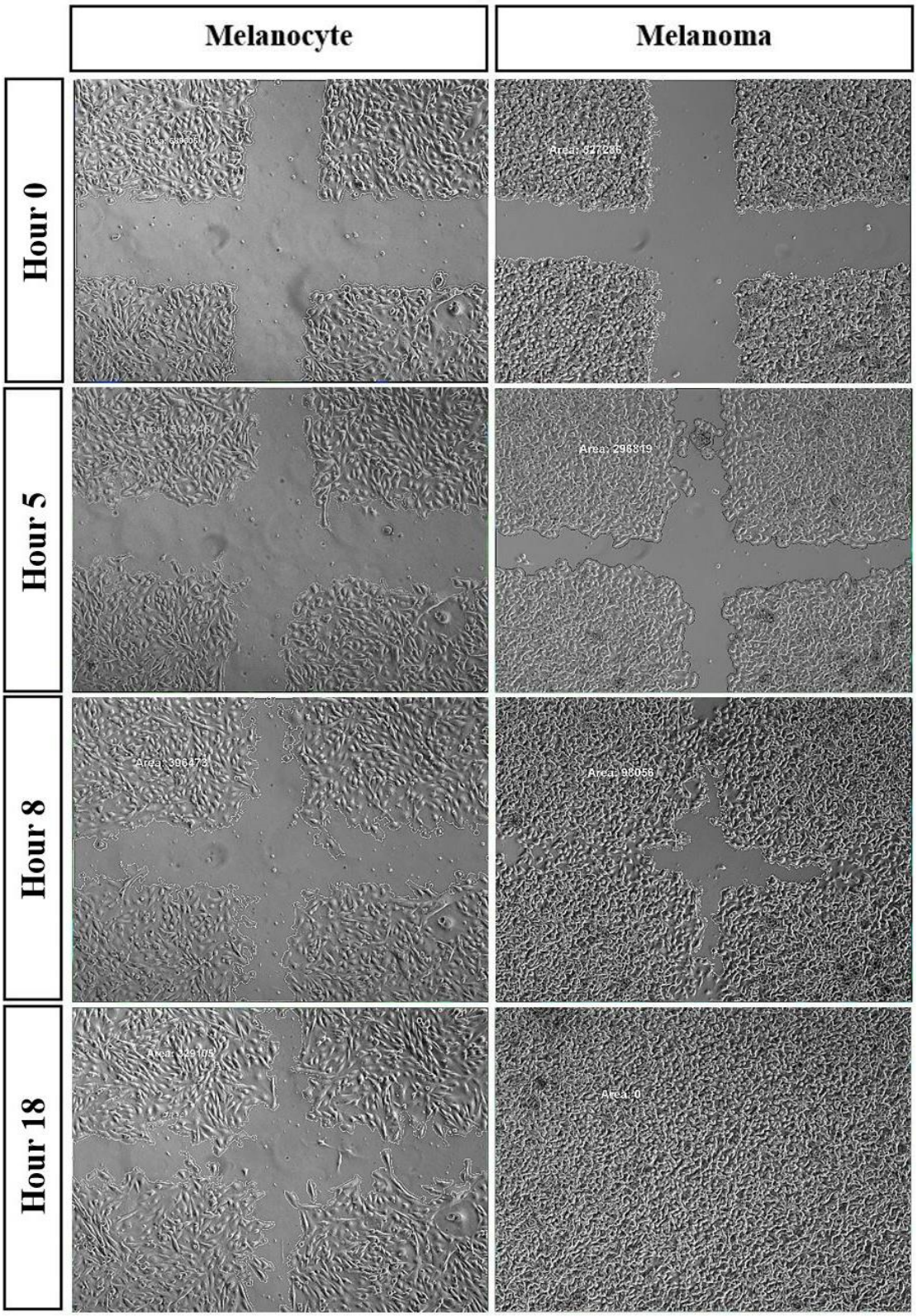

Figure 12: Area analysis of images taken at each time point during an 18-hour wound assay for each untreated cell line A) Melanocytes and B) Melanoma

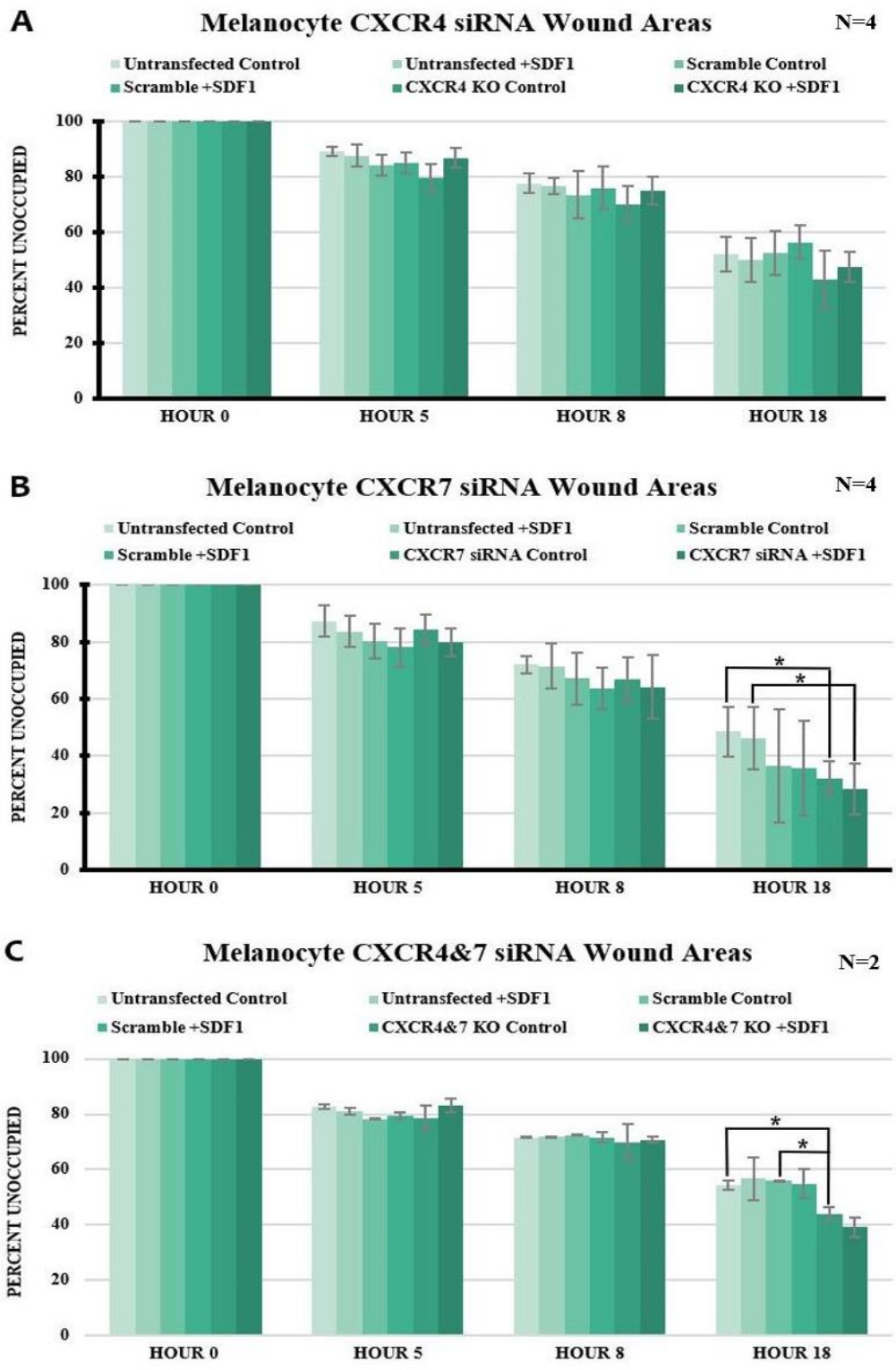

Figure 13: Bar graphs showing progressive healing of melanocyte wounds at each time point over an 18-hour time frame for each set of transfections A) CXCR4 siRNA B) CXCR7 siRNA and C) CXCR4 and CXCR7 siRNAs. Significant p-values are identified by the number of asterisks present: * <0.05, ** <0.005, *** <0.0005, **** <0.0001

Effects of CXCR siRNA Knockdowns on Melanoma Wound Assays

The wound assays with melanoma gave very different results from melanocytes after CXCR4/7 KD (Figure 12, Table 3). The only experiment that showed a statistically significant difference was in CXCR4 KD between untransfected +SDF1 and CXCR4 KD +SDF1. There was a ten percent increase in the area left unhealed, that showed the CXCR4 KD +SDF1 condition was unable to heal the wound as efficently as the untreated +SDF1 condition. These results showed that when CXCR4 was knocked down in melanoma (35% KD CXCR4 and 49% KD CXCR7), these cells moved less, and thus healed less efficiently. This was the opposite of what was observed with melanocytes: they became more motile.

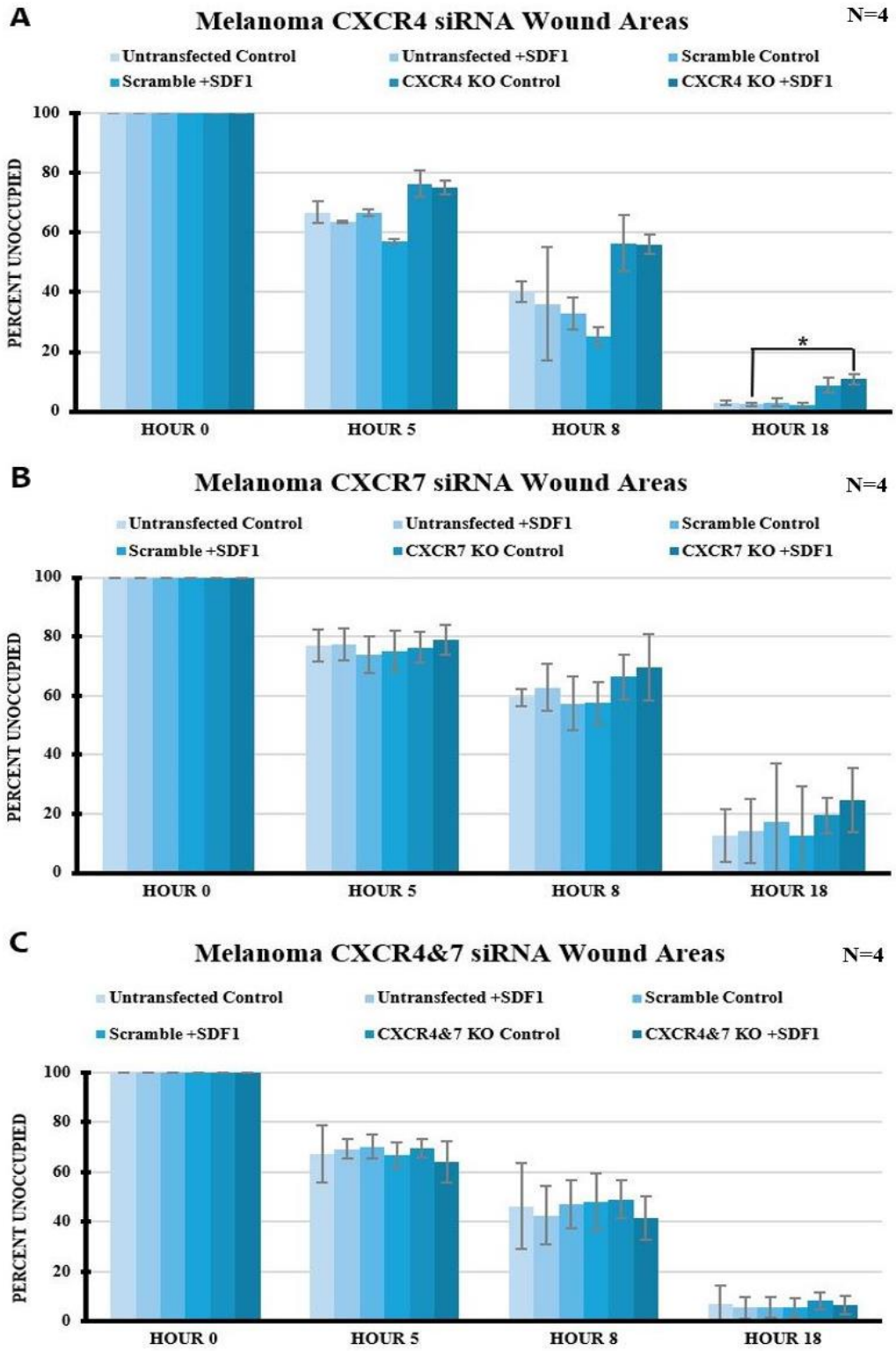

Figure 14: Bar graphs showing progressive healing of melanoma wounds at each time point over an 18-hour time frame for each set of transfections A) CXCR4 siRNA B) CXCR7 siRNA and C) CXCR4 and CXCR7 siRNAs. Significant p-values are identified by the number of asterisks present: * <0.05, ** <0.005, *** <0.0005, **** <0.0001

qPCR and GFP Expression of Each Transfection in Melanocyte and Melanoma

Transfecting CXCR4/7 siRNA into melanocytes and melanomas does not mean necessarily that the receptors protein expression will necessarily be knocked down or to which extent. In order to quantify the level of KD for CXCR4 and CXCR7 after siRNA transfections I performed qPCR analysis as well as took photos of the GFP expression after each set of transfections, in order to confirm the successful transfection of the siRNAs and gene KD. Table 2 summarizes the qPCR results for the melanocyte cell line and Table 3 for the melanoma cell lines, each containing all three sets siRNA transfections with and without the addition of SDF1.

Results show a partial successful knockdown of each CXCR receptor by siRNA transfection. The overall results showed that siRNA transfection was successful at knocking down the corresponding receptor. However, I also noticed that CXCR7 siRNA KD CXCR4 receptor as well (97% KD) while CXCR4 siRNA only KD ~40% of CXCR7. The addition of SDF1 to the cells also affected the receptor KD. In melanoma instances even doubling down the percentage KD.

When I looked at the green fluorescence levels of transfected melanocyte and melanoma cells for knock down effects results were more consistent (Fig.13). Although fluorescence cannot be quantified, these images did allow me to conclude that cell transfection was successful since there was an increase identifiable difference in the GFP expression in the cells after transfection. More recent data using specific CXCR4 or CXCR7 antibodies shows that KD via our siRNA is truly working (data not shown from Tyler Tran).

Melanocyte Conditions	CXCR4 ΔΔCT	CXCR4 % KD	CXCR7 ΔΔCT	CXCR7 % KD
CXCR4 siRNA	0.24	76.3%	0.11	38.7%
CXCR4 siRNA +SDF1	0.11	88.7%	0.32	68.0%
CXCR7 siRNA	0.03	97.0%	0.31	69.6%
CXCR7 siRNA +SDF1	0.71	28.7%	0.60	43.6%
CXCR4&7 siRNA	0.26	74.2%	0.27	73.1%
CXCR4&7 siRNA +SDF1	0.38	62.1%	0.32	68.0%

Table 2: qPCR results of the melanocyte cell line, showing the calculations of the percent knockdown for each condition of the siRNA transfections

Melanoma Conditions	CXCR4 ΔΔCT	CXCR4 % KD	CXCR7 ΔΔCT	CXCR7 % KD
CXCR4 siRNA	0.66	34.7%	0.51	49.1%
CXCR4 siRNA +SDF1	0.33	66.5%	0.47	53.4%
CXCR7 siRNA	0.10	89.8%	0.44	56.4%
CXCR7 siRNA +SDF1	0.66	34.8%	0.55	45.0%
CXCR4&7 siRNA	0.47	69.0%	0.26	74.6%
CXCR4&7 siRNA +SDF1	0.53	46.8%	0.63	37.2%

Table 3: qPCR results of the melanoma cell line, showing the calculations of the percent knockdown for each condition of the siRNA transfections.

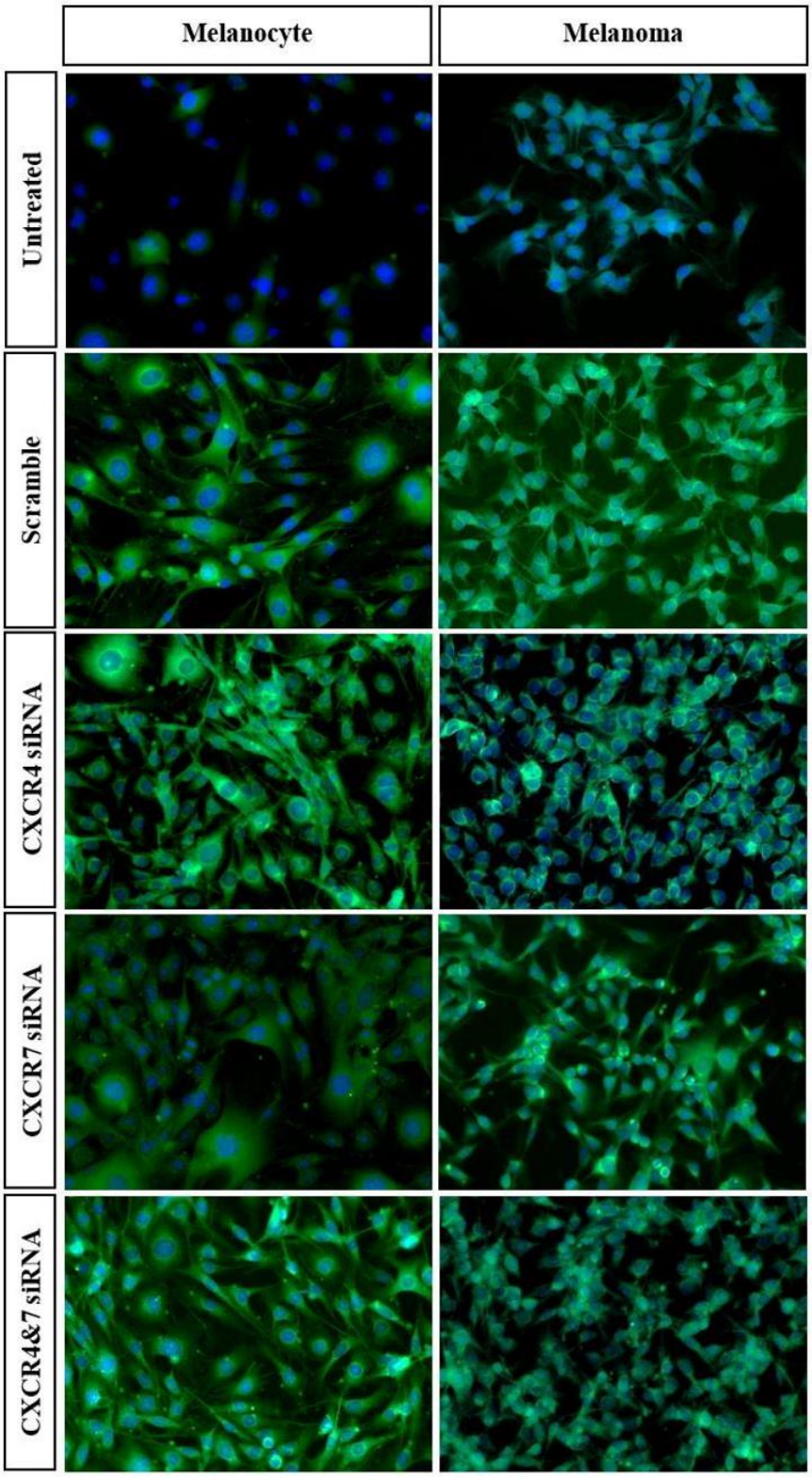

Figure 15: Images taken of melanocyte and melanoma cell lines showing the cell nuclei with DAPI stain (blue) as well as the GFP expression (green) before and after each siRNA transfection.

SECTION 5: DISCUSSION

The overall purpose of this thesis was to find out if CXCR7 is more relevant than CXCR4 in melanoma metastatic capabilities. The overall data presented here may not completely support a hypothesis where CXCR7 has a more relevant role on migratory capabilities than CXCR4, however I can conclude that CXCR4/7 work together to regulate the migratory capabilities of these two cell lines. Knocking-down CXCR7 caused an unintentional knockdown of CXCR4, and vice-versa, perhaps due to the strong homology between these two receptors. This CXCR7 KD with the accidental CXCR4 KD significantly increased melanocyte migratory capabilities as well as significantly decreased melanoma migratory capabilities. On the other hand, CXCR4 KD alone showed similar but lesser significant changes in melanocyte and melanoma migratory capabilities.

A fundamental aspect in directed cell migration is the velocity of migration. Any influences that change the normal velocity of migration does so by disrupting the basic mechanisms of cytoskeletal cell organization (Gail and Boone, 1970). I analyzed the velocity of melanocytes and melanoma upon KD of each CXCR receptor, in order to determine if any effected cell motility. I observed that the most significant increase or decrease in the velocity of melanocytes and melanoma, respectively, was after KD of the CXCR7 receptor, which was accompanied by the unintentional KD of CXCR4 (Figure 6, Table 2 and 3). However, CXCR4 KD alone also affected these cells' motility to a lesser but significant degree. This data supports the theory that the CXCR7 receptor and the CXCR4 receptor work together to play a significant role in the migratory capabilities of melanocytic cells.

Cell migration is the term used to describe the process that individual cells use to move and migrate. The study of the mechanisms that drive cell migration can tell us about how an organism develops to how cancer can progress. Among the cell migration assays, the best known and widely used are the Boyden chamber (Boyden, 1962) and wound assays (Lampugnani, 1999). Boyden chamber assays assess motile cells and their chemotactic abilities upon the influence of soluble substances, whereas wound assays allow researchers to study the chemokinetic transition of cells from a resting state to the migratory state. However, there is a key distinction between stating that a chemokine is a chemokinetic or chemotaxic molecule. Experiments looking at a cell's chemokinetic ability require observing changes in velocity and migrated distance. On the other hand, experiments that affect a cell's chemotaxis response will show changes in parameters such as persistence and chemotaxis index, because chemoattractants will make a cell migrate toward a chemokine source and in a specific direction. Although not necessarily true, many researchers still consider results from Boyden assays reflect perfect chemotaxis or perfect chemokinetic responses. I did not use the Boyden assays preferring to have a clear response in the cells to homogeneous SDF1 presence.

Melanoblasts, the precursors of melanocytes, are known to follow the same cell migratory mechanisms as melanocytes (Mort et al., 2015; Petit and Larue, 2016). There are several types of molecules guiding cells, among the best known are the chemokines. These soluble/secreted proteins exert their effect by binding to their cognate receptors, in my case SDF1 binds to CXCR4 and CXCR7. Among the various effects that chemokines have on melanoblasts are the stimulation of cell proliferation and migration. During development, melanoblasts are homogenously distributed within the epidermis as they migrate to the skin,

eyes and hair follicles in mammals (Luciani et al., 2011; Mort et al., 2016). Research has looked into the presence of specific receptors and ligands in order to discover the possibility that melanoblasts may instruct each other during migration, i.e. use SDF1 which is secreted by melanocytes themselves (Laurent-Gengoux et al., 2018). Furthermore, research has identified that as melanocytes turn cancerous and become melanomas, their migration patterns become aberrant and begin to proliferate extensively leading to malignant cell invasiveness (Bonaventure et al., 2013). When studying normal versus aberrant cell migration, most researchers examine the cell's chemokinetic properties such as velocity and total distance migrated (Laurent-Gengoux et al., 2018; Crawford et al., 2017) or chemotactic properties such as persistence and chemotaxis index during migration towards a chemokine gradient (Lee et al., 2013; Petrie et al., 2009;).

I found that the melanocytes' velocity doubled after what was supposed to be a KD of CXCR7, but which included off target KD of CXCR4 as well. Furthermore, the addition of SDF1 (CXCR7 siRNA +SDF1 condition) to melanocytes caused their velocity to nearly triple (Figure 7). The increase after CXCR4 KD alone, though significant, did not reach twice the velocity of the control. These findings are in opposition to those of Lee et al. who observed that antibody blocking of CXCR7 abolished melanocyte migration, while blocking CXCR4 had no effect on migration (Lee et al., 2013). I believe this difference in results can be explained by looking at the specifics of the experiments both Lee et al. and I performed. First, Lee used Boyden chambers with SDF1 in bottom wells and Ibidi chemotaxis slides. Boyden chambers measure chemokinesis while Ibidis measure chemotaxis. While they measured migrated distance towards SDF1 after 24 hours, they never measured velocity. In contrast, my experiments had a symmetrical addition of SDF1,

and the melanocytes were recorded for several hours and individual cells had their entire pathway tracked. Lee's experiments focused on the chemotaxic effects of SDF1 on melanocyte, whereas I focused on the chemokinetic response. Second, Lee's experiments used neutralizing antibodies to block functional receptors, while my experiments used siRNA. Antibodies would not necessarily block receptor functions, while reducing the amount of receptor in a cell (as siRNA does) will correlate with receptor function better. These two factors show that the two set of experiments are not equivalent in their analysis and therefore can show different results without necessarily contradicting each other.

On the other hand, melanoma cells showed a greater velocity decrease after CXCR7 KD (which included off target CXCR4 KD) than with CXCR4 KD only, compared to control scramble siRNA (Figure 7, Table 3). These findings are in agreement with published research. Li and co-workers found that knocking down CXCR7 in M14 melanoma cells significantly inhibited cell migration and invasion in the Boyden assay (Li et al., 2017). The Boyden chamber experiment performed by Li showed similar results to my experiments. Specifically, Li's experiments showed that the addition of SDF1 increased the number of M14 melanoma cells that passed through the permeable membrane, therefore significantly increasing the invasiveness of the cells. Conversely, CXCR7 KD inhibited the SDF1 effects and caused a significant decrease in the invasive activity of the M14 Melanoma cells. Although Li and I were analyzing different aspects of the cells in our experiments, chemokinesis versus chemotaxic, we observed the same effects in the melanoma cells upon the KD of CXCR7. However, another important difference between Li's experiments and mine, is that Li did not look at the influence of CXCR4 along with the CXCR7 receptor. Here, we see that the CXCR7 receptor, along with the big possibility

of a contributing CXCR4 receptor, is influencing both the chemokinesis and chemotaxic abilities of the melanoma cells. As research continues to determine the significance of CXCR7 versus CXCR4 in metastasis and tumorigenesis, it has been found that it can influence tumor growth in other types of cancers (Wang et al., 2008; Miao et al., 2007). Furthermore, CXCR7 has more ligands than just SDF1, and knocking down this single receptor will inhibit these other pathways as well (Richmond et al., 2018). Previous research on the CXCR family of chemokines has found that different CXCR chemokines can play roles in increasing melanoma tumor cell growth, depending on the location of the tumor in the body (Richmond et al., 2018). The small influence of CXCR4 in my experiments could be due to the increased expression of CXCR4 that is found in early stages of melanoma metastasis in the lungs, bone marrow and liver (Bartolome et al., 2009).

An interesting finding from this thesis was the different response of melanocytes and melanomas after CXCR4/7 KD in wound assays. While the first set of experiments looked at the effects on cell migration of individual cells after CXCR KD, the second set of experiments looked at cells migrating collectively after CXCR4/7 KD. The environment in which a single cell is migrating, with little to no outside influence, such as cell to cell contact, is quite different from the one found when cells are in a monolayer. By looking at melanocytes and melanoma response after KD of CXCR4/7 we can study how the cell changes from normal to aberrant behavior after CXCRs expression changes. When cells are in a much closer proximity to one another, they will communicate and be exposed to each other's signals, which will create a difference in their migratory capabilities. These wound assays are most commonly performed to look at cancerous cell lines to observe their

metastatic capabilities and aggressive nature, such as melanocytes and melanoma (Crawford et al., 2017; Gallagher et al., 2013).

Since the melanocytes were able to “heal the wound” by leaving a smaller area unoccupied after the 18-hour time point, I will propose that this was cause because the melanocytes were developing an abnormal behavior when CXCR7 or both CXCR7 and 4 were KD. Because I could not separate CXCR4 KD from concomitant CXCR7 KD based on my qPCRs, it is difficult to conclude about role of CXCR4 or CXCR7 in melanocytes. As previously mentioned, knocking down CXCR7 caused an unintentional KD of CXCR4 as well. Maybe the development of the abnormal behavior in melanocytes is happening because both receptors are being knocked down. Research investigating microarray signatures of non-cancerous and melanocytic tumors has identified differentially expressed genes as melanocytes develop into melanoma (Koh et al., 2009). Although CXCR4 and CXCR7 were not included in this specific study, they may very well play a similar role as these identified predictive genes. As my experiments show, CXCR7 and CXCR4 could, should be included as one of the outlying genes whose increase in expression can determine predictive melanocytic tumors. There was a ten percent increase in the area left unhealed, that showed the CXCR4 KD +SDF1 condition was unable to heal the wound as efficently as the untreated +SDF1 condition. These results showed that when CXCR4, and off target CXCR7, was knocked down in melanoma, these cells moved less, and thus healed less efficiently. This was the opposite of what was observed with melanocytes: they became more motile.

On the other hand, the smaller response by melanomas in the wound healing assay is likely due to their highly aggressive growth and motility, that by 18 hrs., there was almost

always complete healing. In other words, it is difficult to show reduced motility in highly aggressive cancer cells.

My experiments however brought forth an interesting distinction in how these two cell types respond to CXCRs KD. The motility assay looked at the response of individual cells while wound assays is reflective of a collective response of cells. The motility and wound assays showed increased cell migration after KD of CXCR4 and CXCR7 in melanocytes. In melanoma, the motility and wound assays showed reduced cell migration after KD of CXCR4 and CXCR7, though the wounds portrayed the reduction in a smaller ratio. This suggests that silencing these receptors has the capability of modifying their cytoskeletal rearrangements, causing the increased melanocyte motility and decreased melanoma motility. These changes were not observed in the collective responses via wound assays. Although we found a slight change, it was not as dramatic as when single cells were live tracked.

Most researchers use Boyden chambers to assess motility, which is not as accurate as my experiments that measured velocity of individual cells as well as collective migration responses after CXCR KD (wound assay). The different and seemingly opposing cell responses I observed in melanocytes and melanomas after CXCR KD in the two assays stress the importance of doing motility assessment under different conditions because cells behave differently when in cell-cell contact (close proximity) as in a monolayer environment versus being dispersed, as single, isolated cells (Puliafito et al., 2012). I would like to propose that these findings could be due to multiple factors. First, the KD conditions do affect the cells but it could not be measurable in the wounds given that cells were in a monolayer. Second, we have to remember that these cells also secrete SDF1 in autocrine

manner (Laird et al., 2008), so the response could be a result of larger amounts of SDF1 when cells were in monolayer compared with isolated conditions. Third, there's the possibility of an overproduction of SDF1 in response to the CXCR receptor KD by siRNA. With the cell-cell contact provided in the wound assays, compared to the single cell response, the cells are making up for the lack of available receptor.

Future Directions

For future experiments, Chick Chorioallantoic Membrane (CAM) Assays need to be performed in order to investigate the metastatic effects of the CXCR4 and CXCR7 KDs in-vivo. Next, the addition of Dr. Abrol's mutated receptors (see Figure 1) need to be incorporated into the KD experiments. The addition of the CXCR receptors that have been mutated to recruit either G-protein or β-arrestin can be used to see which pathway has more influence over the onset of melanoma cancer. This will allow for future assays involving mutant receptors where the cells can be transfected with each receptor type (WT or mutant) to establish structural mechanisms of CXCR4/7 signaling in terms of the importance of specific receptor domains. Finally, Fluorescence Resonance Energy Transfer (FRET) can be used in order to see interactions inside the cell using complete mutation of each CXCR site as well as after manipulation of specific domains of each receptor.

CONCLUSION

In summary, I found that first, the CXCR7 and CXCR4 receptors work together in the initiation of a possible transformation into an abnormal phenotype in melanocytes. My observation of the drastic change in the cell velocity and accumulated distance in melanocytes, demonstrates a direct influence of the CXCR7 and CXCR4 receptors in their cytoskeleton. Additionally, the wound assays further support the influence of the CXCR7 and CXCR4 receptors together, as the loss of both together, although unintentional, still caused the melanocytes to increase the speed of wound healing. The changes in melanocytes movement capabilities may ultimately lead to malignancy. Second, melanomas' malignant phenotype diminished after the KD of CXCR7 (which also had off KD target of CXCR4 receptor), as assessed by cell velocity and accumulated distance. This suggests that CXCR7 receptor, along with CXCR4, appears to take a different role in curbing the aggressive malignancy of the melanoma.

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