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HETERODIMERIZATION BETWEEN CHEMOKINE RECEPTORS CXCR4 AND CCR7 AND ITS ROLE IN CANCER

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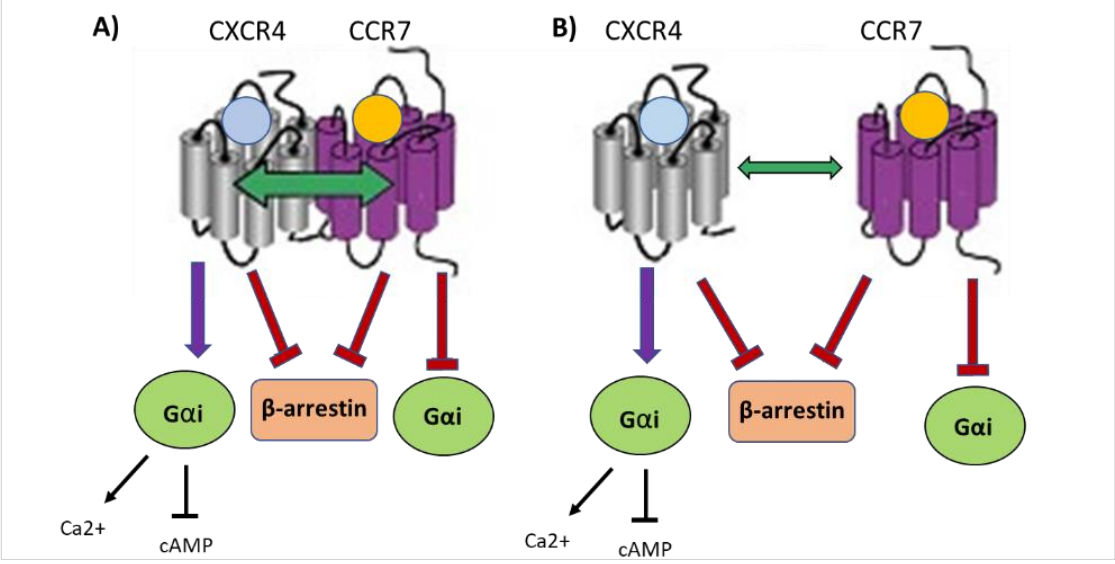

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

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Cover: Proposed model for CXCR4-CCR7 interaction, either via (A) physical interaction or via (B) functional intracellular crosstalk**,** specifically modulating Gαi protein or β-arrestin mediated-pathways in cancer.

(Photo: By Maryam Naveed Muhammad Tariq)

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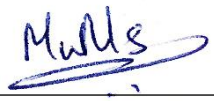
Declaration of Original Work

I, Maryam Naveed Muhammad Tariq, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Heterodimerization between Chemokine Receptors CXCR4 and CCR7 and its Role in Cancer*", hereby, solemnly declare that this is the original research work done by me under the supervision of Dr. Mohammed Akli Ayoub, in the College of Science at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature: 

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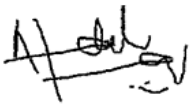
   Signature  Date: 10/11/2022

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Abstract

In physiology, the functional and physical interactions between cell surface receptors for signal molecules such as hormones, neurotransmitters, and cytokines, provides an important mechanism of diversity and regulation of signal transduction. Therefore, investigating the functional interactions between these receptors is of great importance for understanding their role in physiology and pathophysiology with potential application in drug discovery. In this context, numerous evidences support the implication of two chemokine receptors, CXCR4 and CCR7, in many cancer types. In this study, we hypothesized that the functional, and perhaps the physical interaction, occurring between CXCR4 and CCR7 may constitute one of the key molecular mechanisms of their implication in cancer. Therefore, we investigated such CXCR4-CCR7 interaction *in vitro* using different cell lines endogenously (HepG2, MDA-MB231, and HT-29) or transiently (human embryonic kidney (HEK 293)) co-expressing the two receptors. For this, RT-PCR was used for the analysis of the relative expression of CXCR4 and CCR7. Moreover, bioluminescence resonance energy transfer (BRET) technology in live cells combined with SDS-PAGE followed by western blot were applied to study the possible impact of CXCR4-CCR7 interaction on their signaling pathways inside the cells. Our results showed differential expression of the CXCR4 and CCR7 in the different cell lines. More importantly, we revealed an interesting pharmacological and signaling fingerprint supporting the existence of a functional CXCR4-CCR7 interaction. This was mainly characterized by the cross-inhibition of both receptor/G protein coupling as well as receptor/β-arrestin pathway between CXCR4 and CCR7 with an inhibitory impact on the intracellular kinase activation and cell viability. Whether such observations were due to the physical CXCR4-CCR7 interaction (heterodimerization) or simply to an intracellular crosstalk, this needs further investigation. Our study sheds more light on the relationship between CXCR4 and CCR7 and their implication in cell signaling with potential implication in cancer. This may open new perspectives to develop alternative therapeutics by considering the existence of the functional interaction between CXCR4-CCR7 *in vitro*.

Keywords: GPCR, Chemokines, CXCR4, CCR7, Heterodimerization, BRET, G-proteins, Cancer.

Title and Abstract (in Arabic)

التغاير بين المستقبلات الكيميائية CXCR4 وCCR7 ودورها في السرطان

الملخص

في علم وظائف الأعضاء، توفر التفاعلات الوظيفية والفيزيائية بين مستقبلات سطح الخلية لجزيئات الإشارة مثل الهرمونات والناقلات العصبية والسيتوكينات آلية مهمة لتنويع وتنظيم نقل الإشارات. لذلك، فإن دراسة التفاعلات الوظيفية بين هذه المستقبلات له أهمية كبيرة في فهم دورها في علم وظائف الأعضاء والفيزيولوجيا المرضية مع إمكانية التطبيق في اكتشاف الأدوية. في هذا السياق، تدعم العديد من الأدلة تأثير اثنين من المستقبلات الكيميائية، CXCR4 & CCR7، في العديد من أنواع السرطان النقيلي. في هذه الدراسة، افترضنا أن التفاعل الوظيفي، وربما التفاعل المادي أيضًا، الذي يحدث بين CXCR4 & CCR7 قد يشكل إحدى الآليات الجزيئية الرئيسية لتأثيرها في تطور السرطان وتطوره. لذلك، قمنا بالتحقيق في تفاعل CXCR4-CCR7 في المختبر باستخدام خطوط خلوية مختلفة تقوم بالتعبير عن/بتصنيع المستقبلين داخليًا (HepG2 كنموذج لسرطان الكبد، MDA-MB-231كنموذج لسرطان الثدي الظهاري و HT-29 كنموذج لسرطان القولون والمستقيم الظهاري) أو بشكل عابر (الكلية الجنينية البشرية (HEK 293). لهذا، تم استخدام RT-PCR لتحليل التعبير النسبي لـ CXCR4 وCCR7 في خطوط الخلايا المختلفة. علاوة على ذلك، تم تطبيق تقنية نقل طاقة رنين التلألؤ الحيوي (BRET) في الخلايا الحية جنبًا إلى جنب مع SDS-PAGE تليها Western Blot لدراسة التأثير المحتمل لتفاعل CXCR4-CCR7 على مسارات التنشيط والتنظيم داخل الخلايا. أظهرت نتائجنا تعبيرًا تفاضليًا في مستويات CXCR4 و CCR7 في خطوط الخلايا المختلفة. الأهم من ذلك، لقد كشفنا عن بصمة دوائية وإشارات مثيرة للاهتمام تدعم وجود تفاعل وظيفي بين CXCR4 و CCR7 تميز هذا بشكل أساسي بالتثبيط العابر لكل من اقتران المستقبل بالبروتين-G وكذلك مسار المستقبل المقترن بمثبط arrestin بين CXCR4 و CCR7 مع تأثير مثبط محتمل على تنشيط kinase داخل الخلايا وحيوية الخلية. سواء كانت هذه الملاحظات ناتجة عن تفاعل CXCR4-CCR7 المادي (التغاير) أو ببساطة إلى التواصل/التفاعل المتبادل داخل الخلايا، فإن هذا يحتاج إلى مزيد من التحقيق. تلقي دراستنا مزيدًا من الضوء على العلاقة بين CXCR4 وCCR7 و تأثيرهما في إشارات الخلية مع التضمين المحتمل في السرطان. قد يفتح هذا آفاقًا جديدة لتطوير علاجات بديلة من خلال النظر في اكتشافنا حول وجود تفاعل وظيفي بين CXCR4-CCR7 في المختبر.

مفاهيم البحث الرئيسية: GPCR، ناقلات الإشارة الكيميائية، CXCR4، CCR7، التغاير، نقل طاقة رنين التلألؤ الحيوي، البروتين G، سرطان.

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Dedication

To my beloved parents and siblings

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List of Abbreviations

BRET	Bioluminescence Resonance Energy Transfer
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CCR7	C-C Chemokine Receptor 7
C_T	Cycle Threshold
CXCR4	CXC- Chemokine Receptor 4
ECL	Enhanced Chemiluminescent Substrate
ERK	Extracellular Signal-Regulated Kinases
FBS	Fetal Bovine Serum
GDP	Guanosine Diphosphate
GFP	Green Fluorescent Protein
GPCRs	G Protein-Coupled Receptors
GRK	G Protein-Coupled Receptor Kinases
GTP	Guanosine Triphosphate
HEK 293	Human Embryonic Kidney Cells 293
HepG2	Hepatocarcinoma Cells
HT-29	Human Colorectal Adenocarcinoma Cells
MAPK	Mitogen-Activated Protein Kinase
MDA-MB-231	Triple-Negative Metastatic Breast Cancer
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDL	Poly-D-Lysine
PI3K	Phosphatidyl Inositide-3-Kinase
PKA	Protein Kinase A

PKC	Protein Kinase C
PMSF	Phenylmethylsulfonyl Fluoride
PVDF	Polyvinylidene Difluoride
RET	Resonance Energy Transfer
RIPA	Radioimmunoprecipitation Assay Buffer
Rluc	*Renilla* Luciferase
RT-PCR	Reverse Transcription-PCR
RT-qPCR	Real-Time Quantitative PCR
SDF-1	Stromal Cell-Derived Factor 1
SDS-PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
TE buffer	Tris-EDTA
YFP	Yellow Fluorescent Protein

Chapter 1: Introduction

1.1 G Protein-Coupled Receptors (GPCRs)

Transfer of data from sender to receiver becomes useful when the receiver can interpret the data (or signal) into something valuable and respond accordingly [1]. Hence, comprehending how cells can incorporate signals from various data-coding compounds into elaborately structured responses is a significant issue in molecular biology [1]. In complex lifeforms, hormones and neurotransmitters are primarily used for cellular interactions, and cell surface receptors are in charge for identifying signals and transferring information inside the cell [1]. There are three main types of cell surface receptors, namely enzyme-linked receptor tyrosine kinases (RTKs), ion channel receptors and G protein-coupled receptors (GPCRs) [1].

The broadest and most diverse family of cell surface receptors are GPCRs, also known as G protein (guanine nucleotide-binding protein)–coupled receptors, or 7-transmembrane receptors (7TMRs) [2–4]. GPCRs can be found in the cell membranes of many organisms, entailing invertebrates, plants, mammals, and microbes [2]. GPCRs are comprised of three parts: an intracellular C-terminal domain, a short extracellular N-terminal domain and seven transmembrane domains (intermediate region) [2, 4, 5]. These domains are linked by three extracellular and three intracellular coils [2, 4, 5]. There are over 1,000 distinct types of GPCRs encoded by around 4% of the human genome alone [6, 7]. Based on their function and sequence, GPCRs are divided into six classes; rhodopsin-like receptors (Class A), secretin family (Class B), metabotropic glutamate receptors (Class C), fungal mating pheromone receptors (Class D), cAMP receptors (Class E), and frizzled and smoothened receptors (Class F) [8]. These receptors have high affinity to ligands, including hormones, cytokines and growth factors [3]. GPCRs regulate the majority of physiological responses to neurotransmitters, hormones, and environmental stimulants and hence, they are frequently targeted for pharmacological therapies [4]. These cell surface receptors that are the most common drug targets, accounting for around 35% of all drugs that are FDA-approved [9, 10], however, nearly 15% of the 800-1000 human GPCRs are presently such targets [11].

GPCRs can mediate innumerable bodily functions, including hormonal responses, nerve transmission, taste, growth and sensation through activation of various intracellular signaling pathways, in response to an extracellular signal (stimuli) [6, 12].

1.2 Molecular Signaling Pathways of GPCRs

In GPCRs, the intracellular loops and the C-terminal area (Serine Threonine rich) of the receptor are associated with the heterotrimeric G proteins, while the three external hydrophilic loops and the N-terminal domain are engaged in ligand binding, as shown in Figure 1 [13–15]. Heterotrimeric G proteins comprise of an GDP (Guanosine diphosphate)-bound Gα subunit and Gβγ subunits complex, linked together [13–15]. Thirty-five genes encoding G protein subunits (12γ, 5β, and 18α) have been identified [1]. In intracellular signaling transduction, they behave as molecular switches; when G proteins are bound to GTP (Guanosine triphosphate), they are activated, and when bound to GDP, they are deactivated [16].

When a GPCR is activated upon ligand binding, conformational changes occurs where GTP substitutes GDP, hence, separating GTP-Gα from the Gβγ-dimer [13–15]. These two components work along with effector proteins to activate various signal transduction pathways and promote the production of intracellular secondary messengers like phosphoinositides, cAMP (cyclic adenosine monophosphate) and calcium (Figure 1) [5, 13–15, 17]. These pathways are involved in many biological responses, including cell survival, proliferation, immune responses, odor, taste, pain, smell and others (Figure 1) [13–15, 17].

Heterotrimeric G proteins are classified into four categories based on their Gα subunits: $G\alpha_{12/13}$, $G\alpha_{q}$, $G\alpha_{stimulatory}$ ($G\alpha_{s}$) and $G\alpha_{inhibitory}$ ($G\alpha_{i}$) [16, 18]. Though more indiscriminate coupling can also occur at times, GPCRs frequently bind to specific members of the Gα protein family [6]. The heterogeneity of G-protein subfamilies' enables various regulatory processes in signaling transduction. The $G\alpha_{s}$ triggers the adenylyl cyclase (effector enzyme) to generate the second messenger cyclic adenosine monophosphate or cAMP, which activates protein kinase A (PKA) and phosphorylate multiple intracellular proteins that control cellular functions [18, 19]. $G\alpha_{i}$, on the other hand, has an inhibitory impact on adenylyl cyclase, lowering intracellular

cAMP [18, 20]. $G\alpha_q$ stimulates phospholipase C (PLC), which cleaves membrane-bound phosphatidylinositol 4,5–bisphosphate (PIP2) into inositol 1,4,5–triphosphate (IP3) and diacylglycerol (DAG) as second messengers [18, 20]. Ca^{2+} release from the endoplasmic reticulum is aided by IP3 [18, 20]. PKC (protein kinase C) is activated by increased intracellular Ca^{2+} and DAG diffused from the plasma membrane, which promotes cellular signaling [18, 20, 21]. The small GTPase Rho is known to be activated by the G proteins $G\alpha_{12/13}$ [18, 22]. $G\beta\gamma$ dimer, on the other hand, activates ion channels and PLC [18, 20]. Figure 1 shows a schematic diagram of the typical GPCR structure, and the intracellular signaling pathways induced by G proteins ($G\beta\gamma$ and $G\alpha$, including $G\alpha_{12/13}$, $G\alpha_s$, $G\alpha_i$, $G\alpha_q$) upon activation.

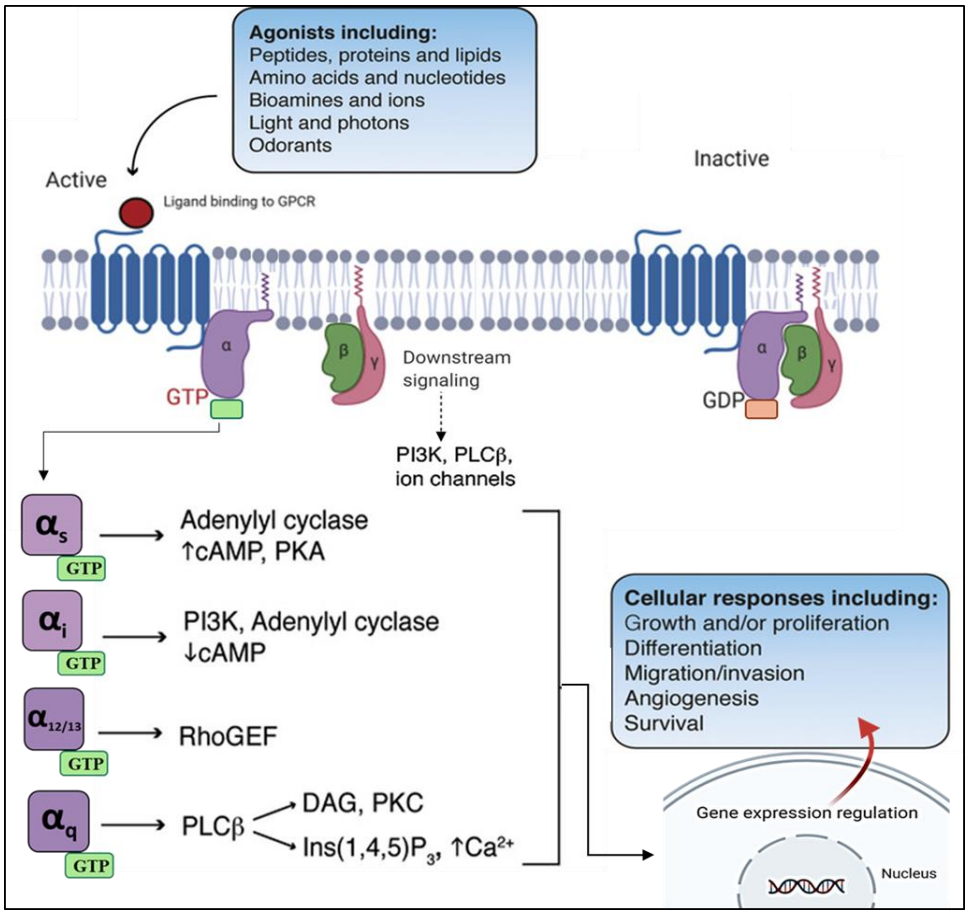

Figure 1: G protein-coupled receptors (GPCR).

The diagram illustrates the typical seven transmembrane structure of GPCRs, their diverse ligands (agonists), and their mode of activation. The binding of the agonist induces the physical coupling with and the activation of various heterotrimeric $G\alpha\beta\gamma$ proteins (G_s, G_i, G_q, and $G_{12/13}$) resulting in the dissociation of $G\alpha$ and $G\beta\gamma$, each to promote specific downstream signaling pathways controlling multiple cell responses.

Hydrolysis of GTP by GTPase leads to the reattachment of GDP-Gα and Gβγ, as the GTPase activity innate to Gα inhibits G protein activation [14, 15]. Inhibiting the heterotrimeric G-protein signaling also entails GRK (G protein-coupled receptor kinases) that phosphorylates numerous serines/threonines in the cytoplasmic tail (C-terminal) of chemokine receptor, and recruits cytosolic adaptor proteins like β-arrestin that attaches to the phosphorylated receptor (Figure 2) [13–15, 23]. Diverse GRKs can uniquely phosphorylate GPCRs that are read as barcodes by β-arrestins, resulting in particular conformational changes to induce specific signaling outcomes [23]. β-arrestins can perform three functions, namely, desensitization, receptor internalization and as signaling transducers (Figure 2) [13–15]. At the very same time that it desensitizes and sterically blocks G-protein signaling and slows the rate of second messenger generation, β-arrestins can also serve as signal transduction units that can promote numerous signaling pathways, including PI3K/Akt (Phosphatidyl inositide-3-kinase - Protein kinase B) and MAPK/ERK1/2 (Mitogen-activated protein kinase - Extracellular signal-regulated kinase) [23]. They also function as adaptors that can facilitate receptor internalization via clathrin-mediated endocytosis of the agonist-activated receptors [13–15, 23]. This requires the ubiquitination of the β-arrestins by E3 ubiquitin ligases, MDM-2 [13–15, 23]. Hence, the receptor can be taken to lysosomes for destruction or recycled back to the plasma membrane [13–15, 23].

Research on β-arrestin based transduction pathways has led to the concept of biased signaling, where a GPCR induces intracellular signaling by preferring one particular pathway (e.g. β-arrestin) over another (e.g. G protein), in response to a biased agonist that causes a specific GPCR conformation, unlike unbiased ligands that can result in both pathways [23, 24]. Likewise, receptors can also be biased [23].

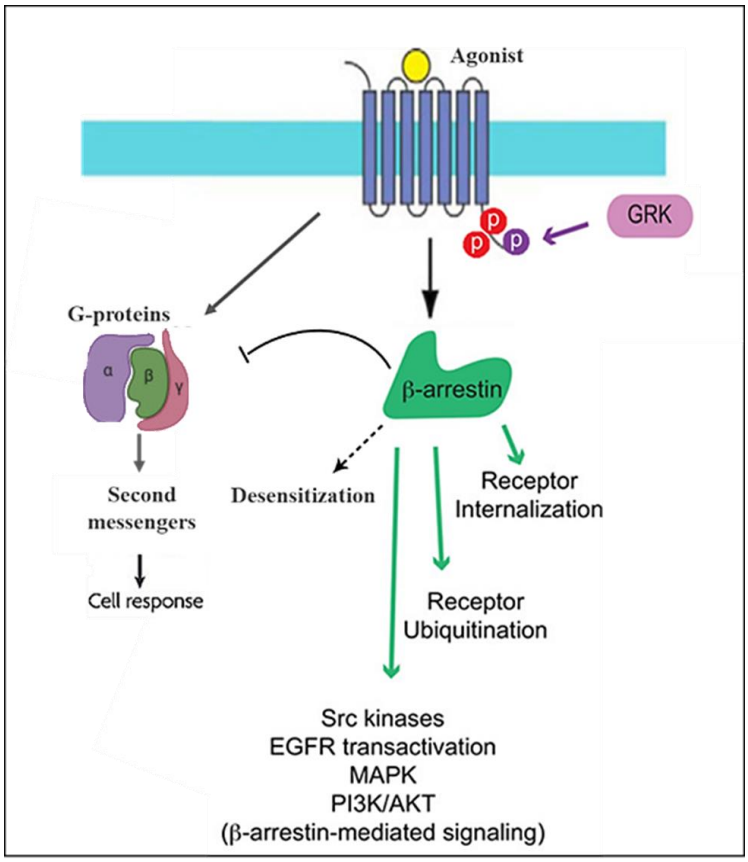

Figure 2: Schematic diagram of the role of β-arrestin in GPCR modulation

The recruitment of β-arrestins by GRK (GPCR kinases) mediates GPCR phosphorylation, leading to desensitization by switching off G protein signaling and consequent receptor ubiquitination and internalization. The stimulation of β-arrestin–regulated signaling (PI3K/Akt, ERK/MAPK etc.) is triggered by β-arrestin's interaction with the receptor.

Typically, human diseases can be caused by both acquired and inborn mutations in genes encoding for GPCRs [6, 12]. These include diseases like diabetes, Alzheimer's, psychiatric disorders, obesity, and cancers. Mutations in specific GPCRs can entail abnormal increase in expression and activity of receptor, mutations in its signal transduction pathways, and/or enhanced secretion and synthesis of receptor activating ligands by cancer and stromal cells in vicinity, for instance [6, 12, 25–28]. Understanding the mechanisms that contribute to abnormal function and expression of GPCR is critical for the formation of novel effective therapeutic methods [29]. Therefore, GPCRs, particularly from Class A are the most widely-studied therapeutic targets due to its available structure, ease of access and central role in diseases [8]. Among class A GPCRs, chemokine receptors are frequently investigated in research studies [8, 13, 14, 17, 30–35].

1.3 Chemokine Receptors: A GPCR Class to Target

The chemokines are small secreted proteins (8–12 kDa) that belong to the chemoattractant cytokine family and have chemotactic properties [14, 17, 31, 33]. These ligands interact with chemokine receptors that are part of GPCRs Class A family [32, 35]. So far, 20 chemokine receptors and about 50 chemokines have been identified [17, 31]. Chemokines are known to control how cells are positioned and recruited into tissues, and they have an important role in immune response, tissue development, and embryogenesis [17]. Depending on their protein sequence, and particularly, the placement of the cysteine (C) residues at their N-terminus, chemokines are categorized into four primary classes: the C-, the CC-, the CXC-, and the CX3C-chemokines ('X' is any amino acid; 'C' denotes the cysteine) (Figure 3) [14, 30, 31, 36]. Similarly, chemokine receptors (CCR, CXCR, CX3CR, and XCR, where 'R' represents receptor) and ligands (XCL, CCL, CXCL, and CX3CL, where 'L' means ligand) are categorized the same way [14, 30, 36].

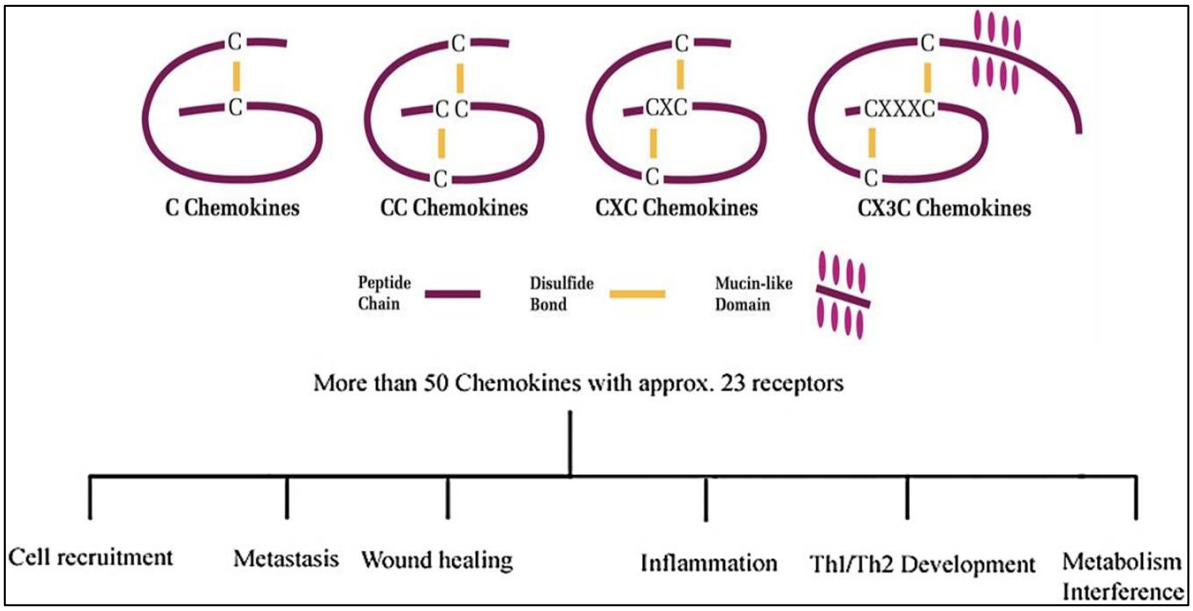

Figure 3: Chemokine types and their typical functions

Chemokines exhibit bidirectional promiscuity, i.e., multiple ligands can activate a single receptor, and many receptors can be activated by one ligand from the same class [14, 37] (Figure 4). Hence, the interaction between chemokines and their receptors are very redundant [14, 37].

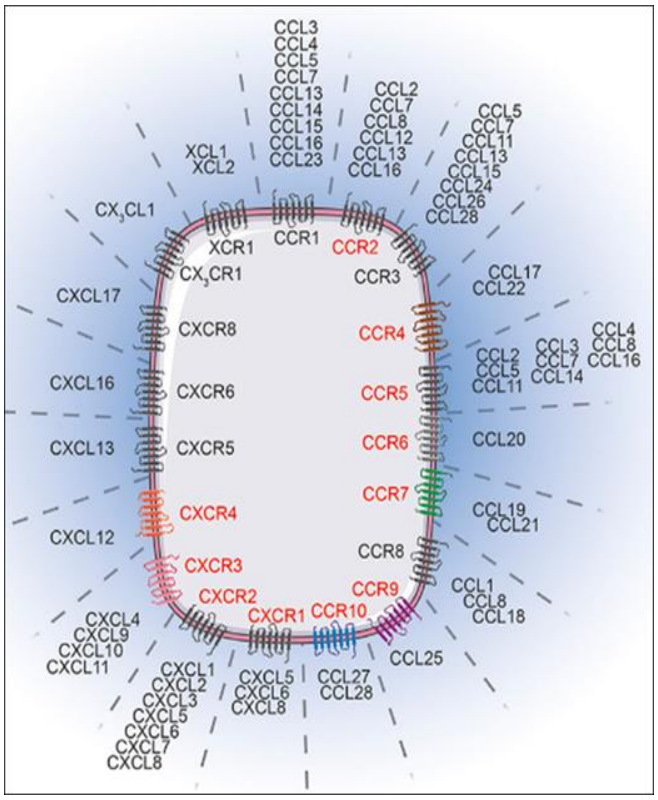

Figure 4: Chemokines and their receptors [14].

Chemokines are widely known for their typical roles in inflammation, immune cell migration, wound healing, and T-helper cells development [38–40]. However, abnormal regulation and expression of chemokine receptors or chemokines are positively linked with numerous disorders, particularly the ones linked with inflammation like cancer [14, 17, 38, 41–44], where the overexpression of chemokine receptor has been positively linked to poor prognosis in majority of malignancies (Figure 5) [5, 45]. Several chemokines and their receptors have actually been found in both primary tumor lesions and metastatic areas [43]. They play major roles in the angiogenesis, growth, proliferation, metastasis and anti-apoptosis of cancer cells [38, 43, 46, 47].

Various types of tumor exhibit a distinct chemokine-receptor pattern in a number of studies involving mice models and human cancer biopsies (Figure 5) [17, 38, 48, 49]. Although numerous chemokines and their receptors are implicated in cancer, the CCR7-CCL19 or CCL21 and CXCR4-CXCL12 are among the most well-known receptor-ligand combinations involved in carcinogenesis in various different forms of cancer [5, 50–58].

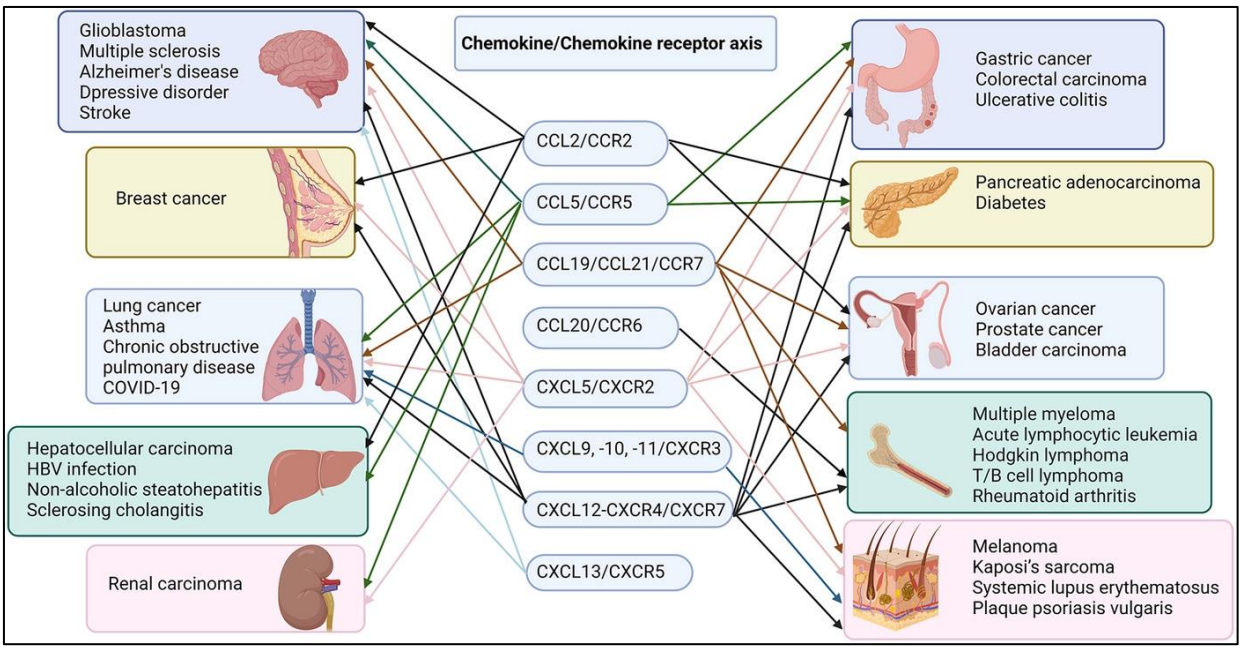

Figure 5: Chemokines and their receptors in inflammatory diseases and cancer [59].

1.4 CXC- Chemokine Receptor 4 (CXCR4) in Cancer

The CXCL12-CXCR4 is one of the most extensively studied chemokine-receptor pair of the CXC family that is essential for enhancing cancer cell survival and directing metastasis [5, 13, 17, 60, 61]

CXCR4 is a rhodopsin-like GPCR, comprised of 352 amino acids [13, 62]. The stromal cell-derived factor 1 (SDF-1) or CXCL12 is the typical ligand that interacts to CXCR4 [13, 63]. CXCR4 is widely expressed in central nervous system and immunological cells and by binding to CXCL12, it is normally involved in mediating hematopoiesis, differentiation of cells, leukocyte trafficking, regeneration of tissue and organogenesis by responding to molecules stimulating inflammation [64, 65]. Moreover, CXCR4 is a key receptor for HIV-1 (human immunodeficiency virus-1) strains that occurs during advancement to AIDS dementia and immunodeficiency [65, 66]. Studies confirmed the notion that CXCR4 and CXCL12 are a biunivocal pair; deletion of either CXCL12 or CXCR4 genes in mice has resulted in aberrant cerebellum and cardiac septum development, fetal lethality, compromised bone marrow myelopoiesis, and abnormal B-cell lymphopoiesis [13, 65, 67].

CXCR4 is frequently expressed in a number of hematological malignancies and solid tumors, such as gastric, prostate, colorectal, lung, ovarian, brain, pancreatic, and breast cancers, esophageal, neck, bladder, and head carcinoma, glioblastoma, melanoma, neuroblastoma, and osteosarcoma, where it is positively linked with metastatic dissemination and poor prognosis [13, 17, 38, 43, 45, 48, 49, 61, 68–80]. The ordinary tissue next to the tumor overexpressing CXCR4, however, exhibits normal or no CXCR4 expression, which raises the intriguing possibility that cancer cells react differently to distinct microenvironmental factors [13, 17].

1.4.1 Molecular Signaling Pathways of CXCR4 in Cancer

Binding of CXCL12 to CXCR4 triggers tumorigenesis via activation of several downstream signaling pathways [15, 37]. Firstly, G proteins dissociate to Gβγ-dimer and GTP-Gα, stimulating pathways Jak-STAT (Janus kinase-signal transducers and activators of transcription), JNK/p38 (c-Jun N-terminal kinase) MAPK, NF-κB (Nuclear factor kappa B), PLC, Ras-MAPK, and PI3K-Akt-mTOR (mammalian target of rapamycin) (Figure 6). These pathways are responsible for various functions like gene expression, cell survival, proliferation, chemotaxis and migration (Figure 6) [15, 37].

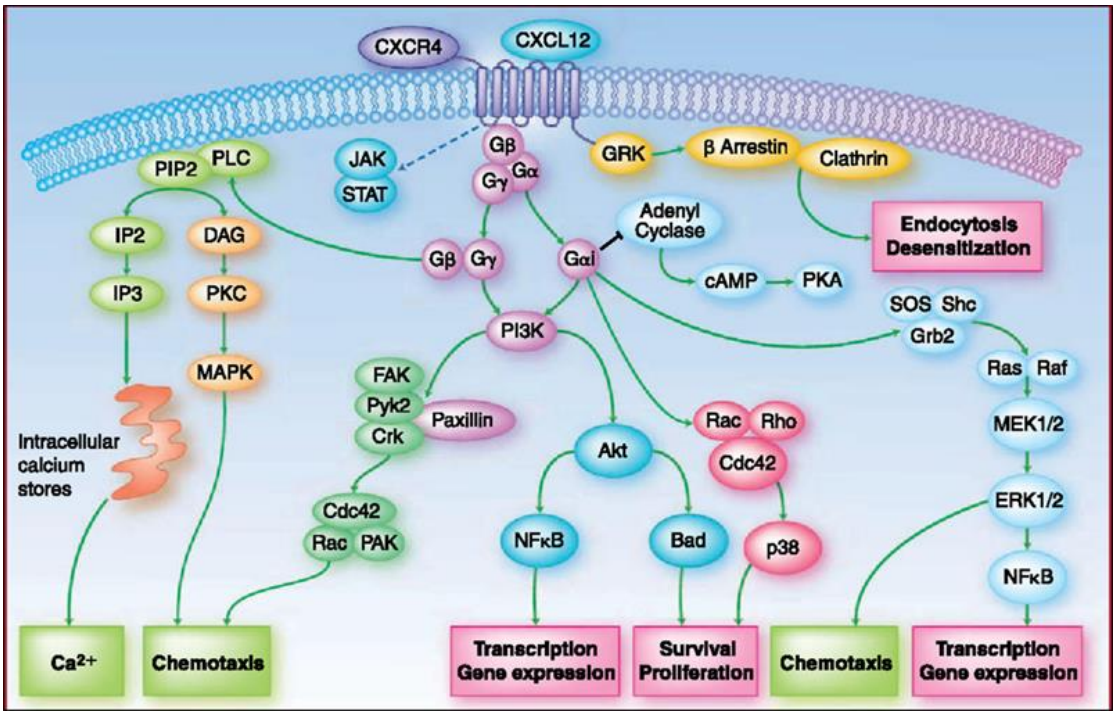

Figure 6: A scheme of the CXCL12/CXCR4 intracellular signal transduction pathways [81].

Desensitization of CXCR4 receptor occurs upon consistent CXCL12 binding, causing phosphorylation of intracellular C terminal domain of CXCR4 at serine sites by GRKs [15, 37, 81]. This causes β-arrestin recruitment and clathrin-regulated endocytosis of receptor. Additionally, β-arrestin can also activate signaling pathways like the MAPK p38 [15, 37, 81].

Several studies show that CXCL12 binding to CXCR4 on different types of tumor cells promotes their growth (*in vivo* and *in vitro*), either through PI3K/Akt or MAPK pathways [13, 82, 83]. Blocking the CXCR4 pathway may impair tumor metastasis, hence, improving the overall survival [13, 82, 83].

1.5 CC- Chemokine Receptor 7 (CCR7) in Cancer

Similar to the CXC chemokine family, the abnormal or upregulated expression of the CC class of chemokine receptors, particularly the CCR7 have also been linked to tumor metastasis and growth [5, 57, 80].

CCR7 is a 378 amino acid GPCR that is expressed in a variety of lymphoid tissues like mature dendritic cells, and T and B lymphocytes [5, 51, 57]. In response to the local synthesis of its respective ligands, namely CCL19 and CCL21, it is implicated in leukocyte trafficking to secondary lymphoid organs (like spleen and the lymph nodes) [5, 51, 57]. Gene knockout studies in mice showed that lack of CCR7 or CCL19/CCL21 displayed substantial decrease in T cell migration to secondary lymphoid organs, indicating its crucial role [84].

The CXCR4-CXCL12 overexpression is associated with the migration of cancer cells to the lymph nodes, lung, bone marrow, and liver, while the CCR7-CCL21/CCL19 overexpression has primarily been linked to lymph node metastasis [5, 53]. It is predominantly involved in the progression of numerous types of malignant tumors like skin (melanoma), colorectal, cervical, gastric, tonsillar, hepatocellular, prostate, breast, thyroid, esophageal, head and neck, lung cancers and chronic lymphocytic leukemia [17, 38, 41, 42, 44, 48, 52, 77, 85–94]. CCR7 expression was linked to greater invasion, metastasis, increased tumor size and poor prognosis in the most of these cancers [5, 17, 48].

However, some studies showed contrasting results. A study by Zhou and colleagues revealed that CCL19 overexpression notably restricted gastric cancer cell tumor development and proliferation, both *in vivo* and *in vitro* by activating a specific CCR7-linked signaling pathway like AIM2 [95]. Another study on comparative analysis of mRNA expression of CCL19 and CCR7 in oral squamous cell carcinoma (OSCC) and normal oral mucosa revealed no link between CCR7-CCL19 expression and microscopic and clinic parameters, hence, CCR7-CCL19 may not be linked with OSCC [96].

1.5.1 Molecular Signaling Pathways of CCR7 in Cancer

CCR7 can trigger downstream signaling pathways via interaction with its cognate ligands, namely CCL19 and CCL21, dissociating G proteins to active Gα subunit and a Giβγ heterodimer and triggering downstream signaling cascades, including MAPK, JNK (c-Jun N-terminal kinase), ERK1/2, PI3K/Akt, JAK/STAT and Rho GTPases that can stimulate the expression and transcription of diverse genes like matrix metalloproteinase (Figure 7) [5]. All of this can induce wide-ranging biological functions involving actin cytoskeleton reorganization, adhesion, chemotaxis, degradation of matrix, cell survival, invasion and proliferation, angiogenesis and migration, as shown in Figure 7 [5].

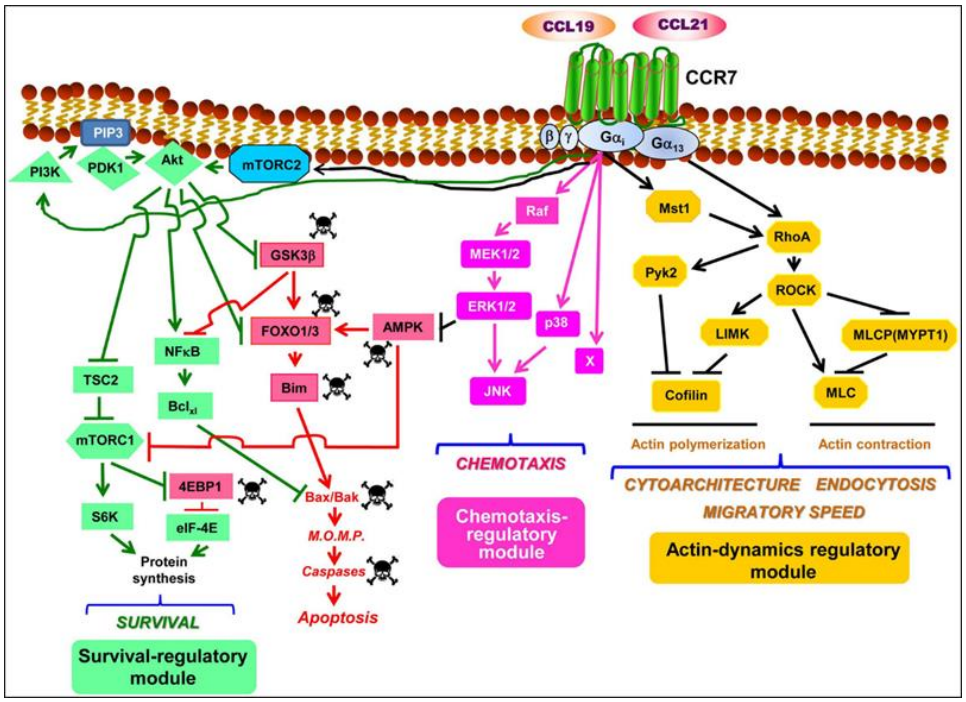

Figure 7: A schematic of the CCL19/CCL21/CCR7 intracellular signal transduction pathways [96].

Although CCL19 and CCL21 have the same binding affinities, they trigger signaling pathways with variable effects [97, 98]. Both stimulate G protein signaling when bound to CCR7, however, CCL19 is a more potent ligand in G protein signaling and stimulates β-arrestin-regulated CCR7 phosphorylation, which results in receptor recycling and internalization [5, 24, 97, 98]. Hence, CCR7-CCL19 mediated cellular responses have a short time duration [97, 98]. Contrarily, CCL21 is known to significantly increase ERK phosphorylation and calcium flux, compared to CCL19 [5, 24, 97, 98].

Chemokine receptors perform signaling particularly through the Gαi subunit that acutely inhibits the adenylyl cyclase-regulated cAMP and induces intracellular calcium flux mobilization [5, 99, 100]. On persistent $G\alpha_i$ subunit activation, elevated cAMP accumulation and intracellular calcium release occurs, indirectly activating the MEK1/2-ERK1/2 and PI3K/Akt pathways [5, 99]. Hence, both CXCR4-CXCL12 and CCR7-CCL19/CCL21 utilize particularly two pathways in cancer, the PI3K/Akt and the ERK/MAPK signal transduction pathways, known to control cell death, survival, proliferation, growth, invasion, and motility [101–106].

Although the role of chemokines and their receptors in human cancers is obvious, the molecular and cellular mechanisms involved are complex and not fully understood. It's critical to note that there is a lack of data from *in vivo* experimental systems about the precise role of individual chemokine receptors in the development of cancer [5].

1.6 Implication of CXCR4 and CCR7 Together in Cancer

In cancer, CCR7 and CXCR4 is usually up-regulated together [5, 50]. Therefore, both CXCR4 and CCR7 receptors have been studied together for their functional roles in metastasis, tumor progression and migration in most cancer cells, including breast, cervical, colorectal, esophageal, gastric, primary central nervous system lymphoma, prostate, pancreatic, non-small cell lung, head and neck cancer, esophageal cancer and so on [5, 14, 50–58, 107–109].

Studies investigating CXCR4 and CCR7 showed that the expression of both receptors were linked with lymph node metastasis in cervical and breast cancers, and could be used as biomarkers for poor prognosis in such cancers [50, 52, 53]. Also, both receptors

were found to promote metastasis by preventing anoikis in breast cancer cells [51]. The sensitivity and potency of chemokine responses have been shown to be enhanced by receptor complex formation, like formation of oligomers between receptors [14, 15, 110]. Hence, targeting and studying plausible interactions of these receptors is of special relevance in cancers as a strategy to restrict tumor formation and to significantly inhibit its metastatic ability.

1.7 Heterodimerization of GPCRs

Several proteins, including β-arrestins and G proteins, communicate with monomeric GPCRs in the plasma membrane, however, numerous studies indicate that GPCRs can also interact with additional receptors to generate dimers or highly elaborate oligomers under physiological settings [1, 111–116]. Such cross-talk can occur between identical (homodimers) or different receptors (heterodimers) [111–113, 115, 116].

However, to affirm heterodimerization between receptors, and their potential functional significance in native tissues, three conditions must be considered [117]. First, these protomers must physically communicate and co-localize [117]. Secondly, they must demonstrate features different from individual receptors, and lastly, loss of heteromers must lead to loss of their particular functional properties [117].

In particular, growing data suggest that receptor heterodimerization form distinctive signal transducing units having unique pharmacological properties [56, 117–119]. The normal functions of GPCRs is typically altered by heterodimerization, which regulates these receptors' activity [111, 113, 116]. Thus, heterodimerization consists of physical and functional interactions between receptors and can cause one protomer to significantly change the function of another protomer, hence, modifying or influencing downstream signaling and activation of second messengers, receptor internalization/recycling, cell surface trafficking, and ligand binding efficacy, as shown in Figure 8 [56, 113, 115].

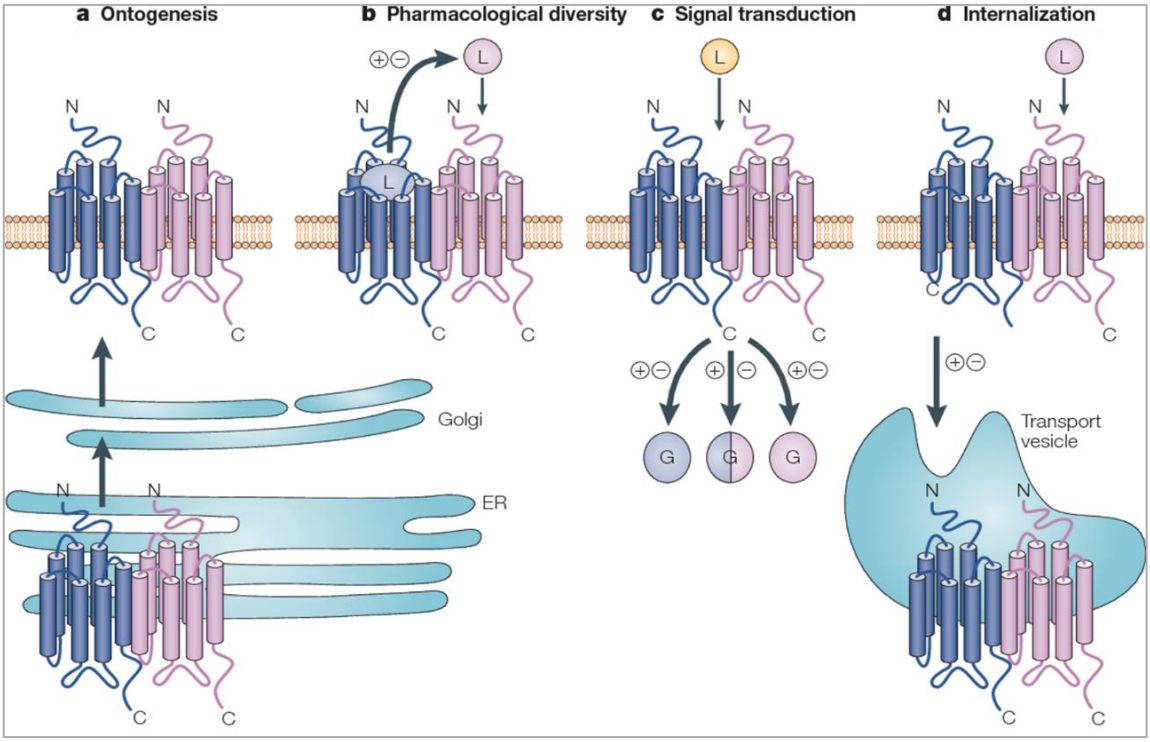

Figure 8: GPCR heterodimerization and its impact of receptor pharmacology, signaling, and trafficking [120].

Notably, heterodimerization between two different protomers may reduce or increase or even change the kind of intracellular signaling that is elicited by a particular stimulus or combination of stimuli [1]. It can lead to modifications in G protein signaling, for instance, by binding to a different subtype of Gα protein [1]. It may stimulate the G protein coupling and strengthen the downstream signaling effect, or vice versa [1]. Moreover, it can alter second messenger synthesis by affecting the way G protein coupling occurs [1]. Furthermore, in heterodimerization, when one protomer is activated by agonist, it can trigger internalization of another protomer (whether agonist bound or no) or both or even modify the outcome of internalized protomer [117]. Although heterodimerization of GPCRs introduces a new level of complexity, it also offers a potential therapeutic benefit for drug discovery from a pharmacological perspective [1].

Heterodimerization has been extensively studied and is well-documented between various chemokine subtypes [121–123].

1.8 Homo- and Heterodimerization in Chemokine Receptors

The concept of oligomerization is well-known among chemokine receptors [37, 124, 125]. Chemokines that are known to homodimerize include CXCR4, CCR7, CCR5, CCR2, CXCR1, CXCR2, while CCR2-CCR5 and CXCR1-CXCR2 form heterodimers, including CXCR4 that can heterodimerize with numerous other chemokines [126]

Several findings have shown that CXCR4 can homodimerize, and even heterodimerize with other GPCRs, namely CXCR7, CCR2, CCR5 and CCR7 (predominantly expressed on immune cells like T lymphocyte surface), augmenting or reducing the signaling capability of its partner receptor [81, 125–127]. In fact, CXCR4 homodimerization is known to cause G protein independent signaling, where β-arrestin is recruited, causing receptor desensitization, internalization and recycling or β-arrestin mediated signaling pathways (via ERK 1/2 pathway) and play role in cell migration, survival and proliferation [81, 127, 128]. It can also induce signaling via the JAK/Stat signaling pathway, resulting in intracellular calcium mobilization and gene transcription, however, this is debatable [81, 127, 128]. In contrast, CCR7 homodimerization by CCL21 is known to induce Src kinase-dependent signaling and facilitate effective cell migration [129].

Furthermore, CXCR4 heterodimerizes with ACKR3 (Atypical chemokine receptor 3), also called CXCR7, which strongly binds to CXCL12 compared to CXCR4 [37, 119, 124, 130]. CXCR4 also heterodimerizes with CCR5, stimulating $G_{q/11}$ signaling pathway and maintaining T lymphocyte activation and immunological synapse stabilization [118, 131]. Additionally, CXCR4 can heteromerize with other GPCRs, including opioid and adrenergic receptors, and this may help to regulate pain and blood pressure [127,p 2, 132, 133]. Figure 9 depicts CXCR4 homodimerization and its heterodimerization with other chemokine receptors, and its associated signaling pathways.

Resonance energy transfer (RET) techniques like FRET (fluorescence resonance energy transfer) or BRET (bioluminescence resonance energy transfer) have been employed to depict physical interaction between GPCR heterodimers [1, 117, 134].

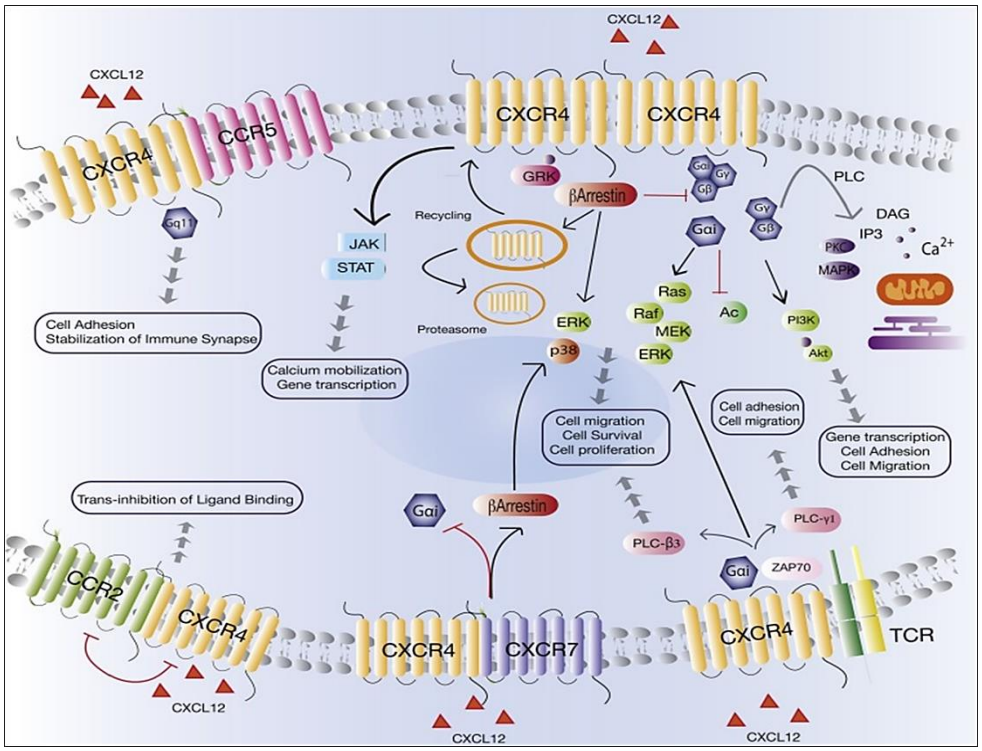

Figure 9: Homo- and heterodimerization of CXCR4.

Schematic representation of CXCR4 in form of homodimer or of heterodimer with other chemokine receptors and their related major signaling pathways [124].

1.9 Technique to Study GPCR Heterodimers via Bioluminescence Resonance Energy Transfer (BRET) - Principle and Applications

Most physiological responses in cell are dependent on protein-protein interactions and trafficking with many mediators [135]. Assessing these procedures in living cells in real time gives valuable insight into the spatiotemporal mediation of various cellular functions [135].

Bioluminescence Resonance Energy Transfer (BRET) is a non-destructive technique that has gained attention as a proximity-based assay to assess conformational rearrangements and protein–protein interactions in a quantitative real-time method, and in living cells [135, 136]. It is based on the principle of energy transfer between a luminescence donor and a fluorescence acceptor through a non-radioactive dipole-dipole interaction [135]. BRET happens when there is less than 10 nm of distance between the acceptor and donor and the energy transfer efficacy between them is inversely related to the sixth power of distance [135, 136]. The energy transfer mechanism, on the other hand,

is also dependent on the overlap of the acceptor's excitation spectrum with the donor's emission spectrum, as well as the acceptor and donor's relative spatial alignment [134, 136].

In BRET analyses, one target protein is coupled to a bioluminescent energy donor, like luciferases from *Oplophorus gracilirostris* or *Renilla reniformis* (sea pansy), and the second protein is attached to a fluorescent energy acceptor, like YFP (Yellow Fluorescent Protein) or GFP (Green Fluorescent Protein), produced by jellyfish [135]. They are then transfected in cells, eventually followed by addition of a substrate [135]. Although different types of BRET exist (QD-BRET, eBRET^{2,} BRET^{3,} BRET^{1}, and BRET^{2}), depending on the substrate type and nature of the acceptor/donor pairs, the original assay is called BRET^{1} that uses coelenterazine h (benzyl-coelenterazine) as a substrate, *Renilla* Luciferase (Rluc) as donor and YFP as acceptor proteins (Figure 10) [136].

Renilla Luciferase (Rluc) oxidizes the substrate, namely coelenterazine into coelenteramide, which emits light at 480nm, which is caught by the YFP [135]. YFP gets excited and generates a green light at 530nm [135]. The BRET machine analyzes the intensity of light emitted by YFP and Rluc, and the BRET signal measurement is attained by dividing acceptor's light signal and the donor's luminescence emission, i.e., YFP to Rluc, or acceptor/donor ratio (Figure 10) [135].

BRET is a phenomenon that happens in nature [137]. For example, in the absence of GFP, the photoprotein aequorin from the jellyfish Aequorea generates blue light, but when GFP and aequorin are coupled *in vivo*, GFP takes the energy from aequorin, releasing green light [137].

BRET technology is an intriguing substitute to fluorescence-based imaging techniques like FRET as the bioluminescent energy donor does not produce autofluorescence, light scattering, photobleaching or phototoxicity because it does not need an exterior source of light for excitation, hence, having a very low background [135]. BRET-assay can directly monitor protein–protein interactions in living cells, unlike most other assays like as pull-down, protein ligation, cross-linking assays, or co-immunoprecipitation assays [135]. When the impact of the cellular environment is being

explored or when interacting proteins characteristics can be modified by purification or extraction, the BRET assay's noninvasive feature proves to be beneficial [135].

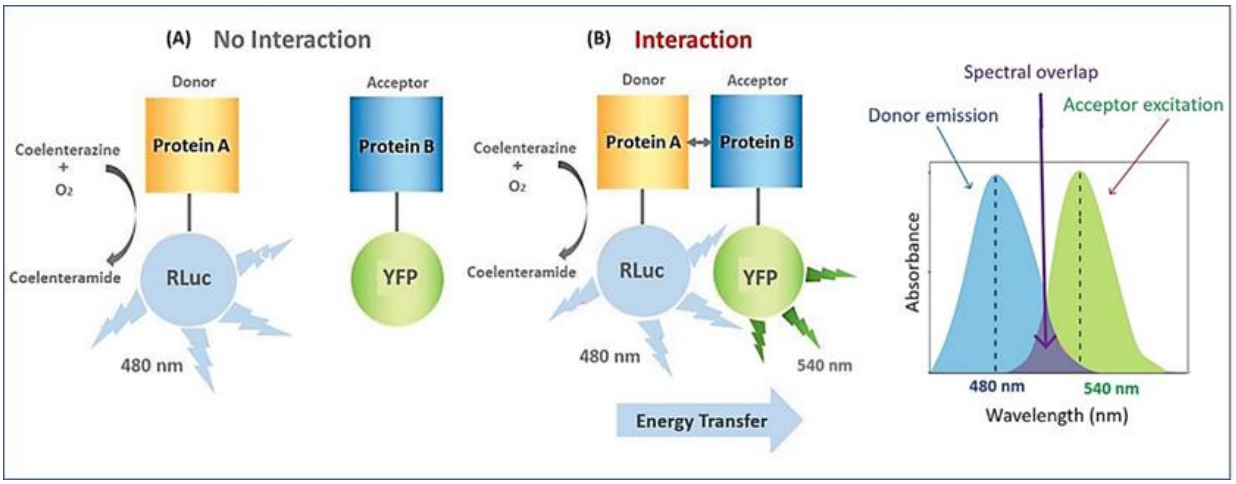

Figure 10: Principle of BRET assay.

In BRET analyses, one target protein (protein A) is fused to a bioluminescent energy donor, namely *Renilla* luciferase (Rluc), and the second target protein (protein B) is fused to a fluorescent energy acceptor, like YFP (for Yellow Fluorescent Protein). These two fusion proteins are then transfected in cells followed by the addition of coelenterazine substrate (Rluc substrate). A) In case of no interaction between proteins A and B (distance >10 nm), the activation of the BRET donor Rluc does not result in the emission of BRET acceptor YFP. B) If both proteins A and B interact when in close proximity (<10 nm), and Rluc oxidizes coelenterazine into coelenteramide, which emits light at 480 nm, caught by the YFP. YFP gets excited and generates a green light at 530 nm. The BRET signal is measured via light intensity emitted by YFP and Rluc (acceptor/donor ratio).

BRET has been widely employed to study the activation, signaling activity and kinetics of GPCRs [135]. Hence, BRET-based experiments have been used to investigate coupling of GPCRs to its regulatory proteins like β-arrestins or G proteins (and study signal transduction), receptor dimerization/oligomerization and post-translational changes like ubiquitination [136]. It has also been employed in ligand-binding studies with an energy donor coupled receptor and fluorophore-coupled ligands [135]

Many studies on immune cells have been performed using BRET to study heterodimerization and the functional effects of CXCR4 and CCR7 cross-regulation [56, 84, 138, 139]. However, evidence is lacking concerning the role or functional interaction of CXCR4-CCR7 heterodimerization in cancer specifically [56]. This is due to restriction

in finding native heterodimers and accessibility of *in vivo* models [56]. Only one study showed the existence of CXCR4-CCR7 heterodimers in advanced primary mammary human and mouse breast cancer cell lines, where high number of heterodimers was linked with cancer severity [56]. The study also showed the development of invasive phenotype in non-metastatic breast cancer cells, caused by the forced CXCR4-CCR7 heterodimerization, which could be used as a potential biomarker for malignant mammary cancers [56].

This constitutes a solid rationale for our hypothesis about the possibility of CXCR4-CCR7 heterodimerization and its implication in cancer development and progression.

Chapter 2: Hypothesis and Objectives

2.1 Hypothesis

In this project, we hypothesize that the heterodimerization between CXCR4 and CCR7 may have implication in human pathophysiology including cancer, as shown in Figure 11.

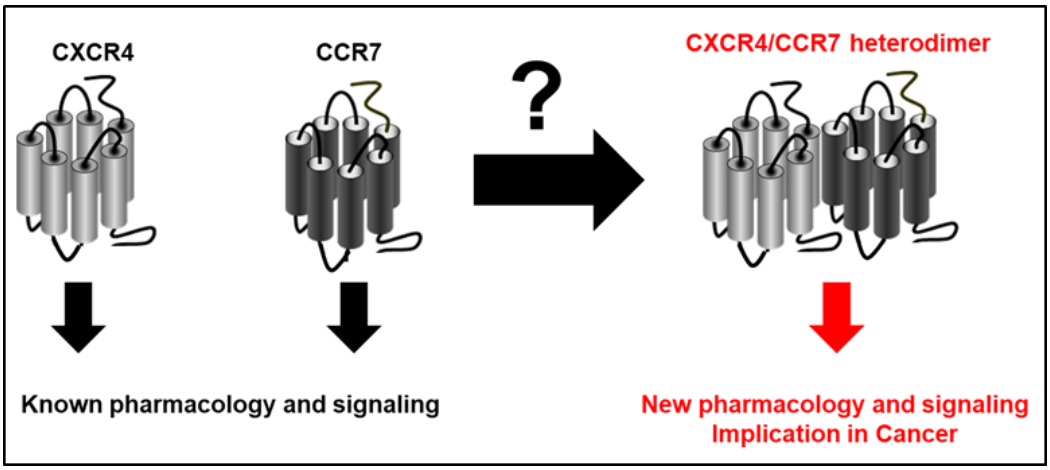

Figure 11: Hypothesis of heterodimerization between CXCR4 and CCR7.

2.2 Objectives

We propose to investigate the *in vitro* physical interaction between CXCR4 and CCR7 and/or its functional consequences of the pharmacology and signaling of the receptors of the cancer cells (Figure 12). For this, two cell models were used: (i) cancer cell lines endogenously expressing the two receptors, including the triple negative MDA-MB-231 (breast cancer), HT-29 (colon cancer) and HepG2 (liver cancer) as well as (ii) the human embryonic kidney (HEK 293) cell line transiently expressing the two receptors.

The specific approaches applied in this project are the following:

1) To perform RT-PCR for evaluating the relative expression levels of CXCR4 and CCR7 in various cancer cell lines.

2) To use BRET technology for investigating the interaction between CXCR4 and CCR7 and the functional consequences of such interaction on the receptor

pharmacology and signaling by investigating; receptor/G protein coupling and receptor/β-arrestin association.

3) To conduct SDS-PAGE, followed by western blot for assessing the phosphorylation of the key intracellular kinases, including ERK 1/2 and Akt.

4) To examine the effect of CXCR4 and CCR7 interaction and activation on cancer cell viability.

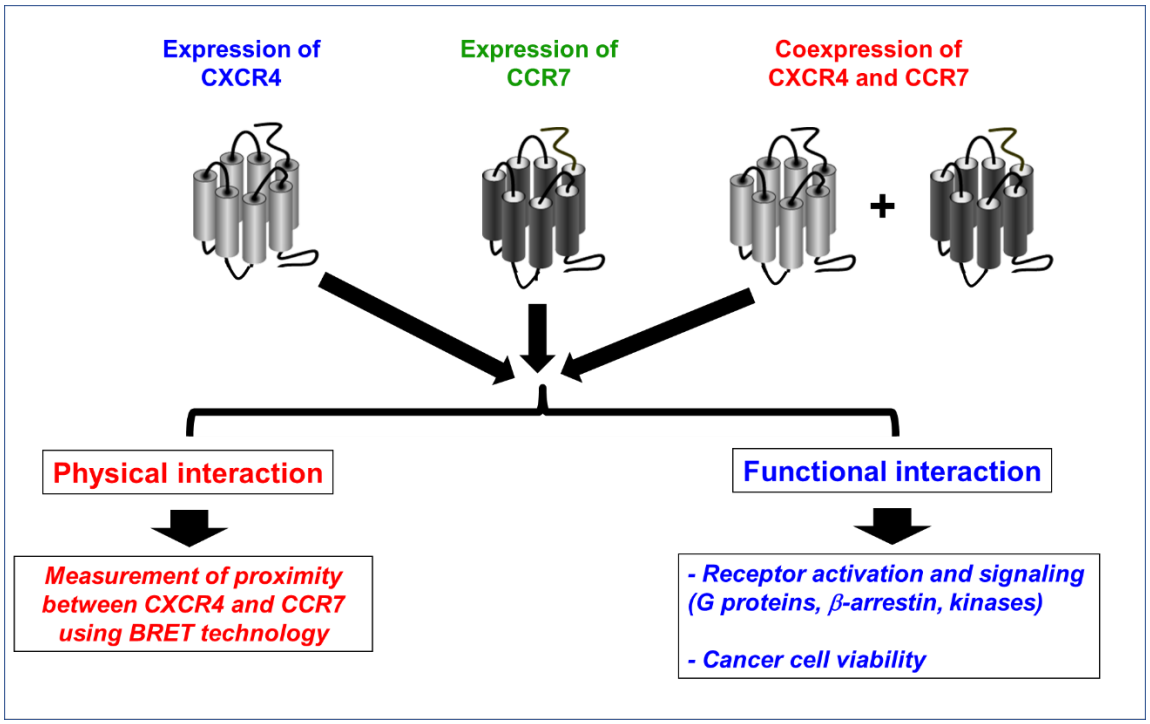

Figure 12: Objectives and the general experimental approach to be used.

Chapter 3: Materials and Methods

3.1 Chemicals, Reagents, and Plasmid

The receptor plasmids like CXCR4-WT (Wild Type), CCR7-WT, CCR5-WT and with donor constructs like Rluc (*Renilla* luciferase)-tagged CXCR4 and Rluc-tagged CCR7 were kindly provided by Prof. Pfleger KD. (Harry Perkins Institute of Medical Research and UWA, Perth, Australia). The ligands, namely CXCL12 (agonist for CXCR4) and CCL19 (agonist for CCR7) (100 nM) were obtained from PeproTech (PeproTech Inc., Rocky Hill, NJ, USA). Plasmid encoding acceptor fused G-protein and β-arrestin constructs, involving Venus (YFP variant)-fused Gαi and Venus-fused β-arrestin 2, were kindly provided by Scott, M. (Cochin Institute, Paris, France).

Fluorescence microscopy was used to visually confirm the appropriate expression of the proteins tagged with YFP and Rluc, and the Tristar 2 plate reader (Berthold Technologies, Bad Wildbad, Germany) was used to read light emission at 540 nm (acceptor) and 480 nm (donor). The luminescent substrate, namely coelenterazine h was purchased from Promega (Promega Corporation, Madison, WI, USA); whereas Lipofectamine™ 2000 was purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA) and used for cell transfection. 96-well white microplates (Optiplate-96) were from Perkin Elmer (Perkin Elmer, Cambridge, Cambridgeshire, UK). For western blot, the antibodies recognizing AKT and ERK1/2 (both native and phosphorylated forms) were from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA, USA) and Pierce BCA (bicinchoninic acid) protein assay kit was purchased from Thermo Scientific (Cat. no. 23225, Thermo Scientific, Rockford, IL, USA).

3.2 RT-PCR

3.2.1 RNA Isolation

RNA Isolation was performed using the RNeasy Mini Kit (Cat. no. 74104, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, cells (1×10^7 cells) were lysed with 350 µL of RLT lysis buffer and 3.5 µL of βME (β-mercaptoethanol) and homogenized further to induce complete lysis. Thereafter, 350 µL of 70% ethanol was added to precipitate RNA and mixed well. Then the samples were transferred to RNeasy

spin column and centrifuged for 1 minute at maximum speed (15000 rpm). The flow-through was discarded, with RNA on the column. DNase I incubation mix (80 µL) was also directly added to spin column membrane and incubated at 20–30°C for 15 minutes. This was followed by a series of washing and elution steps. The RNA was finally eluted in 30-50 µL of RNase-free water. This was further diluted to 80 – 100 µL.

The RNA quality and concentration were determined using NanoDrop ND-2000 (Thermo Scientific, Waltham, MA, USA), where NF (nuclease free) water was used as a blank. The RNA was stored at -20^{o}C until further use.

3.2.2 Two-step Reverse Transcription Polymerase Chain Reaction (RT-PCR)

For acquiring PCR products, a two-step reverse transcription polymerase chain reaction (RT-PCR) was performed, entailing cDNA generation from RNA by reverse transcriptase and gene amplification by Taq DNA polymerase enzymes. A High-Capacity cDNA Reverse Transcription Kit (Cat. no. 4368814, Applied Biosystems, Foster City, CA, USA) was used as per manufacturer's instructions. 1 µg of RNA was used for cDNA synthesis. Conventional reverse transcription PCR was performed in T100 Thermal Cycler (BioRad, Hercules, CA, USA) for 2 hours and 15 minutes. The program was as follows: 25°C for 10 minutes (primer annealing), 37°C for 2 hours (reverse transcription), 85°C for 5 minutes (inactivation) and 4°C for infinity hold. The cDNA was stored at -20^{o}C until further use.

For gene amplification, a PowerUp SYBR Green Master Mix (Cat. no. A25742, Applied Biosystems, Carlsbad, CA, USA) was used as per manufacturer's instructions, with GAPDH (Glyceraldehyde 3-Phosphate Dehydrogenase) as internal control. The primers (Microgen, Daejeon, South Korea) were as follows: CXCR4 (206 bp fragment) forward: 5'-TTCTACCCCAATGACTTGTG-3' and reverse: 5'-ATGTAGTAAGGCAGCCAACA-3'; CCR7 (529 bp fragment) forward: 5'-TCCTTCTCATCAGCAAGCTGTC-3' and reverse: 5'-GAGGCAGCCCAGGTCCTTGAA-3'; GAPDH (164 bp fragment) forward: 5'-GAGTCCACTGGCGTCTTCACC-3' and reverse: 5'-GAGGCATTGCTGATGATCTTGAGG-3'. For a single reaction, 10 µL/well of reaction mixture (SYBR green, primers, NF water and cDNA) was added in 96-well PCR plate.

For negative control, NF water was used instead of cDNA. The plate was inserted in QuantStudio 5 Real-Time PCR instrument (Applied Biosystems. Foster City, CA, USA). The program was run as follows: 95°C for 2 minutes (Initial denaturation), 95°C for 15 seconds x 40 cycles (denaturation), 56°C (for CXCR4) and 62°C (CCR7) for 1-minute x 40 cycles (annealing), 72°C for 1-minute x 40 cycles (elongation). Thermal cycler was operated for 1 hour and 30 minutes to get PCR products. For integrity assessment, the products (23 uL) were run on 1.5% agarose gel, stained with ethidium bromide and visualized under UV light with Gel DocTM EZ Imager (Bio-Rad, Hercules, CA, USA) to get the gel image. Figure 13 summarizes the steps involved in PCR.

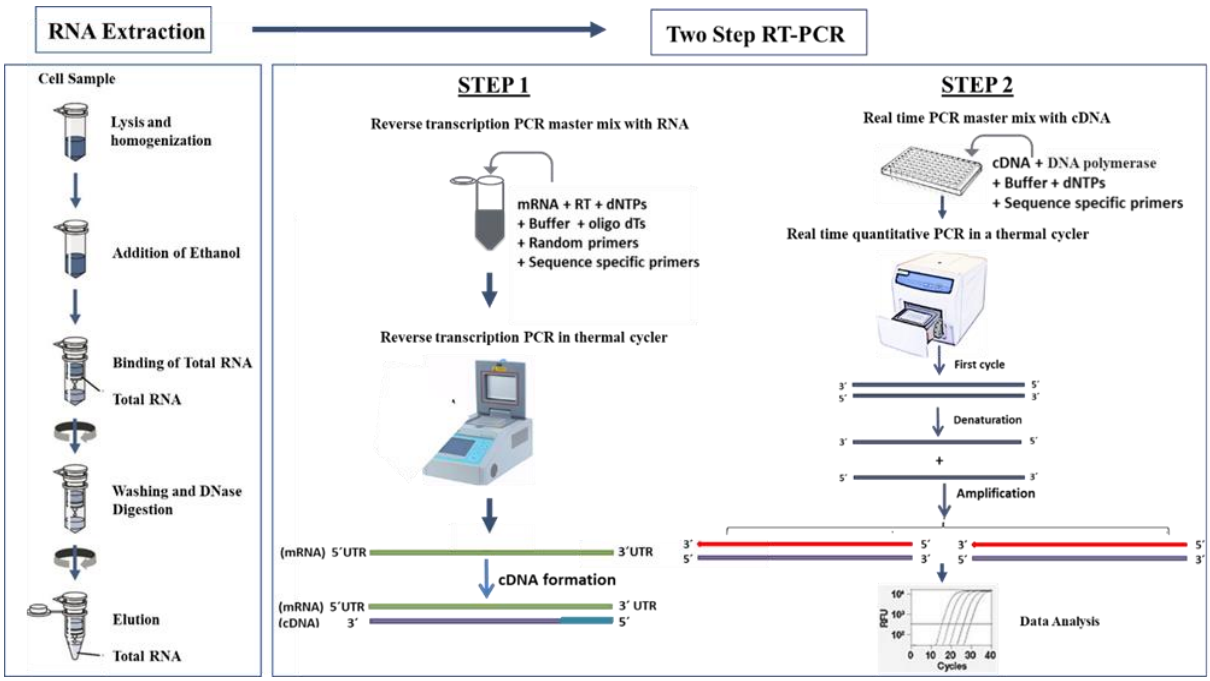

Figure 13: Steps for attaining amplified PCR products, involving RNA extraction and two-step RT-PCR.

3.3 Preparation of Plasmids

Transformation was performed using NEB® 5-alpha Competent *E. coli* cells (New England BioLabs Inc., Ipswich, MA, USA). The respective plasmids, namely CXCR4-Rluc, CCR7-Rluc, and untagged CCR7-WT, CCR5-WT and CXCR4-WT were suspended in 50 µL TE buffer (Tris-EDTA) to get concentrated DNA plasmids and incubated for 30 minutes at 37°C. Each plasmid (1-5 µL having 1 pg-100 ng) was added to bacterial cells (50 µL) in a transformation vial and briefly flicked to mix without vortexing. The mixture

was placed on ice for 30 minutes, followed by heat shock for 30 seconds at 42°C, and keeping back on ice for 5 minutes. Next, 950 µL of pre-warmed SOC Outgrowth Medium (New England BioLabs Inc., Ipswich, MA, USA) was pipetted into each vial with transformed cells and incubated for 1 hour at 37°C in shaking incubator (250 rpm). Centrifugation was performed for 5 minutes at 25°C and 5000 rpm to attain pellet that was resuspended in 50-100 µL of remaining SOC media, while the rest was removed. For plating transformed bacteria, the pellet mixture was evenly spread into the warm LB agar plates with the antibiotic ampicillin (Sigma-Aldrich, Saint Louis, MO, USA) for colony selection and incubated for 12-16 hours at 37°C.

For every plasmid, one colony was taken from the transformed agar plates and dispersed in 200 mL of tryptic soy broth (soybean-casein digest medium) (Neogen Culture Media, Heywood, LA, UK) supplemented with ampicillin. The colony suspension was kept for overnight incubation in rotary shaking incubator (250 rpm) at 37°C. Subsequently, centrifugation was performed at 6000 rpm for 15 minutes to attain pellet of transformed bacteria. The supernatant was discarded and the pellet was stored at -20°C prior to being used for plasmid extraction.

The Qiagen Plasmid Maxi Kit (cat. nos. 12162, 12163 and 12165, Qiagen, Hilden, Germany) was used to extract plasmids from bacterial pellets, as per the manufacturer's instructions. Finally, NF water was used to resuspend the extracted DNA, and the NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) was used to determine the quality and concentration of plasmids.

3.4 Cell Culture and Transfection

All cell lines, namely HT-29 (human colorectal adenocarcinoma), MDA-MB-231 (human triple-negative metastatic breast cancer), Hep-G2 (human hepatocarcinoma) and HEK 293 (Human embryonic kidney 293) cells were maintained in a humidified incubator at 37°C, 5% CO_2 and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 IU/ml penicillin (Gibco, Carlsbad, CA, USA). All experiments were conducted using 70–80% confluent cells.

For BRET, transient transfections were performed in HEK 293 cells in 96-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per manufacturer's

recommendations. Various plasmid combinations (acceptor and donor) were prepared for transfection in different experiments, as follows: (i) Rluc-tagged CXCR4 (50 ng) with either Venus-tagged Gαi or β-arrestin 2 (50 ng) in the presence of absence of untagged CCR7 (50 ng) (ii) Rluc-tagged CCR7 (50 ng) with either Venus-tagged Gαi or β-arrestin 2 (50 ng) in the presence or absence of untagged CXCR4 (50 ng), (iii) Rluc-tagged CXCR4 (50 ng) and Venus-tagged β-arrestin 2 (50 ng) in the absence, or presence of either untagged CCR5 or CCR7 (50 ng) (iv) Rluc-tagged CCR7 (50 ng) and Venus-tagged β-arrestin 2 (50 ng) in the absence, or presence of either untagged CXCR4 or CCR5 (50 ng) (v) Rluc-tagged CCR7 and Venus-tagged β-arrestin 2 in the absence, or presence of increasing amount of untagged CXCR4 (5, 10, 15, 25, 50, 75, 100 ng). Briefly, every respective plasmid combination was mixed with 25 µL of Opti-MEM medium (Gibco, Carlsbad, CA, USA). A solution of 0.5 µL of Lipofectamine 2000 in 25 µL Opti-MEM medium was also prepared. Both reaction mixes were incubated at room temperature (RT) for 5 minutes. Following incubation, the plasmid solutions and Opti-MEM-Lipofectamine mixture were combined and incubated for 20 minutes at RT. The respective transfection mix (50 µL/well) were then seeded in PDL (poly-D-lysine)-coated 96-well white microplate or Optiplate-96 (Perkin Elmer, Cambridge, Cambridgeshire, UK) along with HEK 293 cells (10^4 cells in 150 µL/well) and incubated at in a humidified incubator at 37°C, 5% CO_2 for 48 hours.

3.5 BRET Assays

For BRET, transfected HEK 293 cells transiently co-expressing their respective tagged (donor and acceptor) and untagged protein combinations were starved in serum free media (SFM) for at least 3 hours at 37°C**.** For agonist treatment in BRET, 2X of CXCL12 (for CXCR4) and CCL-19 (for CCR7) (PeproTech Inc., Rocky Hill, NJ, USA) (100 nM chosen for each [139]) were prepared in PBS (Phosphate Buffered Saline). The SFM was aspirated from wells, followed by PBS wash and adding the respective treatments, namely CXCL12, CCL19 and both in designated wells (50 µL total volume/well). PBS of 50 µL was added in each well with no treatment (basal or control). The 96-well microplate was incubated for 30 minutes at 37°C, followed by preparing 6X of coelenterazine h (Promega, Madison, WI, USA) (5 µM) in PBS, and adding it to the treated cells (10 µL/well). The 5 µM of coelenterazine was considered optimum [139].

The 96-well white plate was read using the Tristar 2 multilabel plate reader (Berthold Technologies, Bad Wildbad, Germany) at 480 nm emission and 540 nm excitation to determine BRET signal. Figure 14 illustrates the BRET protocol in brief.

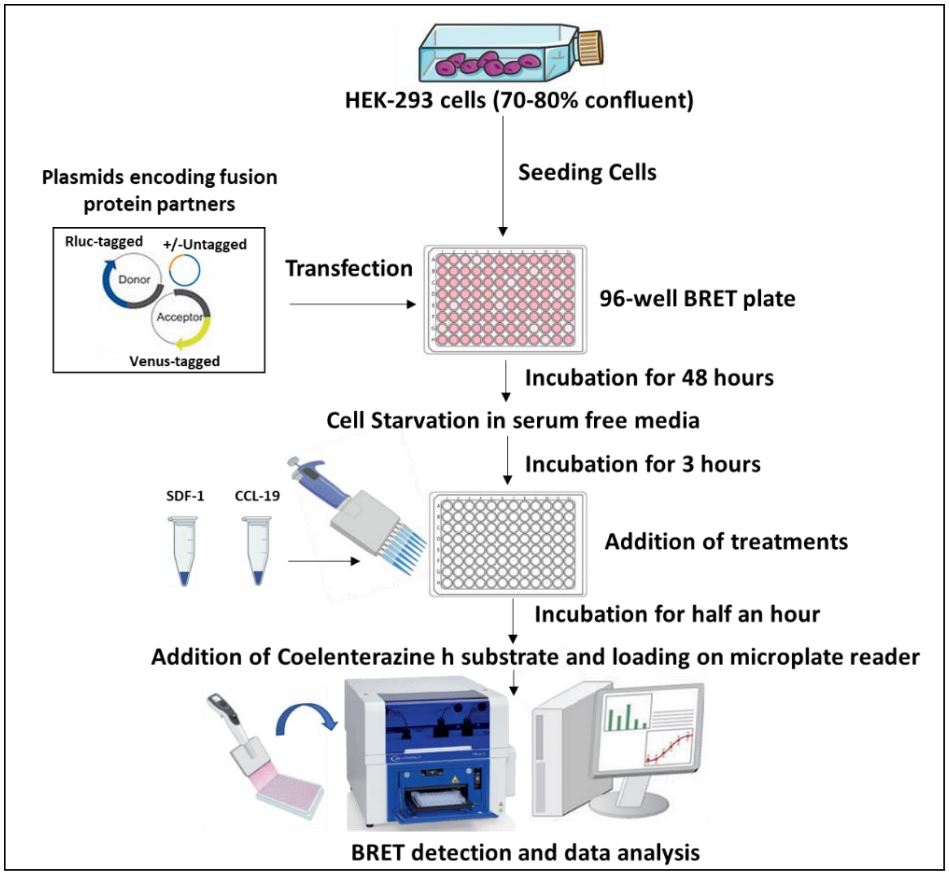

Figure 14: BRET protocol in transfected HEK 293 cells.

3.5.1 BRET Titration

BRET titration was conducted to assess the effect of increasing amount of CXCR4 expression on CCR7-Rluc and β-arrestin 2-Venus coupling. For this, HEK 293 cells were co-transfected with 50 ng CCR7-Rluc and 50 ng β-arrestin 2-Venus in the absence or presence of increasing quantities of untagged CXCR4 (5, 10, 15, 25, 50, 75 and 100 ng), and seeded into 96-well plates. 48 hours post-transfection, cells were starved with SFM for at least 3 hours before conducting BRET experiments. The SFM was aspirated from wells, followed by PBS wash. The reaction was stimulated with 100 nM of CCL19 for 30 min at 37°C and 50 µL as total reaction volume per well was used. PBS of 50 µL was

added in each well with no treatment (basal or control), followed by adding 10 µL coelenterazine h (5 µM), before measuring BRET signal in live cells.

3.5.2 Transfection Efficiency

Transfected HEK 293 cells co-expressing their respective donor and acceptor proteins were first seeded in 96-well white (for BRET and luminescence measurements) and black (for fluorescence 24 measurements) plates. By adding Coelenterazine h substrate and using the Tristar 2 multilabel plate reader to quantify light emission at 480 nm, the expression of Rluc-tagged proteins was determined. In contrast, the acceptor-tagged proteins (Venus) were evaluated using fluorescence microscopy for visual inspection and the Tristar 2 multilabel plate reader for reading light emission at 540 nm.

3.6 SDS-PAGE and Western Blotting

The cell lines (HEK 293 and HT-29) endogenously expressing CXCR4 and CCR7 receptors were seeded in six-well tissue culture plates. Each well was seeded with 1 X 10^5 cells in complete DMEM media and incubated overnight in a humidified incubator at 37°C, 5% CO_2. The cells were then serum starved with SFM incubated for 3 hours. Thereafter, the cells were treated as follows: PBS (control), 100 nM of CXCL12, 100 nM of CCL19 and 100 nM each of both. The concentration was chosen based on previous studies [84, 130]. The treated plates were incubated at 37°C for 15 minutes to detect ERK 1/2 and Akt phosphorylation. A 15 minute time point was selected at which both kinases were found to be activated [109, 127, 140, 141]. Following treatments, cells were washed with cold PBS and thereafter, cells were lysed with 150 µL/well of cold RIPA lysis buffer (Radioimmunoprecipitation assay buffer) (Merck Millipore, Burlington, MA, USA) supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany). Following treatment, the cells were scraped and centrifuged at maximum speed (15,000 rpm) for 30 minutes at 4°C. The supernatant with soluble protein were assembled in fresh pre-chilled 1.5 ml Eppendorf tubes and kept at −20°C until further use.

To determine protein concentration, Pierce BCA Protein Assay kit (Cat. no. 23225, Thermo Scientific, Rockford, IL, USA) was used as per the manufacturer recommended protocol. In brief, the BSA (bovine serum albumin) standards and experimental protein samples were prepared following manufacturers recommended protocol. The absorbance

was read at 562nm using the GloMax Discover plate reader (Promega, Madison, WI, USA). Thereafter, the protein samples (20 µg) were prepared with Laemmli buffer (Bio-rad) containing 8% β-mercaptoethanol, and heated at 95°C for 5 minutes.

For SDS-PAGE (polyacrylamide gel electrophoresis), the protein samples were loaded in 10% SDS-PAGE gel. The electrophoresis was performed at 100 volts for 2.5 hours. Following electrophoresis, proteins were transferred to PVDF (polyvinylidene difluoride) membranes (Bio-Rad, Hercules, CA, USA) via the conventional wet-transfer technique, operated under ice-cold conditions at 100 volts for 100 minutes. The PVDF membranes were thereafter blocked in blocking buffer (5% non-fat milk in 1X PBST, i.e., PBS (Gibco, Carlsbad, CA, USA) containing 0.1% Tween 20 (Bio-Rad, Hercules, CA, USA) using shaker for 1 hour. After blocking, the membranes were washed thrice with 1X PBST, 10 minutes each. The membranes were then probed with primary antibodies against pAkt, pERK1/2, Akt and ERK1/2 (prepared in 5% bovine serum albumin in 1X PBST) and incubated overnight in a shaker at 4°C. Anti-mouse or anti-rabbit IgG conjugated to HRP (horseradish peroxidase) were used as secondary antibodies as per the primary antibodies source. The membranes were incubated for 1 hour in secondary antibodies in rotary shaker at 37°C. All antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA, USA) (Table 1).

Finally, the membranes were developed with Pierce ECL (Enhanced Chemiluminescent Substrate) (Thermo Scientific, Waltham, MA, USA). Images were acquired using the LI-COR C-digit Blot Scanner (LI-COR Biosciences, Lincoln, NE, USA). Figure 15 explains the western blotting protocol in brief.

Table 1: List of antibodies used for Western blotting.

Antibody	Catalog number	Dilution used
Akt	9272	1:1000
pAKT (Ser 473)	9271	1:800
ERK 1/2	4695	1:1000
pERK1/2(Thr 202/Tyr 204)	9106	1:2000
Anti-mouse IgG-HRP	7076	1:3000
Anti-rabbit IgG-HRP	7074	1:3000

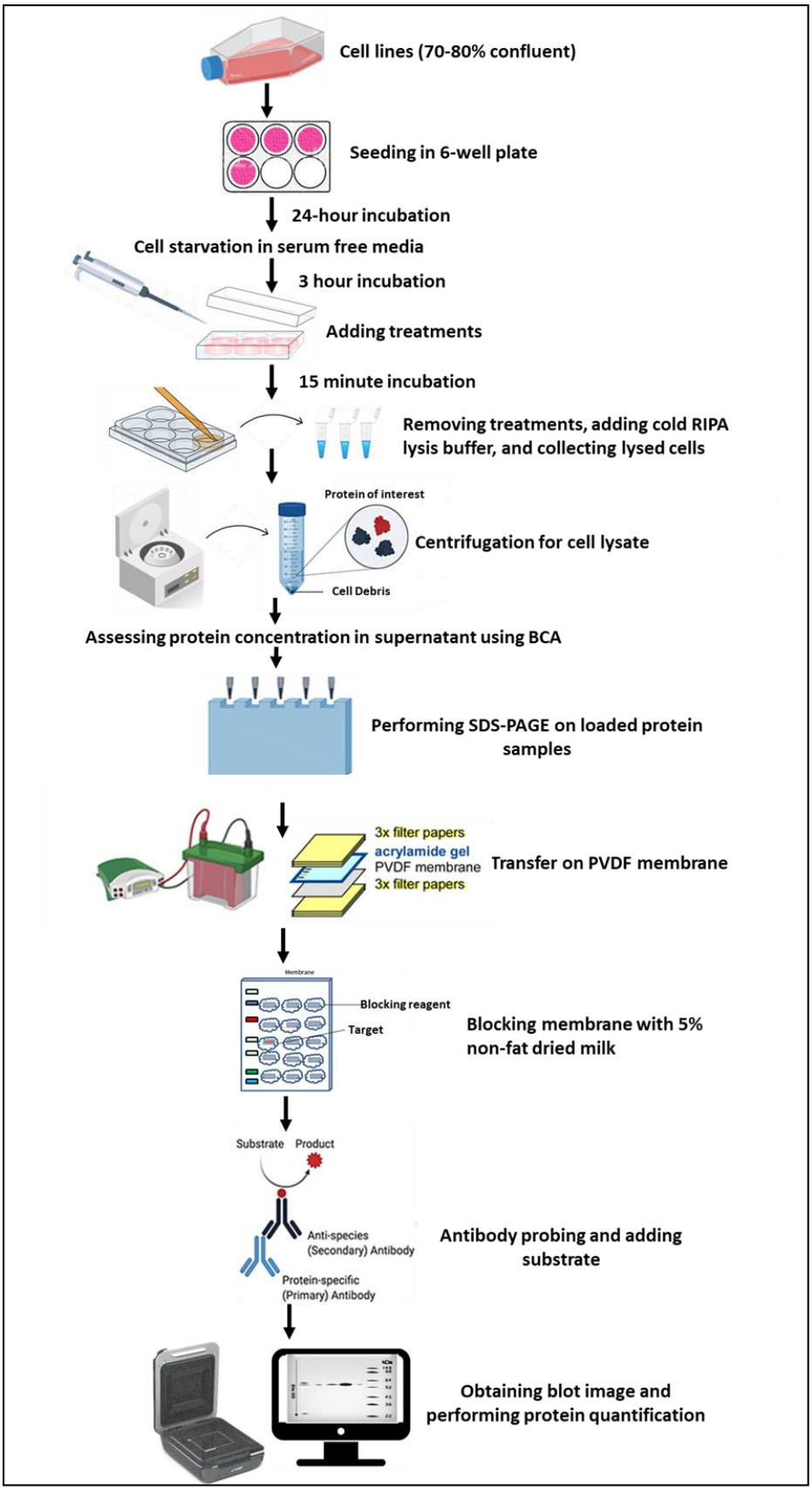

Figure 15: Schematic diagram of the steps involved in Western blotting.

3.7 Cell Viability Assay

Cell viability was determined using the MTT assay kit (ab211091, Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. Briefly, MDA-MB-231 and HT-29 cancer cells (10^4 cells/well) were seeded into the 96-well plates and treated with CXCL12 (100 nM), CCL19 (100 nM), and both and incubated for 48 hours at 37°C. Next, the media was aspirated and substituted with 50 µl of SFM and 50 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reagent per well and incubated at 37°C for another 3 hours. Thereafter, 150 µL of MTT solvent was added per well. Finally, the absorbance values of formazan solution were assessed at 590 nm using the GloMax Discover plate reader (Promega, Madison, WI, USA).

3.8 Data Analysis and Statistics

All experiments on BRET, western blotting and RT-PCR were performed in triplicates and as three independent assays. For RT-PCR, Quantstudio Design and Analysis software v1.3.1 (Applied Biosystems, Foster City, CA, USA) was used for data analysis, where fluorescence data was collected via amplification plot, and cycle threshold (C_T) values were obtained for the samples. The C_T values for the gene of interest was normalized to the internal control's (GAPDH) C_T value. The relative gene expression for CXCR4 or CCR7 with respect to GAPDH was evaluated using the Comparative C_T Method ($\Delta\Delta C_T$ Method) to assess fold change ($2^{-\Delta\Delta CT}$), and the standard error of the mean (SEM) were estimated for each experimental group. Following western blot, the bands were generated and visualized using with Image Studio software, version 5.2 (LI-COR Biosciences, Bad Homburg, Germany). BRET ratios were measured as 540 nm over 480 nm light emissions. To determine the ligand-induced impact, the baseline BRET value (control or PBS treated cells) was subtracted from the treatment values. In BRET data, the values were normalized by taking as 100% the CXCL12 ligand for CXCR4-Rluc (donor) that will initiate the BRET reaction. Graphs were plotted using GraphPad Prism software version 7 (GraphPad Prism Software Inc., San Diego, CA, USA). Statistical analyses were performed with two-way ANOVA and Tukey's multiple comparisons test/Bonferroni test to determine statistically significance between the different treatments and conditions. *P* value of ≤ 0.05 was considered statistically significant.

Chapter 4: Results

4.1 RT-PCR on the Relative Expression of Receptors on Cancer Cell Lines

RT-PCR was performed to assess the relative expression levels of CXCR4 and CCR7 receptors in different cell lines by using the fold change method ($2^{-\Delta\Delta CT}$), which is the comparative C_T method of quantitative gene expression.

By plotting graphs among different cell lines, the relative fold changes of CXCR4 and CCR7 expression were detected. As shown in Figure 16A, CXCR4 was differentially expressed in all cell lines, however, it was upregulated by 8 to 9-fold in HT-29 cells, around 3-fold in HepG2 cells, and by around 1.5 to 2-fold in MDA-MB-231 when compared to HEK 293 cells (control) (Figure 16A). As for CCR7, its expression level was negligible in MDA-MB-231 and HepG2 cell lines, but significant in HT-29 (~3-fold change) cells in comparison to HEK 293 cells (Figure 16B). Hence, the relative expression levels for both receptors were found to be the highest in HT-29 cell lines.

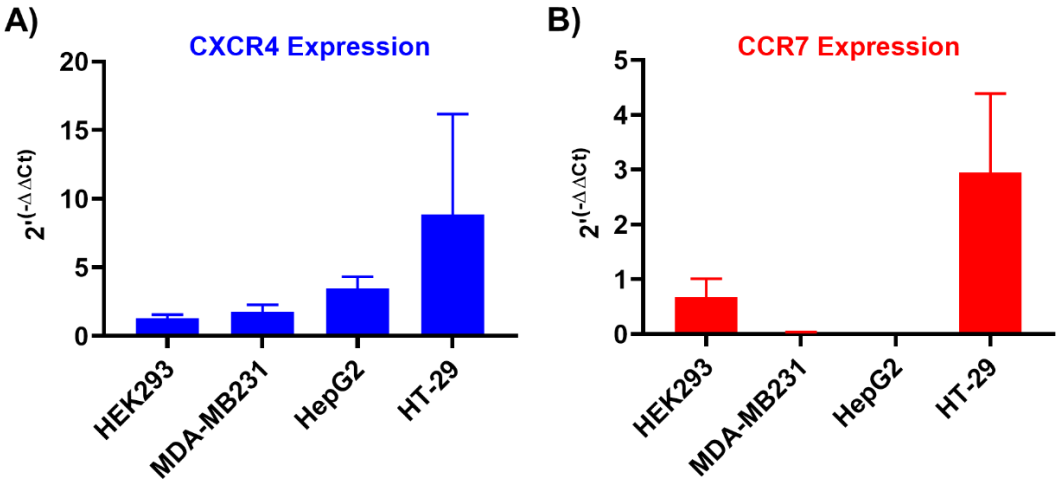

Figure 16: Relative expression levels of CXCR4 and CCR7 in cancer cell lines.

Relative expression levels of CXCR4 (A) and CCR7 (B) analyzed by RT-PCR in MDA-MB231, HepG2, and HT-29 cell lines relative to HEK 293 cells (taken as control). Data are means ± SEM of triplicate measurements.

4.2 Effect of CCR7 Co-Expression on CXCR4/Gαi Coupling and Activation

To assess whether CCR7 has any influence on the functional interaction of CXCR4 and with its cognate Gαi and their activation, BRET was performed after HEK 293 were transiently transfected with CXCR4-Rluc and Venus-Gαi in the absence or presence of untagged CCR7 and treated with either CXCL12, CCL19, or both (Figure 17A).

In the absence of CCR7, BRET was significantly induced with respect to basal upon treatment of cells with CXCL12 (100 nM) as expected, indicating the activation of CXCR4 with respect to Gαi (Figure 17B). By contrast, CCL19 (100 nM) had no significant effect demonstrating the specificity of the BRET response with CXCL12 (Figure 17B). The combination of CCL19 with CXCL12 had no significant effect on CXCL12-induced BRET increase between CXCR4-Rluc and Venus-Gαi (Figure 17B).

Likewise, upon CCR7 co-expression, BRET was also significantly induced upon CXCL12 treatment. More interestingly, we observed that CCL19 also promoted a significant BRET increase between CXCR4-Rluc and Venus-Gαi (Figure 17B). However, the combination treatment with CCL19 and CXCL12 had no significant effect on both ligand-induced BRET responses (Figure 17B). In addition to validating our BRET assay to measure CXCR4 activation in HEK 293 cells, these data suggest the existence of a functional interaction between CCR7 and CXCR4 in HEK 293 cells. Such a functional interaction is associated with CCL19/CCR7 promoting CXCR4/Gαi interaction and coupling.

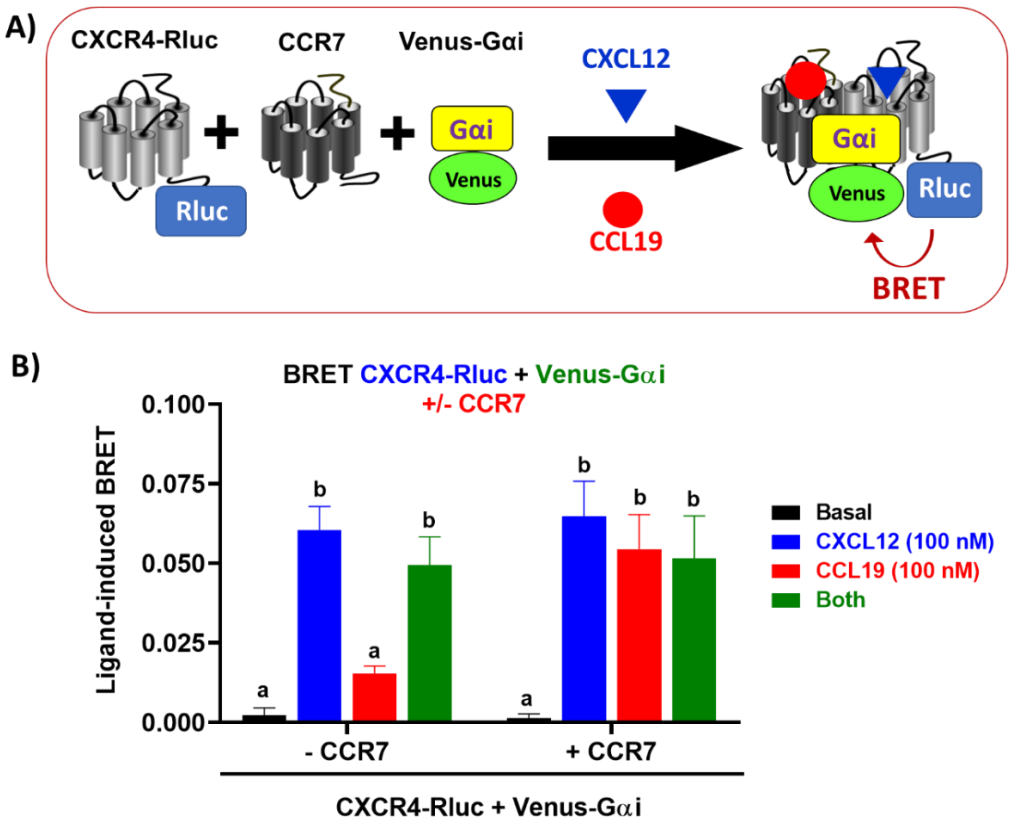

Figure 17: Effect of CCR7 co-expression on CXCR4/Gαi coupling and activation.

A) Schematic representation of the transient transfection for BRET assay in HEK 293 cells. B) BRET data in HEK 293 cells transiently co-expressing CXCR4-Rluc (BRET donor) and Venus-Gαi (BRET acceptor) in the absence or presence of untagged CCR7 and treated for 30 minutes with either CXCL12 (100 nM), CCL19 (100 nM), or both. Data are means ± SEM of ligand-induced BRET of five independent BRET experiments performed in triplicate. The different letters in Panel B indicate statistically significant effects of the different treatments (p-value < 0.05). The same letters indicate no significant difference between the indicated treatments compared to each other.

4.3 Effect of CCR7 Co-Expression on CXCR4/β-Arrestin Interaction

Like for CXCR4/Gαi interaction, we also examined the effect of CCR7 co-expression on CXCR4 and β-arrestin 2 interaction using BRET as shown in Figure 18A.

Like for CXCR4-Rluc/Gαi pair, in the absence of untagged CCR7 co-expression CXCL12 but not CCL19 increased BRET signal between CXCR4-Rluc and β-arrestin 2-Venus indicating CXCR4 activation and β-arrestin recruitment (Figure 18B). Remarkably, when CCR7 was co-expressed, the CXCL12-induced BRET between CXCR4-Rluc and β-arrestin 2-Venus was decreased while CCL19 seemed to induce a significant BRET

response (Figure 18B). Moreover, the combined treatment with CXCL12 and CCL19 led to a lower BRET response as compared to the same condition in the absence of CCR7 (Figure 18B).

Hence, CCR7 co-expression may have an inhibitory effect on CXCR4-Rluc and β-arrestin 2-Venus interaction and pathway induced by CXCL12. This is not consistent with the data on CXCR4/Gαi coupling where CCR7 had no inhibitory effect. This suggests a selective and differential action of CCR7 on CXCR4 depending on the targeted pathway, positive on CXCR4/Gαi and negative on CXCR4/β-arrestin 2. However, it seems like CCL19 activating CCR7 can promote both CXCR4/Gαi as well CXCR4/β-arrestin 2 interaction while it blocked CXCL12-dependent responses.

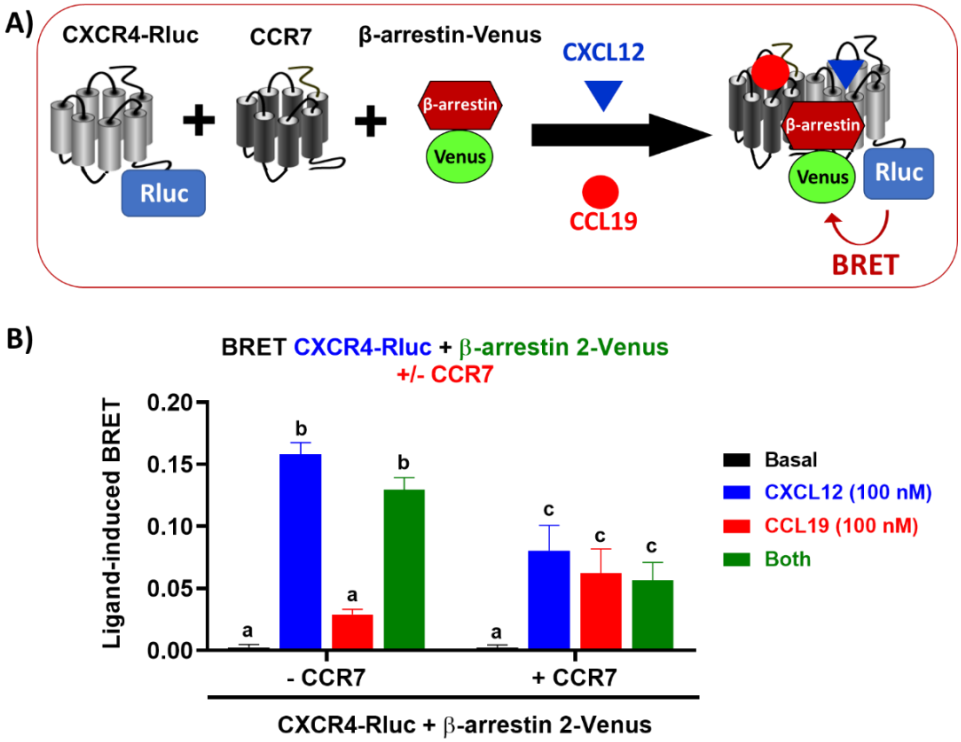

Figure 18: Effect of CCR7 co-expression on CXCR4/β-arrestin interaction.

A) Schematic representation of the transient transfection for BRET assay in HEK 293 cells. B) BRET data in HEK 293 cells transiently co-expressing CXCR4-Rluc (BRET donor) and β-arrestin 2-Venus (BRET acceptor) in the absence or presence of untagged CCR7 and treated for 30 minutes with either CXCL12 (100 nM), CCL19 (100 nM), or both. Data are means ± SEM of ligand-induced BRET of four independent BRET experiments performed in triplicate. The different letters in Panel B indicate statistically significant effects of the different treatments (p-value < 0.05). The same letters indicate no significant difference between the indicated treatments compared to each other.

To further demonstrate the functional effect of CCR7 on CXCR4 activation, we examined the effect of untagged CCR7 on BRET between CXCR4-Rluc and β-arrestin 2-Venus (Figure 19A) in parallel to the analysis of protein expression (Figure 19B). For each transfection, the relative luminescence of CXCR4-Rluc (indicative of CXCR4 expression) and the relative fluorescence of β-arrestin 2-Venus (indicative of β-arrestin 2 expression) were measured Figure 19B. Moreover, we used another chemokine receptor, CCR5, known to heterodimerize with CXCR4 [118, 131], as a control.

As shown in Figure 19A, CCR7 co-expression drastically diminished CXCL12-dependent BRET signal between CXCR4-Rluc and β-arrestin 2-Venus. However, CCR5 co-expression had no effect on that response (Figure 19A). Such effects were not due to any changes in the expression of CXCR4-Rluc (as BRET donor) and β-arrestin 2-Venus (as BRET acceptor) that may lead to BRET changes (Figure 19B). Indeed, the luminescence of CXCR4-Rluc as well as the fluorescence of β-arrestin 2-Venus were comparable between the control condition (in the absence of CCR5 or CCR7) and when both CCR5 and CCR7 were co-expressed (Figure 19B).

Together, the BRET data on CXCR4/Gαi (Figure 17) and CXCR4/β-arrestin (Figure 18) demonstrate the selective inhibitory effect of CCR7 on CXCR4/β-arrestin interaction since it was not observed on CXCR4/Gαi coupling and with CCR5 as another CXCR4-interacting receptor (Figure 19A). In addition, the inhibitory effects of CCR7 on CXCL12-promoted BRET responses cannot be explained by a decrease in the overall protein expression (Figure 19B).

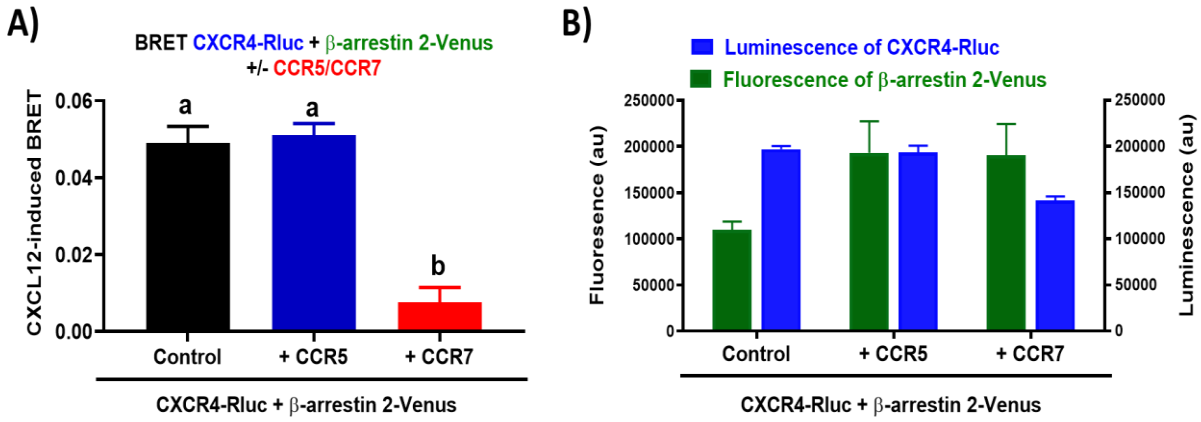

Figure 19: Effect of CCR5 and CCR7 co-expression on CXCR4-arrestin interaction.

A) BRET data in HEK 293 cells transiently co-expressing CXCR4-Rluc (BRET donor) and β-arrestin 2-Venus (BRET acceptor) in the absence (black bar) or presence of either untagged CCR5 (blue bar) or untagged CCR7 (red bar) and treated for 30 minutes with CXCL12 (100 nM). B) The quantification of the expression of the BRET partners via the measurement of the relative luminescence of CXCR4-Rluc and the fluorescence of β-arrestin 2-Venus in each transfection. Data are means ± SEM of CXCL12-induced BRET of three independent BRET experiments performed in triplicate. The different letters in Panel A indicate statistically significant effects of the different treatments (p-value < 0.05). The same letters indicate no significant difference between the indicated treatments compared to each other.

4.4 Effect of CXCR4 Co-Expression on CCR7/Gαi Coupling and Activation

To further characterize the functional interaction between CXCR4 and CCR7 in HEK 293 cells, we used the opposite configuration of BRET assay consisting of studying the influence of untagged CXCR4 co-expression on the interaction and BRET response between CCR7-Rluc and Venus-Gαi, as illustrated in Figure 20A.

As shown in Figure 20B, in the absence of CXCR4 co-expression, CCL19 but not CXCL12 promoted a significant BRET increase between CCR7-Rluc and Venus-Gαi. The combination of CXCL12 and CCL19 had no impact on BRET signal as compared to CCL19 alone (Figure 20B). However, when CXCR4 was co-expressed with CCR7-Rluc and Venus-Gαi, CXCL12 still did not promote any BRET response between CCR7-Rluc and Venus-Gαi, but interestingly, it significantly reduced both CCL19- and combined CCL19/CXCL12-mediated BRET responses (Figure 20B). Such inhibitory effect of CXCR4 on BRET between CCR7-Rluc and Venus-Gαi was not due to any changes in the

expression of CCR7-Rluc (as BRET donor) and Venus-Gαi (as BRET acceptor) that may lead to BRET changes (Figure 20C). Indeed, the luminescence of CCR7-Rluc as well as the fluorescence of Venus-Gαi were comparable between the control condition (in the absence of CXCR4) and when CXCR4 was co-expressed (Figure 20C).

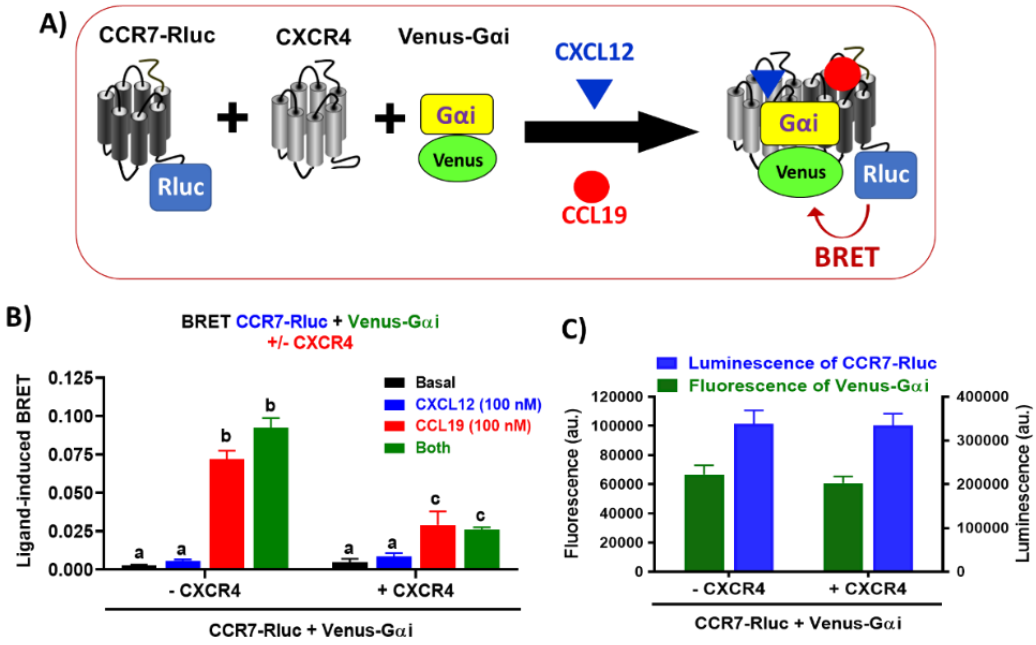

Figure 20: Effect of CXCR4 co-expression on CCR7-Gαi coupling and activation.

A) Schematic representation of the transient transfection for BRET assay in HEK 293 cells. B) BRET data in HEK 293 cells transiently coexpressing CCR7-Rluc (BRET donor) and Venus-Gαi (BRET acceptor) in the absence or presence of untagged CXCR4 and treated for 30 minutes with either CXCL12 (100 nM), CCL19 (100 nM), or both. C) The quantification of the expression of the BRET partners via the measurement of the relative luminescence of CCR7-Rluc and the fluorescence of Venus-Gαi in each transfection Data are means ± SEM of ligand-induced BRET of three independent BRET experiments performed in triplicate. The different letters in Panel B indicate statistically significant effects of the different treatments (p-value < 0.05). The same letters indicate no significant difference between the indicated treatments compared to each other.

4.5 Effect of CXCR4 Co-Expression on CCR7/β-Arrestin Interaction

Next, we examined the influence of untagged CXCR4 on CCR7/β-arrestin interaction using the approach as illustrated in Figure 21A.

In the absence of CXCR4, BRET signal between CCR7-Rluc and β-arrestin 2-Venus was significantly increased upon treatment with CCL19, but not CXCL12 compared to the basal condition (Figure 21B). The combination of CXCL12 and CCL19 had no impact on BRET signal as compared to CCL19 alone (Figure 21B). However, when CXCR4 was co-expressed with CCR7-Rluc and β-arrestin 2-Venus, CXCL12 still did not promote any BRET response between CCR7-Rluc and Venus-Gαi, but interestingly, this completely abolished both CCL19- and combined CCL19/CXCL12-mediated BRET responses (Figure 21B). Such inhibitory effect of CXCR4 on BRET between CCR7-Rluc and β-arrestin 2-Venus was not due to any changes in the expression of CCR7-Rluc (as BRET donor) and β-arrestin 2-Venus (as BRET acceptor) that may lead to BRET changes (Figure 21C). Indeed, the luminescence of CCR7-Rluc as well as the fluorescence of β-arrestin 2-Venus were similar between the control condition (in the absence of CXCR4) and when CXCR4 was co-expressed (Figure 21C).

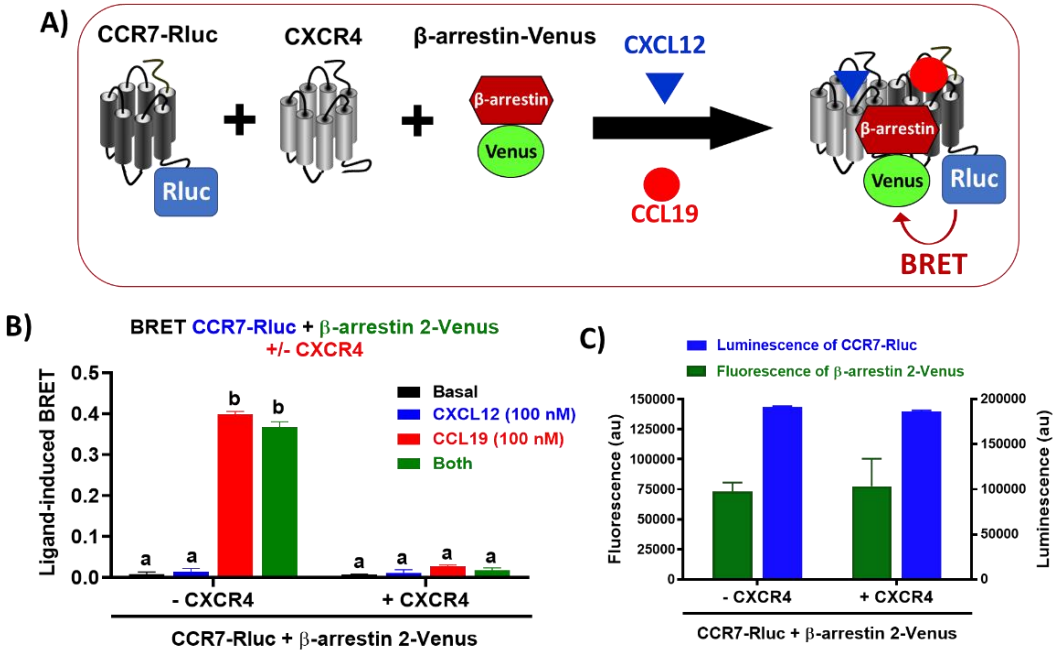

Figure 21: Effect of CXCR4 co-expression on CCR7/β-arrestin interaction.

A) Schematic representation of the transient transfection for BRET assay in HEK 293 cells. B) BRET data in HEK 293 cells transiently co-expressing CCR7-Rluc (BRET donor) and β-arrestin 2-Venus (BRET acceptor) in the absence or presence of untagged CXCR4 and treated for 30 minutes with either CXCL12 (100 nM), CCL19 (100 nM), or both. C) The quantification of the expression of the BRET partners via the measurement of the relative luminescence of CCR7-Rluc and the fluorescence of β-arrestin 2-Venus in each transfection Data are means ± SEM of ligand-induced BRET of three independent BRET experiments performed in triplicate. The different letters in Panel B indicate statistically significant effects of the different treatments (p-value < 0.05). The same letters indicate no significant difference between the indicated treatments compared to each other.

To further demonstrate the specificity of the inhibitory action of untagged CXCR4 on BRET between CCR7-Rluc and β-arrestin 2-Venus, we also used untagged CCR5 as another chemokine receptor known to heterodimerize with CXCR4 but not yet reported with CCR7.

As shown in Figure 22A, CXCR4 co-expression drastically diminished CCL19-dependent BRET signal between CCR7-Rluc and β-arrestin 2-Venus. Interestingly, CCR5 co-expression also inhibited CCL19-dependent BRET signal between CCR7-Rluc and β-arrestin 2-Venus (Figure 22A). Such inhibitory effects caused by the co-expression of CXCR4 and CCR5 were not due to any changes in the expression of CCR7-Rluc (as BRET

donor) and β-arrestin 2-Venus (as BRET acceptor) that may lead to BRET changes (Figure 22B). Indeed, the luminescence of CCR7-Rluc as well as the fluorescence of β-arrestin 2-Venus were comparable between the control condition (in the absence of CXCR4 or CCR5) and when both CXCR4 and CCR5 were co-expressed (Figure 22B).

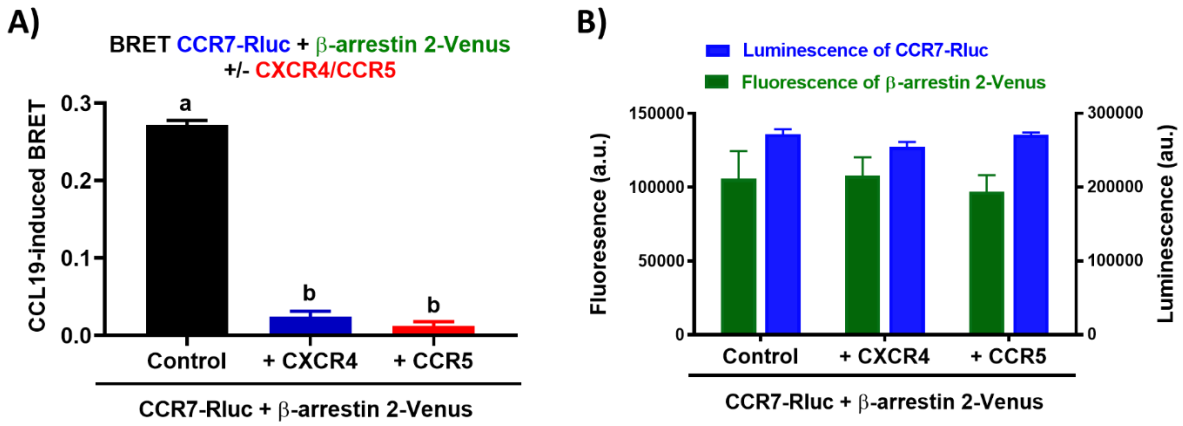

Figure 22: Effect of CXCR4 and CCR5 co-expression on CCR7/β-arrestin interaction.

A) BRET data in HEK 293 cells transiently co-expressing CCR7-Rluc (BRET donor) and β-arrestin 2-Venus (BRET acceptor) in the absence (black bar) or presence of either untagged CXCR4 (blue bar) or untagged CCR5 (red bar) and treated for 30 minutes with CCCL19 (100 nM). B) The quantification of the expression of the BRET partners via the measurement of the relative luminescence of CCR7-Rluc and the fluorescence of β-arrestin 2-Venus in each transfection. Data are means ± SEM of CXCL12-induced BRET of three independent BRET experiments performed in triplicate. The different letters in Panel A indicate statistically significant effects of the different treatments (p-value < 0.05). The same letters indicate no significant difference between the indicated treatments compared to each other.

To further characterize the inhibitory action of CXCR4 on CCR7 activation and its interaction with β-arrestin 2, we tested the effect of increasing amount of untagged CXCR4 in BRET assay. For this, HEK 293 cells were co-expressing a constant amount of CCR7-Rluc (BRET donor) and β-arrestin 2-Venus (BRET acceptor) with increasing levels of untagged CXCR4 as indicated in Figure 23A. Then BRET signals were measured upon stimulation of cells with CCL19. Moreover, the relative luminescence of CCR7-Rluc and the relative fluorescence of β-arrestin 2-Venus were measured in parallel (Figure 23B).

Our data showed CXCR4 co-expression led to a strong inhibition of CCL19-induced BRET response between CCR7-Rluc and β-arrestin 2-Venus even with 5 ng of CXCR4 plasmid (Figure 23A). The inhibition was proportional to the quantity of CXCR4 plasmid co-transfected in the HEK 293 cells until a total inhibition of the response with 75-100 ng of CXCR4 plasmid (Figure 23A). This indicates a specific inhibition of CCR7-Rluc and β-arrestin 2-Venus interaction by the untagged CXCR4. The CXCR4 inhibitory trend was not due to any changes in the expression of CCR7-Rluc (as BRET donor) and β-arrestin 2-Venus (as BRET acceptor) that may lead to BRET changes with CXCR4 amount increase (Figure 23B). Indeed, the luminescence of CCR7-Rluc as well as the fluorescence of β-arrestin 2-Venus were similar regardless of the amount of CXCR4 co-expressed with CCR7-Rluc and β-arrestin 2-Venus (Figure 23B).

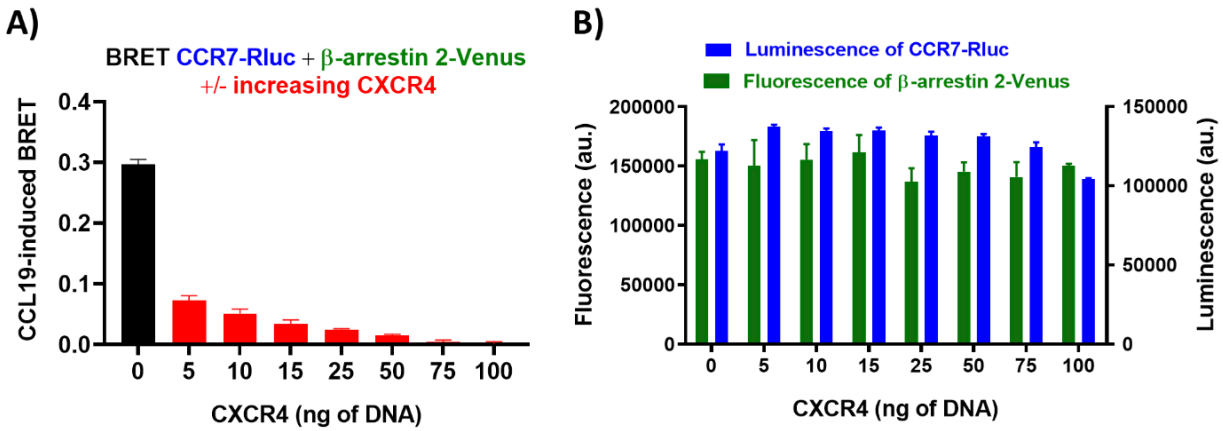

Figure 23: Effect of the increasing amount of CXCR4 co-expression on CCR7/β-arrestin interaction.

A) BRET data in HEK 293 cells transiently co-expressing CCR7-Rluc (BRET donor) and β-arrestin 2-Venus (BRET acceptor) in the absence (black bar) or presence of increasing amount of untagged CXCR4 (5, 10, 15, 25, 50, 75, 100 ng of DNA) (red bars) and treated for 30 minutes with CCL19 (100 nM). B) The quantification of the expression of the BRET partners via the measurement of the relative luminescence of CCR7-Rluc and the fluorescence of β-arrestin 2-Venus in each transfection. Data are means ± SEM of CXCL12-induced BRET of three independent BRET experiments performed in triplicate.

Altogether, the BRET data on the effect of CXCR4 co-expression on BRET between CCR7-Rluc and either Venus-Gαi or β-arrestin 2-Venus demonstrate the inhibitory action of CXCR4 (and also CCR5) on CCR7 activation in HEK 293 cells. This

is very consistent with the BRET data showing an inhibitory action of CCR7 on CXCR4/β-arrestin interaction (Figures 18 and 19).

4.6 Effect of CXCR4 and CCR7 Activation on Kinase Phosphorylation

Phosphorylation levels of key kinases, namely, ERK1/2 and Akt are indicative of the activation of downstream signaling pathways in cells, leading to cell proliferation, growth, survival, development and so on. The potential of GPCR heterodimers to enhance or inhibit downstream signaling that would otherwise occur from the activation of individual constituent receptors is a crucial property in terms of pharmacology. Therefore, we examined whether the simultaneous stimulation of cells with CXCL12 and CCL19 led to variations in the phosphorylation levels of ERK1/2 (pERK1/2) and Akt (pAkt). For this, we performed SDS-PAGE and western blotting in two different cells, HEK 293 since they were used for all BRET assays, and HT-29 cells that were shown to endogenously express high levels of both CXCR4 and CCR7 (as previously depicted by RT-PCR in Figure 16).

In HEK 293 cells, although the basal levels were high for both pERK1/2 and pAkt, treatment with 100 nM of CXCL12 but not CCL19 produced elevated pERK1/2 and pAkt levels as compared to the basal (Figure 24A). CCL19 even showed relatively reduced pAkt and pERK1/2 levels. This is consistent with the RT-PCR data indicating the expression of CXCR4 in HEK 293 cells. Moreover, simultaneous treatments with CXCL12 and CCL19 showed decreased pAkt and pERK1/2 levels compared with CXCL12 treatment alone (Figure 24A). These preliminary observations suggest that CCL19/CCR7 may lead to a slight inhibition of ERK1/2 and Akt phosphorylation and pathways promoted by CXCL12/CXCR4 in HEK 293 cells. This may be consistent with the inhibitory effect of CCL19/CCR7 on CXCR4/β-arrestin interaction.

In the case of HT-29 cancer cells, the basal levels of pERK1/2 and pAkt were also high (Figure 24B). For pAkt, the treatment of cells with either CXCL12 or CCL19 or even both showed a reduction in the phosphorylation level of Akt as compared to the basal with a stronger reduction observed with CCL19 (Figure 24B). However, both CXCL12 and CCL19 slightly promoted an increase of ERK1/2 phosphorylation as compared to the basal (Figure 24B). Interestingly, the combination of CXCL12 and CCL19 led to a slight reduction of pERK1/2 when compared to the response of both chemokines applied alone.

This observation suggests the inhibition of pERK1/2 and to less extent pAkt pathways in HT-29 cells upon dual activation of CXCR4 and CCR7. This may be also consistent with our BRET data in HEK 293 cells showing a reciprocal inhibition of CXCR4 and CCR7 activation especially with respect to receptor/β-arrestin interaction.

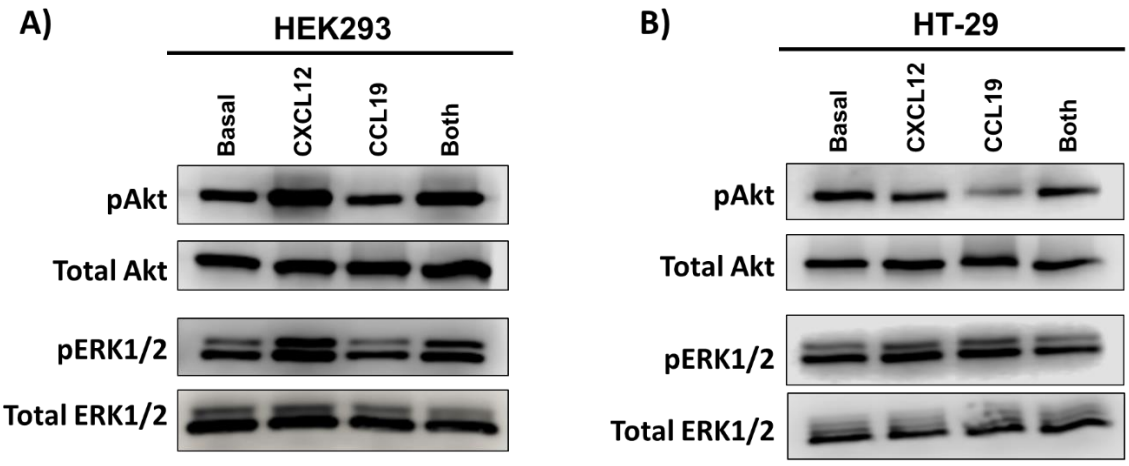

Figure 24: Effect of CXCR4 and CCR7 activation on kinase phosphorylation.

For this, serum starved HEK 293 (A) and HT-29 (B) cells were treated for 15 minutes with either CXCL12 (100 nM), CCL19 (100 nM), or both. Then the phosphorylation status as well as the total Akt and ERK1/2 were detected by SDS-PAGE and western blot as described in Materials and Methods. ERK1/2 and AKT ensured equal loading of proteins in each lane. The data are representative of three independent experiments.

4.7 Effect of CXCR4 and CCR7 Activation on Cancer Cell Viability

Finally, we wanted to link our results and observations in BRET and ERK1/2 and Akt phosphorylation in HEK 293 and HT-29 cells with one integrated cell response which is cell viability. Indeed, GPCRs and CXCR4/CCR7 are known to control cell proliferation and viability via G protein-dependent and/or β-arrestin-dependent pathways. In addition, ERK1/2 and Akt pathways are known for their pivotal role in the control of cell proliferation and survival, respectively. For this, we used MTT assay was in two different cancer cell lines, MDA-MB231 (breast cancer) and HT-29 (colon cancer) treated or not for 48 hours with either CXCL12 (100 nM), CCL19 (100 nM), or both.

In MDA-MB-231 cell line, CXCL12 but not CCL19 significantly increased cell viability (around 115%) (Figure 25A) despite the low level of CXCR4 detected by RT-

PCR in MDA-MB-231 cells (Figure 16A). Remarkably, the combination of CXCL12 and CCL19 did not increase cell viability over the control indicating an inhibitory action of CC19 on CXCL12-promoted cell viability (Figure 25A) despite no detectable CCR7 in MDA-MB-231 cells (Figure 16B).

In HT-29 cell line where both CXCR4 and CCR7 were found highly co-expressed (Figure 16), the picture is different and somehow contrasted with the cell viability data in MDA-MB-231 cells. Indeed, in HT-29 cells CCL19 but not CXCL12 slightly but significantly increased cell viability (around 110%) (Figure 25B). Moreover, such CC19-promoted cell viability vanished upon combination of CCL19 with CXCL12 (Figure 25B).

Together, our preliminary observations on cancer cell viability suggest inhibitory effects of CXCL12 and CCL19 when combined. This may be linked to the possible functional (and potentially physical interaction) between their respective receptors CXCR4 and CCR7.

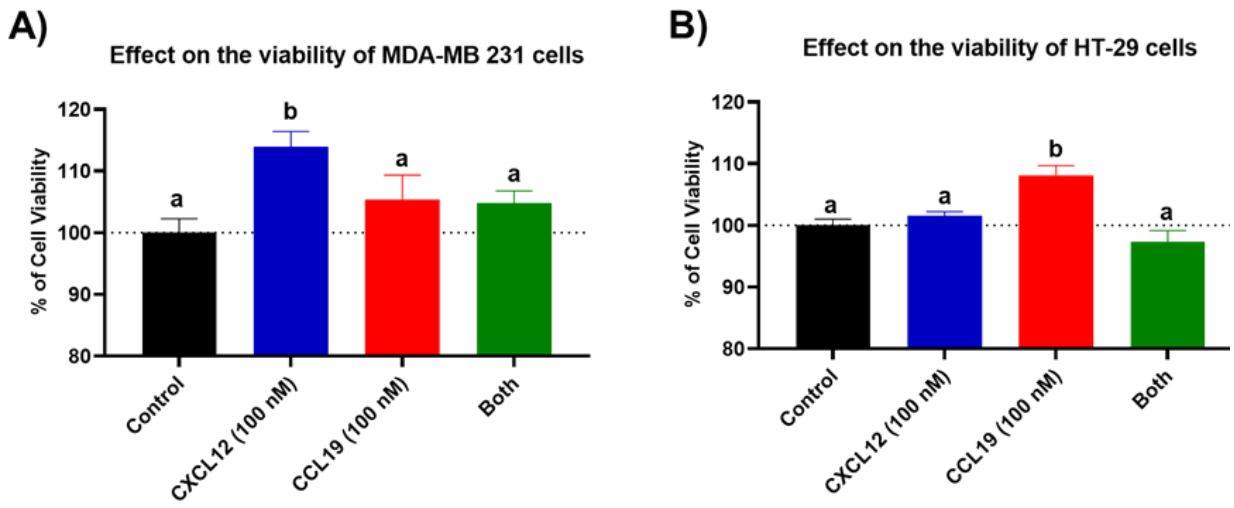

Figure 25: Effect of CXCR4 and CCR7 activation on cancer cell viability.

For this, MDA-MB231 (A) and HT-29 (B) cancer cell line were treated with either CXCL12 (100 nM), CCL19 (100 nM), or both, for 48 hours before cell viability was measured as described in Materials and Methods. Data are means ± SEM of percent of cell viability relative to the control condition performed in triplicate measurements. The different letters indicate statistically significant effects of the different treatments (p-value < 0.05). The same letters indicate no significant difference between the indicated treatments compared to each other.

Chapter 5: Discussion and Conclusion

In this project. we investigated *in vitro* the possibility of functional interaction between two chemokine receptors, CXCR4 and CCR7, and its implication in cancer. Indeed, CXCR4 and CCR7 are known for their implication in different stages of tumorigenesis and their functional/physical interaction in such process has been suggested in previous studies [50,14,142,53,139]. For this, we used BRET technology in HEK 293 cells along with western blotting depicting kinase phosphorylation and cell viability assays in various cancer cell lines.

For BRET assay in live HEK 293 cells, we measured receptor/Gαi protein as well as receptor/β-arrestin interaction for CXCR4 and CCR7 when only one or the two receptors were co-expressed. Upon transfection, cells were then treated or not with either CXCL12, CCL19, or both. Our BRET results showed that CCR7 co-expression may have an inhibitory effect on CXCL12-promoted CXCR4/β-arrestin 2 interaction, but interestingly not CXCL12-promoted CXCR4/Gαi interaction. This trend was the same when both receptors are co-activated with both ligands. In addition, we observed that CCL19 promoted CXCR4/Gαi interaction similarly to CXCL12 and this occurred only when CCR7 was co-expressed. These BRET observations suggest that CCR7 may have two opposite functional effects on CXCR4, activating CXCR4/Gαi while inhibiting CXCR4/β-arrestin interaction, which further support our conclusion of functional CXCR4-CCR7 interaction in HEK 293 cells. Such inhibitory action of CCR7 on CXCR4 was specific since it was not observed when CCR5 was co-expressed with CXCR4 although CXCR4 and CCR5 were also reported to heterodimerize in many models [118, 131]. Interestingly, CXCR4 co-expression was also found to inhibit both CCR7/Gαi coupling as well as β-arrestin recruitment to CCR7 upon CCL19 stimulation, and also stimulation with both ligands. This further supports the existence of functional interaction between CXCR4 and CCR7 which could indicate the possibility of reciprocal inhibition or cross-inhibition between them.

Our BRET data in HEK 293 cells were supported by our preliminary observations on the receptor downstream signaling pathways by assessing the phosphorylation of ERK1/2 and Akt in HEK 293 and HT-29 cells. Indeed, the combination treatment with

CXCL12 and CCL19 seems to reduce the phosphorylated levels of ERK1/2 and Akt if compared to the individual treatments with either CXCL12 or CCL19. This further suggests the inhibitory trend of CXCR4- and CCR7-dependent downstream signaling pathways when both receptors were present and co-activated. In contrast, study by Décaillot et al. investigating CXCR4-CXCR7 heterodimers reported high and sustained pERK1/2 levels in transfected HEK 293 cells when both CXCR4 and CXCR7 were co-expressed and treated with CXCL12 [130]. Moreover, individual treatments with CXCL12 or CCL19 induced robust ERK1/2 and Akt phosphorylation in cancer and other cell lines [84, 144, 145]. For instance, a study detected elevated pAkt expression with CCL19 treatment, implying activation of Akt pathway in MDA-MB-231 cells, in contrast to our study [145]. Another study on H9 cells showed transient and highest phosphorylation of both ERK1/2 and Akt at 0.5–1 min post CCL19 treatment, contrasting our study in HEK 293 cells [84]. Furthermore a study showed good ERK1/2 and Akt phosphorylation upon treatment with CXCL12 for 5 min in HeLa cells, similar to our observation in HEK 293 and HT-29 cells [144]. This affirms the activation of ERK1/2 and Akt in CXCR4- and CCR7-mediated signaling pathways at variable time intervals. However, heterodimerization can modify the individual properties of these receptors and enhance, reduce, or completely inhibit their signaling pathways, as depicted in our study.

Finally, in cell viability assay performed in MDA-MB-231 and HT-29 cancer cells, we also observed that the combination treatment with CXCL12 and CCL19 seems to inhibit CXCL12- or CCL19-mediated increase of cell viability. This is similar to a study that performed cell viability assay by treating two gastric cancer cell lines with CCL19, however it showed variable patterns during the respective time-frame (from 24, 48 to 72 hours) [95]. Another study on MDA-MB-231 cell lines showed similar cell viability results, where cell samples treated individually with CXCL12 and CCL21 had at least two times as many surviving clones as untreated cells [51]. So far, not study has been performed on the impact of cell viability when both CXCR4 and CCR7 are activated with their respective ligands. The fact that our results showed no effect on cell viability, despite being induced by ligands needs further assessment.

Our results showed the possibility of reciprocal inhibition or cross-inhibition between CXCR4 and CCR7-mediated signaling pathways. Indeed, a study by Sohy and

colleagues using BRET technology also showed that CCR2-CXCR4 heterodimerization in transfected HEK 293 cells resulted in trans-inhibition in terms of ligand binding [146]. This study performed functional tests using primary CD4+ T cells and CHO-K1 cell lines expressing CXCR4 or/and CCR2 and showed that binding of CXCL12 to CXCR4 induced a conformational change that reduced the coupling of CCL2 ligand to CCR2 and vice versa [146]. Use of CXCR4 and CCR2-specific antagonists also inhibited agonist-regulated binding to CCR2 and CXCR4 [146]. Additionally, CD4+ T cells endogenously expressing CXCR4 and CCR2 also showed inhibitory effects [146]. Previous studies were performed on CXCR4-CCR7 heterodimerization with varying results [84, 138, 139]. Hayasaka et al., showed that CCR7 formed constitutive heterodimerization with CXCR4, and was reliant on CXCR4 for T-cell activity and expression [138]. Without changing CCR7 expression levels, CXCL12-ligand induced CXCR4 signaling promoted CCR7 ligand binding affinity and elevated CCR7 homo- and CXCR4-CCR7 heterodimers [138]. Contrastingly, a study by Kobayashi et al., showed that unlike CCR7 homodimers, CXCR4-CCR7 heterodimers did not improve CCL19 binding affinity and CCR7-reliant T cell migration [84]. Also, a study by Mcheik and colleagues used various approaches including BRET and showed that CCR7 upregulation during B cell development favored the formation of CXCR4–CCR7 heterodimers, where the ability of CXCR4 to stimulate Gαi1/2 signaling was compromised by CCR7 (in contrast to our study) and also β-arrestin recruitment was entirely abolished (similar to our study), indicating that CCR7 in B cells selectively inactivated CXCR4 [139]. Hence, the differences in the interaction between CXCR4 and CCR7 and its associated signaling pathways may depend on certain cell type and environmental factors. Indeed, due to the importance of both CXCR4 and CCR7 as well as their differential expression profiles in different types of cancer, our findings may be of great importance. The functional and reciprocal inhibitory interaction between CXCR4 and CCR7 probably occurring through their physical heterodimerization may be one of the molecular mechanisms of regulation of these two receptors in physiology. Therefore, any change in their expression levels (increase or decrease) as reported in cancer may impact their function and their related cell responses leading to tumorigenesis.

We also performed RT-PCR which revealed highest relative expression levels for CXCR4 and CCR7 in HT-29 cell line, among other cancer cell lines like MDA-MB-231

and HepG2 cells. Likewise, a study investigating expression levels of CCR7 and CXCR4 in different colorectal carcinoma cell lines found strong CXCR4 expression and intermediate CCR7 expression in HT-29 cell line, among others [58]. Also, studies assessing expression of all chemokine receptors in different human breast cancer cell lines particularly showed high expression CXCR4 and CCR7 in general, with higher CXCR4 expression compared to CCR7 in MDA-MB-231 cell line [77, 147]. Schimanski et al., also reported variable CCR7 and CCR4 expression intensities on different types of human hepatoma cell lines, showing intermediate CXCR4 and strong CCR7 expression in HepG2 [90, 148]. Hence, both CXCR4 and CCR7 are readily co-expressed in cancer cells, though at variable levels.

Taken together, our results revealed an interaction between CXCR4 and CCR7 using BRET technology in live HEK 293 cells as a direct evidence and other functional assays (kinase phosphorylation and cell viability) in cancer cell lines as indirect evidence. Such an interaction may occur either via their physical interaction at the plasma membrane or their functional intracellular crosstalk, as illustrated in Figure 26.

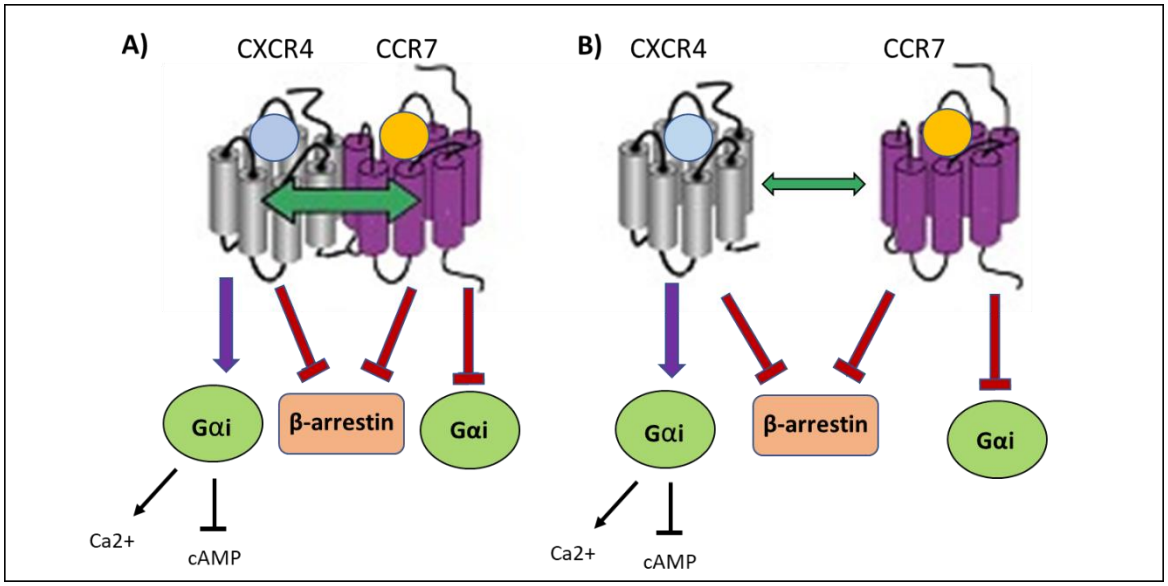

Figure 26: Proposed model for CXCR4-CCR7 interaction.

Our data suggests the functional interaction between CXCR4 and CCR7 may occur either via their (A) Physical interaction at the plasma membrane or their (B) Functional intracellular crosstalk. This resulted in specific modulation of receptor/Gαi protein coupling as well as and receptor/β-arrestin interaction.

Nevertheless, our data did not prove a physical association between CXCR4 and CCR7 and more studies need to be performed to prove this using appropriate approaches and models. This may include BRET saturation and competition assays, co-immunoprecipitation, and confocal microscopy. Moreover, our BRET data with Gαi protein and β-arrestin need to be linked with more functional analysis such as the measurement of their related second messenger production (i.e. cAMP, calcium) and downstream signaling proteins. Finally, the impact of CXCR4-CCR7 interaction on cancer cells and its implication in cancer as a disease requires further studies to investigate the effects on cancer cell proliferation and migration. Indeed, by dissecting the mechanisms of CXCR4-CCR7 interaction and its consequences will surely help to understand their involvement in cancer. Importantly, this should have significant applications in the pharmacological and clinical fields which may help designing new therapeutic strategies to treat cancer by specifically targeting CXCR4 and CCR7 individually or CXCR4-CCR7 as a pharmacological entity.

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The present project is the first study investigating *in vitro* the possibility of physical and functional interaction between two chemokine receptors, CXCR4 and CCR7, and its implication in cancer. For this, we used BRET technology on transfected HEK-293 cells. Indeed, the interaction between CXCR4 and CCR7 in cancer has not been investigated before. Hence, our study suggests the possible implication of these two receptors, possibility through heterodimerization, in different stages of tumorigenesis

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