Chemokine Receptor CXCR7 in Vascular Biology and Disease

By

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Abstract

Objective-Initially CXCR4 was thought to be the exclusive receptor for SDF-1. However, the discovery of a new SDF-1 receptor CXCR7, made the picture more complex. Whether CXCR7 is a "scavenger" receptor or not is still in debate. The biased signaling pattern on CXCR7 has not been determined. The aim of my study is to prove that CXCR7 is not just a scavenger receptor but instead, it can induce cell signaling in a time- and ligand-dependent biased mechanism, unveiling potential role of CXCR7 in vascular biology and disease.

Methods and Results-Here my research work show that a CXCR7-selective agonist TC14012 induced ERK1/2 inhibition in Human Coronary Artery Endothelial Cells, which can be mimicked in 1321 N-1 cell line transfected with human CXCR7 gene. We also successfully transfected CXCR7 into EA.hy926 and Raw 264.7 cells, both of which naturally do not express any detectable CXCR7 mRNA and protein. Then, a series of CXCR7 endogenous and exogenous ligands were screened and their effects on multiple cellular signaling pathways were determined. In addition, when we tried to use Cre-LoxP method to obtain myeloid-specific CXCR7 knock-out mice, surprisingly, CXCR7 mRNA and protein expression were dramatically upregulated in CXCR7-floxed$^{+/+}$/LyzM-Cre$^{+/+}$ mice as compared with the littermate controlled CXCR7-floxed$^{+/+}$/LyzM-Cre$^{-/-}$ mice, both in bone marrow-derived macrophages and in peritoneal macrophages.

Conclusions- CXCR7 is not just a scavenger receptor as proposed in the literatures; instead, it can induce cell signaling, but in a time- and ligand-dependent biased mechanism. Using the Cre-LoxP method for myeloid-specific deletion of CXCR7 in mice may lead to CXCR7 upregulation through a potential compensatory mechanism.

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List of Abbreviations

Cardiovascular diseases (CVDs)

Low density lipoproteins (LDLs)

Extracellular matrix (ECM)

Vascular smooth muscle cells (VSMCs)

Oxidized LDLs (ox-LDLs)

Intravascular ultrasound (IVUS)

Intimal media thickness (IMT)

Positron emission tomography (PET)

Single photon emission computed tomography (SPECT)

Angiotensin-converting enzyme (ACE)

G-protein-coupled receptor (GPCR)

Atypical chemokine receptors (ACKRs)

Calcitonin gene-related peptide (CGRP1)

Vasoactive intestinal peptide (VIP)

Stromal-cell derived factor (SDF-1)

Pre-B-cell growth stimulating factor (PBSF)

Phosphatidylinositol 4, 5-bisphosphate (PIP2)

Diacylglycerol (DAG)

Inositol 1, 4, 5 triphosphate (IP3)

Phosphoinositide 3-kinase (PI3K)

Mitogen activated protein kinase (MAPK)

T cell α-chemoattractant (I-TAC)

Macrophage inhibitory factor (MIF)

Adrenomedullin (ADM)

Calcitonin receptor-like receptor (CLR)

Receptor activity-modifying protein (RAMP)

Bovine adrenal medulla 22 (BAM22)

Cyclic AMP (cAMP)

Human herpesvirus 8 (HHV-8)

Kaposi's sarcoma-associated virus (KSHV)

Kaposi's sarcoma (KS)

Primary effusion lymphoma (PEL)

Multicentric Castelman disease (MCD)

Endothelial progenitor cells (EPCs)

Hematopoietic stem cells (HSCs)

Uridine triphosphate (UTP)

Vascular endothelial growth factor (VEGF)

Fetal bovine serum (FBS)

American Type Culture Collection (ATCC)

Dulbecco's modified eagle medium (DMEM)

Human coronary artery endothelial cells (HCAEC)

Dimethylsulfoxide (DMSO)

Extracellular signal-regulated kinase 1/2 (ERK)

Protein kinase B (AKT)

P38 mitogen-activated protein kinase (p38)

c-Jun N-terminal kinase (JNK)

Chemokine (C-X-C motif) receptor 7(CXCR7) or Chemokine Orphan Receptor 1 (RDC1)

Chemokine (C-X-C motif) receptor 4 (CXCR4)

Phosphate buffer solution (PBS)

Revolutions per minute (RPM)

Macrophage colony stimulating factor (M-CSF)

Polymerase chain reaction (PCR)

Reverse transcription polymerase chain reaction (RT-PCR)

Sodium dodecyl sulfonate (SDS)

Tris-Buffered Saline and Tween 20 (TBS-T)

Bovine serum albumin (BSA)

Polyvinylidene difluoride (PVDF)

Red fluorescent protein (RFP)

Ethylene diamine tetraacetic acid (EDTA)

Protein kinase C (PKC)

Ventriculap septal defect (VSD)

Chapter 1. Introduction

1.1. Cardiovascular diseases

Cardiovascular diseases (CVDs), just as its name implies, involve some malfunctions in the heart and blood vessels. For those which involving the heart includes: cardiomyopathy, hypertensive heart disease, heart failure, pulmonary heart disease, cardiac dysrhythmias, inflammatory heart disease, valvular heart disease, congenital heart disease and rheumatic heart disease. For those which involving the blood vessels includes: coronary artery disease, peripheral arterial disease , cerebrovascular disease, renal artery stenosis and aortic aneurysm. Except Africa, cardiovascular diseases are the leading cause death all over the world [1]. The pathogenesis of heart disease includes: gender, age, lack of physical activity, smoke, obesity, high fat diet, alcohol, hypertension, diabetes, hyperlipidemia, familial inheritance, psychosocial factors and air pollution [2][3][4]. The majority population suffer from CVDs are older adults. The average age of death from coronary artery disease is around 80 in developed countries whereas this number can be decrease to 68 in developing countries [2]. Averagely, a man can get CVDs seven to ten years earlier than a woman [1].

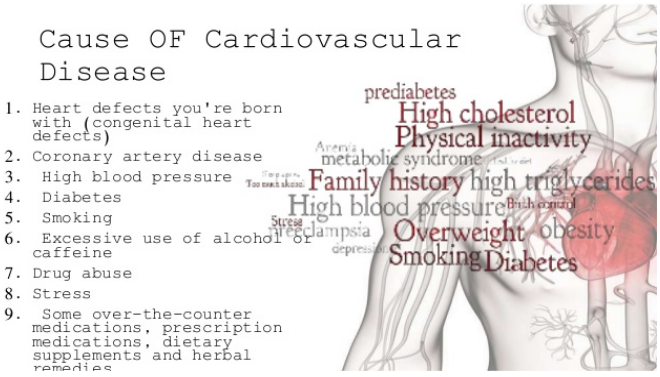


Figure 1.1. Causes of Cardiovascular Disease

Adapt from Muntasir A, Sifat Ullah and Sayed Fahmid Ahmed. *Health & Medicine*. 2015.

1.2. Atherosclerosis

Atherosclerosis is a disease with an artery wall thickness. This is caused by the invasion and accumulation of white blood cells, forming an atheromatous plaque. At the early stage, LDLs enter into the intima and lead to endothelial activation/dysfunction. Then the vascular endothelium gradually lose the ability of controlling the diffusion of plasma molecules [5]. Next, LDL particles permeate and accumulate in the ECM. Then, vascular endothelium will produce some endothelial molecules. These molecules will attract the circulating monocytes and direct them to attach to vascular endothelium. After this attachment, these monocytes further migrate to sub-endothelial area and differentiate into macrophages at the same time. Some injury in vascular endothelium promotes platelets activation and adhesion. Both the adhered platelets and injured endothelial cells secrete chemokines to activate leukocytes and vascular smooth muscle cells (VSMCs) in the intimal layer, which promote the formation of plaque [6][7]. LDLs stayed in the ECM will be oxidized to ox-LDLs and they can trigger different kinds of pro-inflammatory reactions in the intima. These ox-LDLs attract the attached macrophages to scavenge them. As this process goes on, the macrophages load too much lipid and become foam cells. These foam cells retain in blood vessel wall and release cytokines and growth factors to promote VSMCs migration. Moreover, these foam cells can produce collagen which facilitates the formation of a fibrous cap [7]. Once this process remains untreated, apoptosis in the attached macrophages will be induced and leads to releasing of cholesterol, pro-thrombotic molecules into blood vessel wall [8]. The number of VSMCs will be decreased with progression of atherosclerotic plaques. As time goes by, more and more immature and leaky blood vessels are formed, which leads to atherosclerotic plaques more prone to rupture. After these plaques get disrupted, platelets accumulate on vascular surface and trigger the coagulation cascade, leading to thrombus formation [9].

Atherosclerosis is hard to diagnose at early stages because arteries expanded at all plaque locations so that it will not influence blood flow [10]. Although some plaques already get ruptured, symptoms may still not appear until arteries narrowed enough. Patients are always not able to that realize they have atherosclerosis only when they experience other symptoms including coronary artery disease, stroke or heart attack. As atherosclerosis progress, coronary arteries become narrowed and prevent bringing oxygenated blood to the heart. This will cause the following symptoms: chest pain, nausea, shortness of breath, dizziness, sweating and palpitations. Atherosclerosis may start from childhood. However, symptomatic atherosclerosis

always happens in men over 40s and women over 50s. Since atherosclerosis can begin in a very early stage, early screening of children for cardiovascular diseases could be really beneficial.

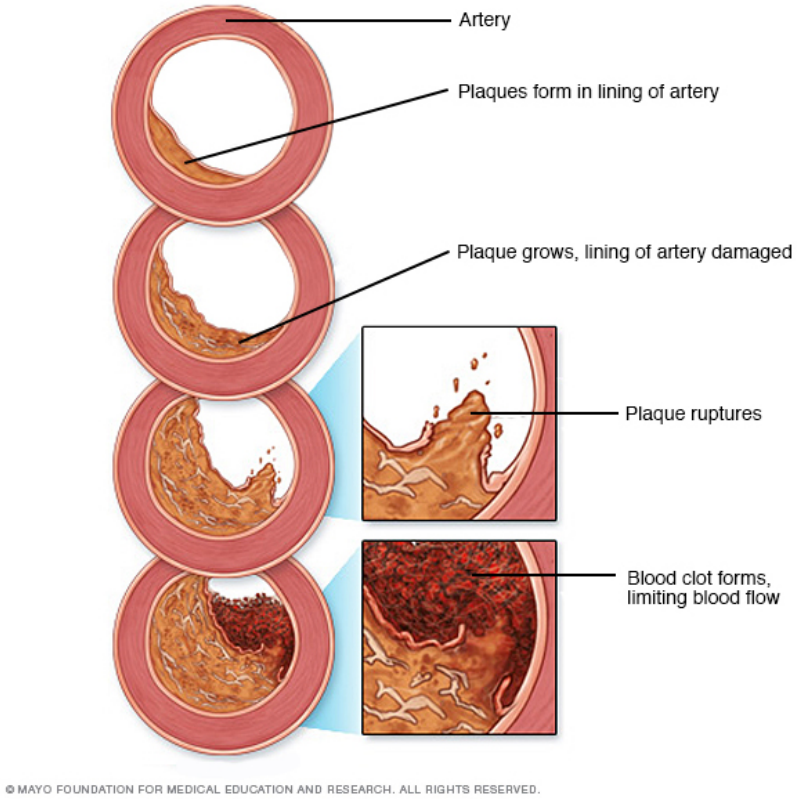


Figure 1.2. Development of Atherosclerosis

Adapt from Mayo Clinic (https://www.mayoclinic.org/diseases-conditions/arteriosclerosis-atherosclerosis/symptoms-causes/syc-20350569)

The exact risk factors of atherosclerosis are still unclear. Personal habits, environment, diet and other certain conditions may increase the possibility of developing atherosclerosis. Some of the factors are modifiable. These are diabetes, dyslipidemia, tobacco smoking, trans-fat, obesity, hypertension and insulin resistance. Other factors are non-modifiable such as advanced age, gender, genetic abnormalities and family history. In addition, there are some uncertain factors including air pollution, alcohol, saturated fat, chronic stress and so on. Researchers have a great interest in studying the role of food-based compounds in preventing atherosclerosis.

Early diagnosis for atherosclerosis is difficult. Clinically angiography has been widely used for detecting severe narrowing or stenosis in blood vessels. However, this technic is unable to detect underlying atherosclerosis disease. Most severe events in locations with heavy plaque occur suddenly. Within just several seconds to minutes, plaque rupture can block artery lumen which leads to permanent debility or sudden death. During the process of plaque rupture, the lesion always breaks at the shoulder of the fibrous cap. Collagen, a mainly thrombogenic component released by plaque will promote thrombus formation. Thrombus then travels downstream through blood flow and form blood clots somewhere else in blood vessels. Since red blood cells play the oxygen carrier role in human body. Once blood flow is completely blocked, the nearby cells are going to die due to lack of enough oxygen supply. This can result in life-threating diseases such as heart attack and stroke in brain. In the past, over 75% of lumen stenosis was considered as the hallmark of clinically significant disease. However, clinical data showed that only 14% cases of patients had more than 75% stenosis. Since sudden plaque rupture always happened in cardiovascular diseases, more attention is paid to "vulnerable plaque" from 1990s [11]. Besides angiography and stress-testing, other new techniques including anatomical detection and physiologic measurement have been developed for earlier detection of atherosclerosis. For anatomical detection methods, there are coronary calcium scoring by CT, intravascular ultrasound (IVUS) and carotid intimal media thickness (IMT) measurement by ultrasound. For physiologic measurement methods, there are lipoprotein subclass analysis, hs-CRP, HbA1c, and homocysteine. For the past few years, nuclear imaging techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) have been invented. Both PET and SPECT are able to estimate the severity of atherosclerotic plaques.

Although cardiovascular diseases are the leading cause of death in many countries, 90% of them are preventable [12][13]. Taking actions to control risk factors will help avoid or delay atherosclerosis and its related diseases. These actions includes but not limited to: healthy diet, physical activity, quit smoking and weight control. Following a healthy diet is important, which include whole grains, lean meats, fruits, vegetables, poultry without skin, seafood, and fat-free or low-fat milk and dairy products. Physical activities always help combat atherosclerosis by improving circulation and functionality of the vessels. Stop smoking can prevent or alleviate

angiosclerosis. Weight control will help a patient to avoid obese, lower blood pressure, and decrease cholesterol.

Alleviating symptoms of atherosclerosis is important although focusing on decreasing underlying atherosclerosis is a more effective way [14]. Non-pharmaceutical means are usually the first method of treatment. Lifestyle changes, such as stopping smoking, eating a healthy diet and practicing regular exercise are often the most appropriate treatment for atherosclerosis. Sometimes, medication or surgical procedures may be recommended as well [15][16][17]. Making changes in diet will help prevent atherosclerosis. A meal consists of more fruits and vegetables will be beneficial [18]. Also, some evidence shows that a diet contains dairy products including milk, cheese and yogurt can reduce risks of cardiovascular diseases [19]. Studies focus on different diet styles over the world implied that a Mediterranean diet may become a better choice comparing to a low-fat diet because it brings about long-term changes to cardiovascular risk factors such as blood pressure and cholesterol level [20].

Medication treatment can also slow or even reverse the effects of atherosclerosis. These include Anti-platelet medication, such as aspirin, which prevents platelets clump in narrowed arteries; Beta blocker medications, which decrease heart rate and blood pressure, reduce the risk of heart attack and some heart rhythm problems; Angiotensin-converting enzyme (ACE) inhibitors, which help slow the progression of atherosclerosis by lowering blood pressure and producing other beneficial effects on the heart arteries; Calcium channel blockers and diuretics, which lower blood pressure.

At present, the most widely prescribed and effective way to treat or prevent atherosclerosis is a group of medications referred to as statins. In pharmacological terminology, statins are called HMG-CoA reductase inhibitors. HMG-CoA is a time-limiting enzyme in the process of endogenous cholesterol biosynthesis. HMG-CoA reductase is the first committed enzyme of the mevalonate pathway. The structure of statins are similar to HMG-CoA so that they can fit into the enzyme's activate site and compete with HMG-CoA. This competition will decrease the rate by which HMG-CoA reductase can produce mevalonate and eventually the level of cholesterol in blood flow will be reduced. There are some fungi such as *Penicillium* and *Aspergillus* can produce natural statins as their secondary metabolites to inhibit HMG-CoA reductase in bacteria and fungi [21]. Through inhibiting HMG-CoA reductase, statins strongly limit the cholesterol-

synthesizing pathway in the liver which is significant because most circulating cholesterol is from internal manufacture. When liver produces much less cholesterol, levels of cholesterol in blood stream will get strongly decreased. Since most cholesterol synthesis happen at night, it is beneficial for patients to take statins with short half-lives at night to maximize their effect [22]. Besides inhibiting cholesterol synthesis, statins can also increase LDL uptake [23] and decrease specific protein prenylation [24]. In a nut shell, researchers hypothesize that statins prevent cardiovascular diseases via the following mechanisms: improve endothelial function, modulate inflammatory responses, maintain plaque stability and prevent thrombus formation [25]. Although statins seems very useful in prevention and treatment atherosclerosis, they remain ineffective for two thirds of patients [26] and long term survival rate is still below expectation.

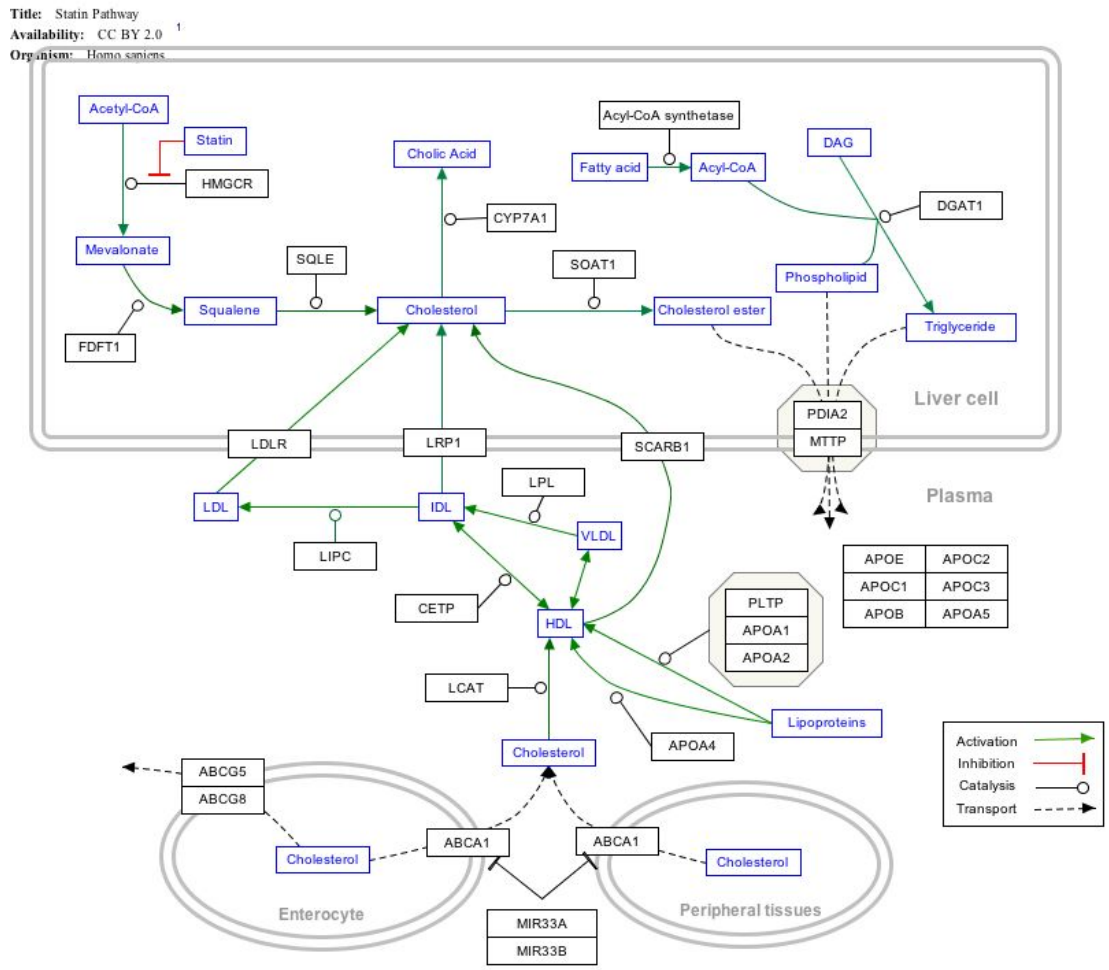


Figure 1.3. Statin Pathway

Adapt from Nathan Salomonis, Alexander Pico, Kristina Hanspers, Thomas Kelder, et al.

If a more severe case of atherosclerosis such as irreversible ischemia happened, surgical procedures are needed. Angioplasty and stent placement are always the first choice. In this procedure, a long catheter will be inserted into the narrowed part of patients' artery. Another catheter attached with a deflated balloon on its tip is then passed through the catheter and delivered to the narrowed area. After that, the balloon is inflated, compressing the deposits against patients' artery walls. A mesh tube is usually left in the artery to help keep the artery open. In some extreme cases, an endarterectomy is necessary, fatty deposits will be surgically removed from the walls of a narrowed artery. If patients' arteries are blocked by blood clots, clot-dissolving drugs will be used to break them apart. In addition, doctors may also choose bypass surgeries for treatment of atherosclerosis. During this process, a doctor creates a graft bypass using a vessel from another part of patients' body or a tube made of synthetic fabric, this allows blood to flow around the blocked or narrowed artery. It is worth mentioning that coronary artery bypass grafting without manipulation of the ascending aorta is a better way to reduce rates of postoperative stroke and mortality compared to traditional on-pump coronary revascularization [27].

1.3. Chemokines

Chemokines which measure from 8 to 12kDa, consist of about 50 small peptides in this superfamily [28]. These widely expressed chemoattractant cytokines play an important role in regulating cell traffic processes [29]. Subsequently their role is extended to other pathological and physiological conditions including angiogenesis, hematopoiesis, atherosclerosis and cancer [30]. According to the conserved cysteine residues from N-terminal, chemokine receptors can be classified into four subfamilies: (CXC, CC, CX3C, and C). Furthermore, the conserved ELR-motif is shared by some members of CXC-chemokines which exert angiogenic effects while those lacking ELR-motif are angiostatic [31]. Chemokine receptors can also be classified based on their function: homeostatic and inflammatory, or both. The constitutively expressed

homeostatic chemokines play a key role not only in development but also in maintenance of immune systems, whereas inflammatory chemokines get induced when they are stimulated [32].

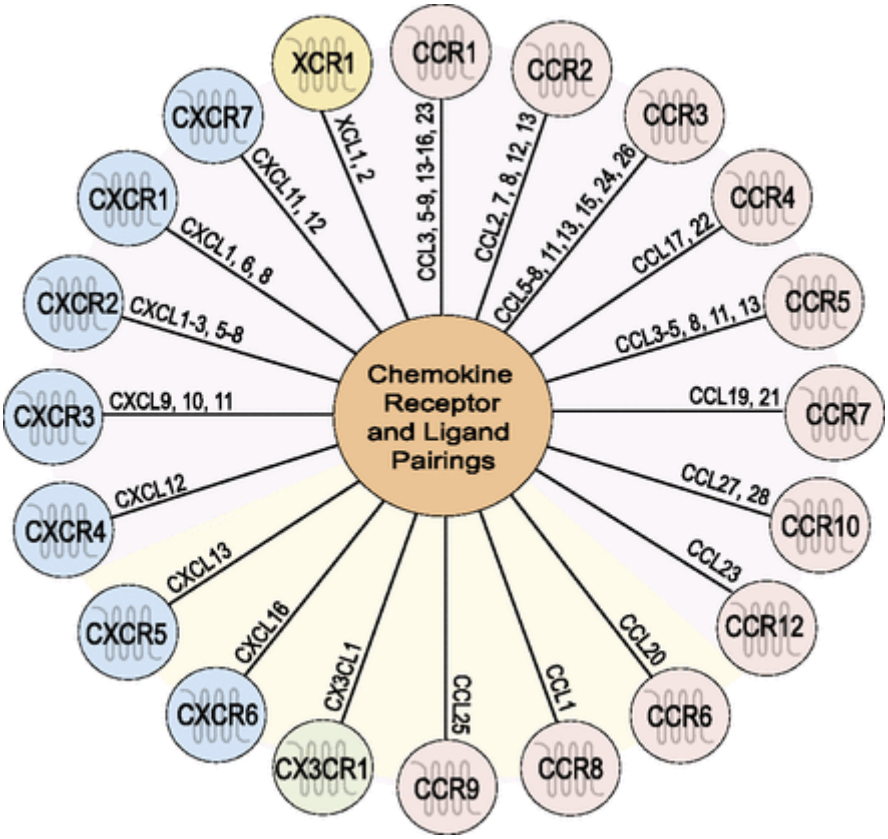


Figure 1.4. Chemokine Receptor and Ligand Pairings

Adapt from Gemma E, *et.al*, *Pharmacological Reviews.* 2013.

1.4. CXCR7

CXCR7 is a seven-transmembrane G-protein-coupled receptor (GPCR). CXCR7 has another name called ACKR3, implying it belongs to the atypical chemokine receptors (ACKRs) family. Most ACKRs lack functional domains for G_i signaling [33]. The gene sequences of CXCR7 remain highly conserved among humans, dogs, mice and rats [34]. Originally it was cloned from a dog thyroid and named RDC-1 [35]. In 2005, considering the similarity of its structure to CXC

receptors, it was renamed CXCR7 according to chemokine receptor nomenclature [36][37]. The gene of human CXCR7 is located on the region of 2q37.3 whereas in mice it is at 55.6 cM in the chromosome [38]. Although CXCR7 encodes two exons, only the last exon is the solely translated coding region [39]. Originally CXCR7 was considered as a receptor for calcitonin gene-related peptide (CGRP1) and vasoactive intestinal peptide (VIP) but studies have not been able to prove this [40][41]. Unlike typical GPCRs, CXCR7 is not able to activate heterotrimeric G proteins. Most chemokine receptors share a conserved motif DRYLAIV at the N-terminus of the second intracellular loop which is critical for calcium signaling and Gαi protein coupling. The CXCR7 sequence is altered to DRYLSIT (A to S and V to T) and this structural difference was considered the reason CXCR7 is unable to induce cell signaling transduction through Gαi [37]. At this point, how CXCR7 mediates activation of intracellular pathways remains controversial. Some studies have indicated that CXCR7 is a scavenger or decoy receptor which does not couple to G-proteins [42][43]. Other evidence suggests that CXCR7 participates in signal transduction only when it forms a dimer with CXCR4 [44][45]. In addition, a few studies demonstrate that CXCR7 can independently induce cell signaling via β-arrestin in certain cell lines [46][47]. None of these is conclusive, however.

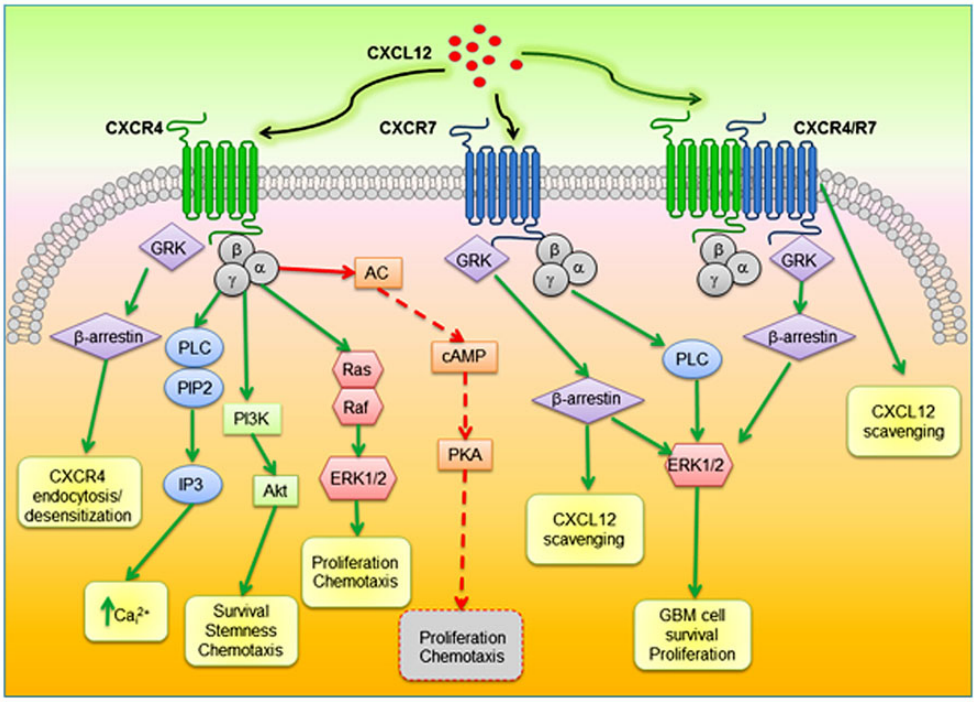


Figure 1.5. CXCR7 Signaling Patterns [48]

Adapt from Sezione DF, *et.al*, *Front. Cell. Neurosci.* 2014.

1.5. Biased Signaling

Now there appears a popular saying called “biased signaling or functional selectivity”. It occurs when a 7TM-receptor preferentially activates one of several available pathways. Biased signaling can be divided into three major types. They are: ligand bias, receptor bias and tissue or cell bias. For ligand bias, it is a situation where different ligands bind the same receptor, but induce different responses. For receptor bias, it means that the same ligand induces different responses on different receptors. For tissue or cell bias, this describe the phenomenon that even the same ligand binds to the same receptor, they can still activate different pathways in a tissue or cell specific manner [49]. The existence of biased signaling does bring redundancy in cell signaling pattern study, however this phenomenon may help unify the conflicting results on CXCR7 study.

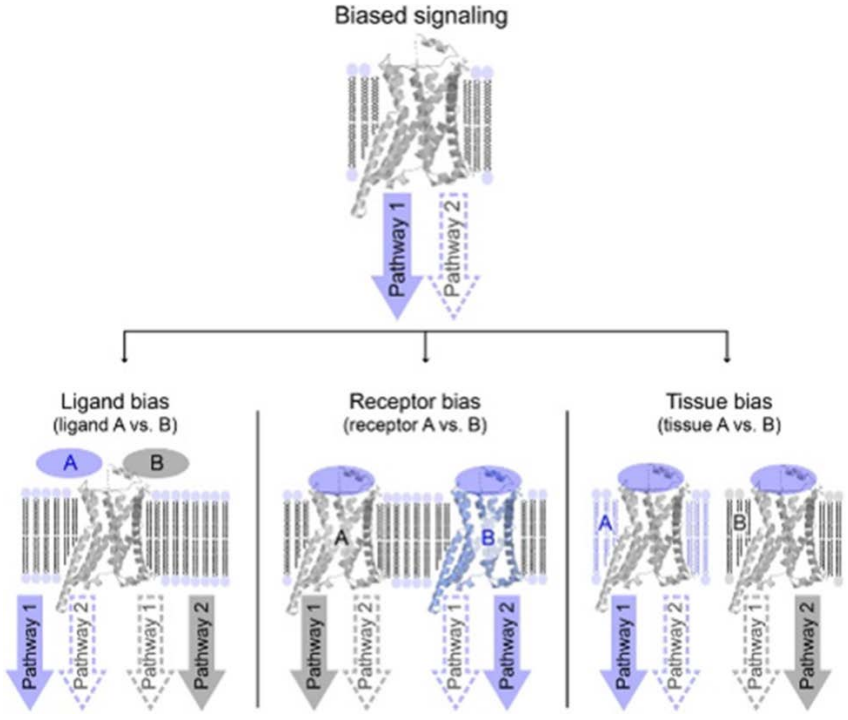


Figure 1.6. Overview of Different types of Biased Signaling [49]

Adapt from Anne, Olav Larsen, Stefanie Thiele and Mette M Rosenkilde. *Front Immunol*. 2014.

1.6. Ligands of CXCR7

SDF-1(CXCL12)

Stromal-cell derived factor (SDF-1), also known as CXCL12, is a pleiotropic chemokine [50] widely expressed among different organs including bone marrow, liver, heart, kidney, thymus, stomach, lymph nodes, pituitary gland, and brain [51]. Beyond these, SDF-1 may be highly induced under certain pathological conditions including ischemia, inflammation, hypoxia, cancer and autoimmune diseases [52][53]. At first, SDF-1 was regarded as a soluble pre-B-cell growth stimulating factor (PBSF) which facilitates progenitor proliferation of bone marrow B cells [54]. Later on its interaction with CXCR4 was discovered [55]. More recently, research found that SDF-1 can take part in homing of progenitor leukocytes into the marrow microenvironment and adaptive immune processes [56][57].

Initially, CXCR4 was thought to be the exclusive receptor for SDF-1. Later on, CXCR7 was found to be a second receptor for SDF-1, which displays 10-fold higher affinity rate compared to CXCR4 [36]. The activity of the SDF-1 signaling pathway is crucial in neural, vascular, and cardiac development and craniofacial organogenesis. SDF-1 binds to CXCR4 and CXCR7 with the formation of homo (CXCR4/CXCR4,CXCR7/CXCR7) or heterodimers (CXCR4/CXCR7), the expression levels of CXCR4 and CXCR7 contribute to the relative abundance of homodimers and heterodimers [44]. When binding to CXCR4, SDF-1 changes its three-dimensional conformation and initiates exchanging from GTP to GDP and dissociates into α- and βγ-subunits, then activates several cell signaling pathways [58]. Through modulating adenylyl cyclase activity, α_i subunits inhibit cAMP formation; α_q can induce PLC-β, which in turn hydrolyzes PIP2 (phosphatidylinositol 4, 5-bisphosphate) facilitating the production of DAG (diacylglycerol) and IP3 (inositol 1, 4, 5 triphosphate). These secondary messengers lead to release of Ca^{2+} from ER and activate protein kinase C. SDF-1 can activate PI3K (phosphoinositide 3-kinase)/Akt, IP3, and MAPK (mitogen activated protein kinase) through CXCR4 to regulate cell survival, chemotaxis and proliferation. In addition, SDF-1 also activates JNK and p38 to control cell

survival [59][60][61]. In our lab we found that CXCR7 was detected in macrophage positive area of aortic atheroma of ApoE-null mice, but not in healthy arteries. Consistent with this, we found that during monocyte-to-macrophage differentiation process, CXCR7 was induced both at mRNA and protein levels. This CXCR7 induction caused a SDF-1 signaling switch from pro-survival pathways (Akt, ERK1/2) to pro-inflammatory pathways (p38, JNK), leading to increased macrophage phagocytosis [62].

At one point CXCR7 was regarded as a scavenger or decoy receptor for SDF-1 [63][64].The findings in zebrafish indicate that CXCR7 functions primarily by sequestering CXCL12 [65]. Consistent with this, although binding with CXCR7, SDF-1 is not able to mediate calcium mobilization [66]. Besides, β-arrestin is more likely to be activated by SDF-1 [47]. Sometimes CXCR4 and CXCR7 form heterodimers, in which case the conformation of CXCR4/G-protein complexes got changed by CXCR7 and thus its signaling will be blocked [44]. The formation of CXCR4-CXCR7 heterodimers can also enhance SDF-1 induced intracellular Ca^{2+} mobilization and ERK1/2 activation [67]. Through activation of SDF-1, the CXCR4-CXCR7 complex increases cell signaling by recruiting β-arrestin including ERK1/2, p-38 and SPAK [68][69]. Apart from these, CXCR7 can independently induce Akt and ERK activation via G-protein or β-arrestin [70].

I-TAC (CXCL11)

IFN-inducible T cell α-chemoattractant (I-TAC), also called CXCL11, is mainly expressed in the pancreas, peripheral blood leukocytes, thymus, liver, spleen and lung. To a lesser degree, it is expressed in the prostate, placenta and intestine [71]. Just like SDF-1, I-TAC, binds the two chemokine receptors CXCR3 and CXCR7. Interferons have the ability to induce I-TAC production among several cell lines including leukocytes, endothelial cells and fibroblasts. The level of I-TAC will get up-regulated during an infection or cancer process. There are two variants of CXCR3: CXCR3-A and CXCR3-B. When binding with CXCR3-A or CXCR7, I-TAC will promote proliferative signals. In contrast, it inhibits the effects in cell growth while binding to CXCR3-B [72]. Similar to SDF-1, when binding with CXCR7, I-TAC is not able to induce calcium signaling or activate ERK1/2 or Akt [73]. However, some scientists have demonstrated that I-TAC can promote ERK and Akt phosphorylation in $CXCR4^{+}CXCR7^{+}CXCR3^{-}$ cell lines [74]. The contradictory results may have been because of

the different cell lines used (tissue specific biased signaling). Furthermore, binding of I-TAC to CXCR7 also recruits β-arrestin-2 which indicates that subsequent signaling is regulated by β-arrestin. Further studies of I-TAC and CXCR7 interactions are needed to clarify these issues.

MIF

MIF (Macrophage inhibitory factor) acts as a pro-inflammatory cytokine with chemokine-like functions which regulate innate immunity. Originally, it was regarded as a T-cell derived factor which plays a role in inhibiting macrophage migration [75]. It is widely expressed by several cell lines including eosinophils [76], endothelial cells [77], epithelial cells [78], lymphocytes [79] and macrophages [80]. Under the dysregulated conditions, MIF participates in a series of diseases including rheumatoid arthritis, systemic lupus erythematosus, sepsis, glomerulonephritis, atherosclerosis and cancer [81][82][83][84].

Several receptors can regulate MIF functions. The first is CD (cluster of differentiation) 74. CD74 is a single-pass type II transmembrane protein which is also known as the plasma membrane form of the major histocompatibility class II invariant chain (Ii) [85]. Binding to CD74 mainly mediates survival and proliferative functions of MIF on immune and tumor cells. With the help of co-receptor CD44, MIF can induce signaling through CD74 to activate tyrosine kinases and the PI3K/AKT pathway [86][87]. There is also evidence that MIF interact with CD74 and subsequently activates ERK1/2 pathways [86]. Apart from CD74, MIF is also identified as a non-cognate ligand for two chemokine receptors, CXCR2 and CXCR4, which regulate cell signaling through their cognate ligands CXCL1/CXCL8 and CXCL12, respectively. CXCR2 and CD74 can form heterodimers which is very important in MIF-regulated atherogenic leukocyte recruitment [88]. Besides, CXCR4/CD74 complexes are able to activate AKT pathways through interaction with MIF [89]. Interestingly, signaling pathways and pathogenic effects which are regulated by MIF have been found to interact with CXCR7. Both MIF-CXCR4 and MIF-CXCR7 axes play an important role in Rhabdomyosarcoma tumor cell migration. Furthermore, in platelets, MIF is able to activate Akt signaling pathway through CXCR7 to limit apoptosis [90][91]. In addition to these, MIF is also found to promote CXCR7 internalization, induce B-cell chemotaxis, and ERK1/2 activation [92]. These discoveries indicate that CXCR7 is a new receptor for MIF.

ADM (Adrenomedullin)

As a peptide, ADM was first identified as a potent vasodilator [93]. ADM is a mitogenic hormone with 52 amino acids which plays a crucial role in lymphatic vascular and cardiac development [94][95][96]. It can activate cognate receptors consisting of CLR (calcitonin receptor-like receptor) and RAMP (receptor activity-modifying protein). There is also evidence that CXCR7 is able to bind to ADM with a K_d of 1.9×10^{-7} M [97]. A recent study proved that the genetically overexpressed ADM ligand caused hypertrophic heart development during embryogenesis in $Adm^{hi/hi}$ mice [98]. In keeping with the crucial role of ADM in promoting lymphangiogenesis, it was discovered that $CXCR7^{-/-}$ presented lymphatic vascular defects. These results indicated that CXCR7 acts as a decoy receptor for ADM in controlling cardiac and lymphatic development [95][96][99][100]. Whether or not ADM can induce any signaling transduction through CXCR7 needs to be determined by further studies.

BAM22 (bovine adrenal medulla 22)

As one of the cleaved products of proenkephalin A, BAM22 was initially isolated from the bovine adrenal medulla [101][102]. In mammals, BAM22 is widely distributed in the central nervous system [103][104]. BAM22 has the classical opioid YGGFM motif and exerts both opioid and non-opioid actions [105]. Through activating three major opioid receptors，μ-，δ- and κ- [106][107][108], BAM22 not only inhibits contractions of ileum [109], vas deferens [110], and bladder [111] but also nociceptive response [112], which is sensitive to naloxone, a medication used to block the effects of opioids. It is quite important that as a unique endogenous peptide, BAM22 got a high affinity when binding to the human Mas oncogene-related gene (Mrg) receptors, which are restricted to small-diameter DRG neurons in humans and rodents [113][107]. Research has proven that altered BAM22 expression in DRG or spinal dorsal horn in complete Freund's adjuvant (CFA)-induced chronic pain and morphine tolerance which suggested that BAM22 participates in the nociceptive process [114][115]. Recently it was found that CXCR7 is a high-affinity receptor for BAM22 which promoted glucocorticoid secretion. BAM22 can recruit both β-arrestin1 and β-arrestin2 at nanomolar concentrations as well as SDF-1 and I-TAC. Consistent with previous studies of CXCR7, BAM22 elicited neither a cyclic AMP (cAMP) nor a calcium response in H295R cells and 293T cells, both of which express high levels of CXCR7.

Intriguingly, the interaction between BAM22 and CXCR7 is also insensitive to naloxone [116]. Further studies on cell signaling transduction between BAM22 and CXCR7 need to be done.

vCCL2/vMIP-Ⅱ

Viruses are grouped into different families such as herpesviruses, retroviruses and poxviruses. These pathogens encode chemokine-binding proteins, chemokine receptors, and chemokine analogues that hijack cellular chemokine receptors [117][118]. Human herpesvirus 8 (HHV-8), also called Kaposi's sarcoma-associated virus (KSHV) is a good example. HHV-8 can lead to Kaposi's sarcoma (KS) which is closely related to immunodeficiency. It can also cause two rare proliferative disorders-primary effusion lymphoma (PEL) and multicentric Castelman disease (MCD).

vCCL2, also known as vMIP-Ⅱ,is a viral CC chemokine encoded by HHV-8. This chemokine was first discovered from the HHV-8 genome which was isolated from a KS biopsy [119]. Three of the ORFs in HHV-8 were predicted to encode the chemokine homologs including vCCL1 (vMIP-Ⅰ), vCCL2 (vMIP-Ⅱ), and vCCL3 (vMIP-ⅢI) respectively, plus one CXC chemokine receptor homolog ORF74 [120]. vCCL2 yields in mature format as a 70-aa chemokine (7.9 kDa), and its 94-aa precursor endows a 23-aa N-terminal signal peptide and a C-terminal arginine [120][121].

As an active chemokine, vCCL2 is able to bind with four classes of receptors. There are: CC family: CCR1, CCR2, CCR3, CCR5, CCR8, CCR10; XC family: XCR1; CX3C family: CX3CR1; and CXC family: CXCR4 [122][123][124]. In addition, it has the ability to downregulate the activity of ORF74 [125]. In most cases vCCL2 is regarded as an antagonist chemokine whereas it can also act as agonist towards CCR3 and CCR8 [122]. One thing noteworthy is that recently vCCL2 was found to act as a partial agonist of CXCR7 which can induce β-arrestin recruitment to the receptor. vCCL2 triggers MAP kinase and PI3K/Akt signaling through other chemokine receptors which can be reduced by CXCR7 [126]. This study provided new insights into the interaction between viral chemokines and atypical chemokine receptors.

VUF11207 and VUF11403

From a styrene-amide scaffold, 24 derivatives were synthesized. These CXCR7 ligands were evaluated with pK_i values ranging from 5.3 to 8.1. With the help of SAR studies, two key compounds, VUF11207 and VUF 11403, were found to have high affinity with CXCR7. These two CXCR7 agonists are able to recruit β-arrestin2 and reduce CXCR7 surface expression. Taken together, these two ligands have great value in CXCR7 study [127].

AMD3100

AMD3100 is a small molecule which belongs to the bicyclam family. Initially AMD3100 was found to have antiretroviral effects and later on was shown to bind with CXCR4. Through interaction with CXCR4, AMD3100 expresses strong and selective inhibition effects of X4-tropic HIV replication in vitro [128]. In vivo, the anti-HIV effect of AMD3100 was proved in the immunodeficiency (SCID)-Hu Thy/Liv mouse model [129]. Based on these proprieties, AMD3100 is widely used as a tool for demonstrating the interaction between SDF-1 and CXCR4. As CXCR4 plays an important role during the hematopoietic stem cell homing process, singly administrated high doses of AMD3100 caused a huge release of these cells into peripheral blood. So AMD3100 and its derivatives are undergoing testing for treatment of cancer [128].

Using a homodimeric receptor BRET sensor, researchers also found AMD3100 to be a ligand for CXCR7. Different from the antagonism effect on CXCR4, AMD3100 enhances SDF-1 binding with CXCR7. In addition, AMD3100 alone is able to recruit β-arrestin to CXCR7 but inhibits recruiting to CXCR4. Thus, AMD3100 is regarded as an agonist for CXCR7 [130].

TC14012

T140 is a peptidomimetic which is derived from horseshoe crab polyphemusin and described as an inverse agonist of CXCR4 [131][132]. TC14012, as a serum-stable compound which is derived from its parent compound T140, is able to recruit β-arrestin2 to CXCR7 [133]. Compared to AMD3100, TC14012 showed much higher potency with CXCR7 (EC_{50} of 350nM for TC14012 vs 140μM for AMD3100) and only one log weaker than SDF-1 (35nM) [134]. Great similarities were evident between the binding mode of TC14012 to CXCR7 and CVX15 to CXCR4 [135]. This provided deep insight into the interaction between ligand and receptor. Thus, TC14012 can be an ideal tool for studying interactions between CXCR7 and its synthetic ligands.

CCX771

CCX771 is a selective small molecule agonist and binds to human CXCR7 with an IC_{50} of 4.1 nM. The structure of CCX771 is now unavailable [66]. It has been reported that CCX771 is highly selective for CXCR7 and had no effect on SDF-1 binding to CXCR4 in NC-37 tumor cells [66]. At present CCX771 is widely used in researches for studying the role of CXCR7 in different cell lines. For instance, CCX771 was able to inhibit tumor growth, lung metastasis and tumor angiogenesis in vivo. This help researchers unveil that SDF-1-CXCR7 autocrine loop affects tumor endothelial cells proangiogenic properties [136].

1.7. CXCR7-related diseases

Cancer

The expression level of SDF-1 and its receptors is described in several kinds of solid tumors and tumor cells including lung, prostate, breast and pancreatic cancers [137][138][139][140]. The particular microenvironment of tumors controls CXCR7 expression. For instance, under hypoxic conditions, the transcription level of CXCR7 in human microvascular endothelial cells and the translation level of CXCR7 in glioma cell lines were increased [141][142]. Consistently, results showed that hypoxia-inducible factor 1 alpha leads to up-regulate CXCR7 transcript levels in mesenchymal stem cells [143]. Tumor suppressor genes get silenced when DNA is methylated, thus there is evidence that the transcriptional level of CXCR7 is regulated by a cancer 1 (HIC1) tumor suppressor [64]. For example, in prostate cancer cells, HIC1 negatively regulated the CXCR7 promoter [144]. The expression level of CXCR7 is controlled by MiRNA-430 in zebrafish which suggests that the overexpression of CXCR7 is caused by a lack of miRNA mediated regulation [145]. Consistent with this, downregulation of miRNA-430 caused a high expression level of CXCR7 in a bladder cancer cell line [146]. Furthermore, the restoration of an important tumor-suppressive miRNA named miRNA-101, inhibited CXCR7 protein synthesis in normal hepatocyte-derived cell lines, different hepatocellular carcinoma cell lines, primary hepatocytes and xenograft mice models [147].

Many tumor cells have the ability to produce SDF-1, whose extracellular bioavailability can be modulated by the cell-surface expressed CXCR4 and CXCR7. This was proved in in vivo imaging which showed that tumor cells expressing CXCR7 decreased the concentration of SDF-1 in the primary tumor microenvironment [148]. Monomeric or dimeric forms of SDF-1 can make huge differences. Among different types of cancers, the dimeric form of SDF-1 can produce opposite effects. For instance, the dimeric SDF-1 is more potent than monomeric SDF-1 in promoting β-arrestin 2 recruitment and chemoattractive activity in a model of human breast cancer [149].In contrast, when it moved to a human colon carcinoma cell line, SDF-1 induced calcium mobilization, β-arrestin 2 recruitment and cell migration from monomeric form whereas it only weakly induced chemotaxis and β-arrestin 2 recruitment [150]. The differences between monomeric and dimeric SDF-1 forms are still not clear enough for unquestionable conclusions.

Depending on different tumor types, CXCR7 may or may not co-express with CXCR4. These two receptors can be distinctively expressed in glioma and breast cancer [151][148]. In contrast, CXCR7 and CXCR4 often co-express in human pancreatic cancer tissues [68]. The single expression of these two receptors on certain cell populations restricts their ability for paracrine regulation. By comparison, co-expression of these two receptors on the same cell population made the direct interaction between CXCR4 and CXCR7 and controlled signaling pathways reciprocally possible.

The SDF-1 mediated chemotactic function was changed when both CXCR4 and CXCR7 expressed on the same cell line. For instance, when CXCR7 was transfected into breast cancer cell line MDA-MB-231, the SDF-1 induced chemotactic function was increased [69]. What's more, under the condition of upregulated CXCR4 expression, CXCR7 enhanced chemotaxis to SDF-1 in rat mammary adenocarcinoma cell line MTLn3 [152]. With an opposite result, CXCR7 limited chemotaxis effects through CXCR4 and SDF-1 interaction in human neuroblastoma cell lines [153].

The key element for promoting angiogenesis and malignant cell migration is the distribution of endothelial progenitor cells (EPCs), which is controlled by transendothelial migration. Since CXCR7 is highly expressed on EPCs, it determines the survival effect of SDF-1 on EPCs. In addition, CXCR7 also influences these SDF-1 regulated processes including transendothelial migration, proliferation, adhesion and tube formation of EPCs [154]. The increased expression of

CXCR7 is beneficial for angiogenesis in cancers which indicates the key role of CXCR7 in controlling the angiogenic process. Under hypoxic conditions, CXCL8 and VEGF were highly increased in the tumor microenvironment, which amplifies CXCR7 expression through the positive feedback mechanism [155]. In breast cancer, CXCR7 activation promotes primary tumor growth through increasing VEGF production and microvessel density [152]. When applied to osteosarcoma and associated lung metastasis, the evidence that CXCR7 is significantly expressed on tumor-associated vessels confirmed its critical role in the metastatic process [156]. In hepatocellular carcinoma, CXCR7 is able to enhance endothelial cell proliferation, migration and VEGF production which mediate angiogenesis and tumor growth [157]. In a nut shell, CXCR7 is very important for angiogenesis and metastasis in tumor cells.

Whether the binding of SDF-1 to CXCR7 can directly induce cell migration is still in debate, so in non-small lung cancer cells, CXCR7 has not been conclusively implicated in SDF-1-regulated behavior [158]. However, the SDF-1 effects regulated through CXCR4 for promoting metastasis can be affected by CXCR7. Under certain conditions, CXCR7 has the ability to impair CXCR4-regulated effects. In breast cancer derived from an immunodeficient mouse model, CXCR7 prevented tumor cell invasion and spontaneous lung metastasis formation [152]. Also, the interaction between SDF-1 and CXCR7 did influence the CXCR4 mediated transendothelial migration of human tumor cells [66][159]. On the contrary, the activation of CXCR7 was able to promote metastasis in the breast cancer model [160]. The role of CXCR7 in tumor migration is still unclear and further studies on its role with CXCR4 are needed.

The discovery of CXCR7 has provided human beings a viable target for anti-tumor and anti-metastatic drugs. Using the model of mice engrafted with breast and lung cancer cell lines, inhibiting CXCR7 with selective antagonists showed that CXCR7 is able to promote tumor growth [160]. The main purpose for developing CXCR7 antagonists is to decrease spreading of tumor cells, metastasis, and angiogenesis. As an example, CCX771, a synthetic CXCR7 antagonist, showed high potential of inhibiting transendothelial migration compared to AMD3100, a CXCR4 antagonist. In a lymphoblastic leukemia model, CCX771 also recruited β-arrestin to CXCR7 [66]. Furthermore, both expressions of CXCR4 and CXCR7 responded to SDF-1 to greatly increase human lymphoma cells' migration, indicating that CXCR7 can be an efficient target for cancer treatment [159].

Since the expression of CXCR7 may be able to direct hematopoietic stem cells (HSCs) to the niches which sustain their migration capacity, CXCR7 is an ideal target for HSCs mobilization-inducing agent development [161]. Although the ability of CXCR7 to regulate the BMSC niche is still in debate, studying CXCR7 antagonists is a hot spot among HSCs mobilizers because this may provide patients with an alternative treatment when other mobilization protocols fail [162].

The structural model for CXCR7 is very helpful for elucidating the pharmacology and potential therapeutic utility of CXCR7 antagonists. Unfortunately there are no detailed structures available at present and only a limited number of ligands for CXCR7 have been reported [163][130][127]. Therefore, approaches such as virtual screening and GPCR homology modeling which have been used in the previous studies of CXCR4 can be promising tools for new CXCR7 ligand identification [164]. A number of pharmacological studies focus on the small molecules of CXCR7 antagonists. These antagonists endow reasonable affinities but researchers lack structure information[165][66][151][166]. The synthetic, modeling and pharmacological effort on small molecules targeting CXCR7 was described in a recent paper [127].

The molecules which can block CXCR4 and CXCR7 simultaneously, represent an ideal pharmacological approach because both receptors are involved in cancer malignancy and GBM angiogenesis [167]. However, the current available data governing the binding of these two receptors seems rather complex. Some antagonists did not bind to CXCR4 or CXCR7 exclusively, but bound to the other one, too. And even when they didn’t act as antagonist, partial agonist activity did show up. This happened on AMD3100, a CXCR4 antagonist which may also act as CXCR7 a partial agonist [130]. In addition, the expression level of CXCR4 got down-regulated through CXCR7 agonists which selectively activated β-arrestin [168]. These complex biological responses may be due to cell type specific biased signaling. For GBM, CXCR4 was mainly expressed in CSCs whereas CXCR7 is mainly distributed in differentiated cells and endothelia [151][169]. In other situations these two receptors are often co-expressed in the form of heterodimers. Thus the study of ligands which can interact with both CXCR4 and CXCR7 must be evaluated in specific cell type while considering agonist/antagonist properties of the molecule. Blocking both CXCR4 and CXCR7 through influencing their shared ligands will help researchers better understand the interaction mode between the ligands and CXCR4/CXCR7 receptors. Synthetic compounds derived from the family of chalcones, have high affinity binding

with SDF-1 and can prevent SDF-1 from interacting with CXCR4 and CXCR7. Such a process is able to inhibit inflammatory responses in eosinophils [170].

In addition, there is an RNA oligonucleotide named NOX-A12 which can bind and neutralize SDF-1 with high affinity [171]. Because of the antitumor activity, NOX-A12 is now clinically available for treatment of leukemia and multiple myeloma. It is also noteworthy that, in an in vivo model of GBM, NOX-A12 was effective in inhibiting or delaying recurrences following irradiation [172].

Cardiovascular Diseases

CXCR7 is expressed in the developing heart and associated with defects in the cardiovascular system. Knocking out CXCR7 can lead to several phenomenon of hyperproliferation including thickened pulmonary, aortic valves, and partially overridden aortas [67][173][174]. These abnormalities may have a relationship with disturbed endothelial cell migration because those hyperproliferative defects were reproduced in endothelial specific CXCR7 knock out mice [67]. CXCR7 is regarded as a scavenger receptor for SDF-1 in heart valves so one proposed hypothesis is that CXCR7 prevents the over-interaction between SDF-1 and CXCR4 through sequestering SDF-1 during heart development, which could lead to hyperplasia [175]. This also implies that CXCR7 independently transduces cell signaling in some vascular cell types. To summarize, SDF-1-CXCR4 axis is credited with controlling the migration and proliferation of EPCs (endothelial progenitor cells) whereas SDF-1-CXCR7 mainly maintains the survival of EPCs and promotes these cells adhering to endothelial cells [176]. It is also important to note that, CXCR7 can transduce cell signaling through β-arrestin to promote the migration of vascular smooth muscle cells [47], strengthening the viewpoint of the importance of biased signaling on CXCR7. The formation of a CXCR4-CXCR7 heterodimer indicates that these two receptors may have mutual functions during angiogenesis. Either deletion of CXCR4 or CXCR7 leads to ventricular septum defects, showing that these two receptors are ideal therapeutic targets for cardiovascular system diseases [177][67].

The very important role of SDF-1 in the cardiovascular system makes itself and its receptors a hotspot of cardiovascular disease research. During the past few years, scientists focused on the SDF-1-CXCR4 axis in myocardial infarction and heart ischemia whereas very few studies

mentioned the function of CXCR7. Of the few studies available, one study discovered that SDF-1β protects cardiac cells via CXCR7 [178] and evidence implied that CXCR7 signaling is able to take part in the regeneration process after myocardial infarction [67][176]. Since we recently reported that during monocyte-to macrophage differentiation, CXCR7 is dramatically induced and mediates SDF-1 signaling independent of CXCR4, which leads to increased macrophage phagocytosis [62]. Furthermore, we reported that CXCR7 induction is suppressed by atorvastatin treatment, leading to decreased macrophage migration in response to SDF-1 [179]. These indicated that CXCR7 can be a potential therapeutic target for cardiovascular diseases, such as atherosclerosis.

1.8. Aim of Study

Although lipid-lowering drugs like statins and technology like interventional cardiology have reached great success in this field, long-term patient survival rate is still below expectation. Thus, new pharmacological targets need to be discovered so that identifying CXCR7 as a new molecular target to combat atherosclerotic cardiovascular disease will greatly impact patients' diagnosis and treatment.

Studies have focused on the interactions between SDF-1 and CXCR7; however, other ligands which are able to bind with CXCR7 have been ignored. Consider biased signaling is a complex phenomenon and it exists to a large degree in the chemokine system, more CXCR7 ligands need to be investigated. We hypothesize that CXCR7 independently couples to the inhibition of the ERK1/2 pathway in a ligand- and cell type-specific mechanism. Also, the in vivo roles of CXCR7 remain poorly understand. To test the above hypothesis and to better understand the role of CXCR7 in vivo, two specific research aims were addressed:

Aim1: To determine whether CXCR7 independently couples to inhibition of the ERK1/2 pathway in vascular endothelial cells and during monocyte-to-macrophage differentiation in vitro.

Aim2: To determine the role of endothelial CXCR7 versus macrophage CXCR7 in the pathogenesis of atherosclerosis.

Chapter 2. Materials and Methods

2.1 Materials

The commercial sources of the reagents used in this study are listed in Table 2.1.

Table 2.1. Chemicals

Reagents	Catalog Number	Company
TC14012	4300	Tocris
Resazurin	AR002	R & D
H_2O_2	BDH3540 – 2	BDH
Rottlerin	557370	Calbiochem
UTP	06625	Sigma
VEGF	V7259	Sigma
FBS	1500 – 500	Seradigm
Human SDF-1α	300 – 28A	Peprotech
AMD3100	3299	Tocris
AMD3645	4179	Tocris
IT1t	4569	Tocris

WZ811	3951	Tocris
Human I-TAC	300 − 46	Peprotech
Murine I-TAC	250 − 29	Peprotech
VUF11207	4780	Tocris
Thioglycolate	211716	BD
Human MIF	300 − 69	Perpotech
ADM	22 − 2 − 10	American Peptide
BAM 22P	1650	Tocris
Blasticidin	J61883	Alfa Aesar
Carbenicillin	BP2648	Fisher Scientific
Polybrene	TR-1003-G	Specialty Media

2.2. Cell Culture and Differentiation

2.2.1. Cell Line and Culture

All the cells used in this project include HCAEC, THP-1, 1321N1, Raw 264.7 and EA.hy926 cells. They were purchased from American Type Culture Collection (ATCC). THP-1 cells can be continuously cultured in suspension while other cells can be cultured in adhesion. The culture media used for THP-1 cells is RPMI-1640 (HyClone, Thermo). For HCAEC, the culture media used is EBM-2 supplemented with VEGF, FGF, EGF, IGF, ascorbic acid, GA 1000 (Lonza). For other cells, the culture media used is DMEM (HyClone, Thermo). All the culture media used were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Thermo), 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza). All the cells were cultured at 37 ℃ in Forma Series ii Water Jacketed incubator (Thermo) in a humidified atmosphere with 5% CO_2.

2.2.2. Cell Passage

For THP-1 cells, they were collected and centrifuged at ~1100 rpm for 5 min and subsequently re-suspended in a concentration of ~ 2-4*10^5 cells/ml. For Raw 264.7 cells, they were maintained in 75 cm^2 flask, detached by accutase (Stemcell) and then centrifuged at ~1100 rpm for 5 min and subsequently re-suspended in a concentration of ~ 2-4*10^5 cells/ml. For HCAEC and EA.hy926 cells, they were maintained in 75 cm^2 flask, detached by trypsin (HyClone) and then centrifuged at ~1100 rpm for 5 min and subsequently re-suspended in a concentration of ~ 2-4*10^5 cells/ml.

2.2.3. Long-term Storage

For long-term storage, all the cells used in this project were collected and centrifuged at ~1100 rpm for 5 min. Then pellets were re-suspended in full culture media containing 10% (v/v) DMSO (EMD Milipore) in 2 ml cryovials. Then the cryovials were transferred into an isopropanol freezing container (VWR) to reach 1 °C/min cooling rate required for successful cryopreservation of cells, then the container was stored in -80 °C overnight. Next, the cryovials were transferred to a liquid nitrogen (-196 °C) tank (taylor-wharton) at the following day. To resuscitate these frozen cells, the cryovials were rapidly removed into a 37 °C water bath and gently swirl the vials till the medium starts to thaw. Then dilute the cells suspension with pre-warmed growth medium and centrifuge at ~1100 rpm for 5 min. After the centrifugation, re-suspend the cells in complete growth medium into the appropriate culture vessel at the appropriate culture environment.

2.2.4. Starvation

For THP-1 cells, they were starved for 4h in RPMI-1640 without FBS. For HCAEC, they were starved in EBM-2 without FBS. For 1321N1, Raw 264.7 and EA.hy 926 cells, they were starved overnight in DMEM without FBS. Cells were pretreated with inhibitors or antagonists for 30 min before stimulation.

2.2.5. Cell Viability

To measure the cell viability and cytotoxicity, Resazurin, a non-toxic, water soluble, redox-sensitive dye, was used. It changes from blue/non-fluorescent state to a pink/highly-fluorescent state via reduction by viable cells. Generally, resazurin was added directly to cultured cells in serum-supplemented medium to reach a final concentration of 10% (v/v). Fluorescence was read by Varioskan Flash Multimode Reader fluorescence (Ex/Em: 544/590).

2.2.6. Cell Activation

To determine TC14012's effect in HCAEC, cells were stimulated for 40 min at the indicated concentrations: 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M and 3×10^{-5} M. Alternatively, cells were stimulated with 3×10^{-5} M at the indicated time points: 0 min, 1 min, 2 min, 5 min, 10 min, 20 min, 40 min and 90 min. To determine whether the stimulatory effects of SDF-1, UTP and VEGF on ERK1/2 pathway can be blocked by TC14012 in HCAEC, cells were pretreated with TC14012 (2×10^{-5} M) for 30 min. Then cells were separately stimulated with 10% FBS, UTP (10^{-4}M), SDF-1 (100ng/ml) and VEGF (3ng/ml). To determine whether the upstream kinases of ERK1/2 can be blocked by TC14012 in HCAEC, cells were pretreated with TC14012 (2×10^{-5} M) for 40 min. Then cells were stimulated with SDF-1 (100ng/ml) for 10 min. To determine the effects of TC14012 and other CXCR4 antagonists on ERK1/2 pathway in HCAEC, cells were stimulated with TC14012 (20uM), AMD3100 (10uM), AMD3645 (10uM), IT1t (1uM) and WZ811 (10uM) for 40 min. To determine the effects of TC14012 and other CXCR4 antagonists on ERK1/2 pathway in THP-1 cell, cells were pretreated with AMD3100 (10uM), AMD3645 (10uM), IT1t (1uM) and WZ811 (10uM) for 30 min and then added SDF-1 (100ng/ml) for 10 min. To determine TC14012's effects in 1321N1 cell, both wild type and CXCR7-transfected cells were treated by TC14012 (2×10^{-5} M), SDF-1(100ng/ml), I-TAC (300ng/ml) and VUF11207 (10^{-5} M) at the indicated time points: 10 min, 20 min and 40 min. To determine the effects of different CXCR7 ligands in EA hy.926 cells, both wild and CXCR7-transfected cells were stimulated with TC14012 (20 μM), SDF-1 (100 ng/ml), h-ITAC (300 ng/ml), m-ITAC (300

ng/ml), VUF11207 (10^-5 M), MIF (100 ng/ml), ADM (10^-7 M) and BAM22 (3×10^-6 M) for 10 min. To determine the effects of different CXCR7 ligands in Raw.264.7 cells, both wild and CXCR7-transfected cells were stimulated with TC14012 (20 μM), SDF-1 (100 ng/ml), h-ITAC (300 ng/ml), m-ITAC (300 ng/ml), VUF11207 (10^-5 M), MIF (100 ng/ml), ADM (10^-7 M) and BAM22 (3×10^-6 M) for 10 min.

2.2.7. Generation of CXCR7-floxed$^{+/+}$/LyzM-Cre$^{+/+}$ and CXCR7-floxed$^{+/+}$/LyzM-Cre$^{-/-}$ Mice

We bought C57BL6J homozygous CXCR7-Flox$^{+/+}$/Cre$^{-/-}$ and Flox$^{-/-}$/LyzM-Cre$^{+/+}$ mice from Jackson Lab. Those mice were housed in specific pathogen-free animal facilities of Auburn University. Mice were kept under 12 h dark / 12 h light cycles and were fed with fresh water and pellet food. These original mice were crossed to obtain heterozygous Flox$^{+/-}$/LyzM-Cre$^{+/-}$ mice. Next, these heterozygous mice were subsequently crossed and resulted in Flox$^{+/+}$/LyzM-Cre$^{+/-}$ and Flox$^{+/-}$/LyzM-Cre$^{+/+}$ mice. Then the Flox$^{+/+}$/LyzM-Cre$^{+/-}$ and Flox$^{+/-}$/LyzM-Cre$^{+/+}$ mice were continuously backcrossed to obtain homozygous Flox$^{+/+}$/LyzM-Cre$^{+/+}$ and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice. All mice were maintained in a facility free of well-defined pathogens under the supervision of the Biological Resource Unit at Auburn University. All animal protocols were approved by Auburn Institutional Animal Care and Use Committee; and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

2.2.8 Isolation of Mouse Peritoneal Macrophages

Mice used in this experiment were fed with normal chow diet for 16 weeks. Before euthanizing mice, 2 ml of 3% thioglycolate medium was injected into the peritoneal cavity of each mouse using 3 ml syringes attached to 23 G needles. 72 h after injection, mice were euthanized and the abdomen of each mouse was washed with 75% ethanol. Then, 10 ml of cold PBS was injected into the peritoneal cavity of each mouse using 10 ml syringes attached to 23 G needles. After this,

perform a gentle massage on the peritoneal cavity and then aspirate the fluid carefully without puncturing any organ. Remove the needle and dispense the peritoneal fluid into 15 ml conical centrifuge tubes. Then, centrifuge 10 min at 1100 RPM in a refrigerated centrifuge. These freshly isolated cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin at 37 ℃ in a humidified atmosphere with 5% CO_2. The peritoneal macrophages were used in experiments after being stabilized 3 h.

2.2.9 Isolation of Mouse Bone Marrow Derived Cells

Mice used in this experiment were fed with normal chow diet for 16 weeks. They were euthanized and disinfect the hind limbs with ethanol (75%). Then remove skin and muscles and separate the bones with a sterile scalpel. After that, wash femur and tibia with 96% ethanol for a minimum of 90 sec to guarantee aseptic cell preparation. Continue the washing process with sterile PBS to rinse off remaining ethanol. For harvesting of bone marrow, cut the proximal and distal end of each bone with a pair of fine scissors to gain access to the femoral and tibial shafts. Flush the bones with warm starvation DMEM medium. Then, hold the bone with a fine forceps and rinse one by one until it turns semi-translucent. Carefully apply pressure to the end of the syringe plunger in order to minimize cell stress. Following with filter the bone marrow through a 70 µm nylon web and gather the filtrate. Next, centrifuge the cell suspension at 1100 RPM for 10 min at room temperature. These freshly isolated cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin at 37 ℃ in a humidified atmosphere with 5% CO_2. To obtain polarized macrophages, bone marrow derived cells were treated with 20 µg/mL of M-CSF and differentiated up to 7 days.

2.3. PCR Analysis

2.3.1. Isolation and Measurement of RNA and DNA

Cells were grown in six-well plates until confluence. The total RNA and DNA were extracted from HEK293 cells, 1321N1 cells, mice peritoneal macrophages, mouse bone marrow derived macrophages, EA.hy 926 cells and Raw 264.7 cells according to manufacturer's protocol for the RNeasy and DNeasy kits, respectively (Qiagen).

2.3.2. cDNA Synthesis

For the synthesis of the first strand of cDNA, 1μg of total RNA after DNase (Ambion) treatment was reverse-transcribed using Taqman reverse transcription reagents (Applied Biosystems) using the recipe in Table 2.2.

Table 2.2. Reaction composition for cDNA synthesis

Component	Volume/reaction
10xTaq RT buffer	5 μL
25mM $MgCl_2$	11 μL
10mM dNTP	10 μL
oligo dT	2.5 μL
Rnase inhibitor	1 μL
Reverse Transcriptase or RNase free H_2O	1.25 μL
RNA	variable
RNase free H_2O	variable
Total Volume	50 μL

The mixture of all the components was incubated at 25 °C for 10 min; 48 °C for 30 min; 95 °C for 5 min and then held at 4 °C.

2.3.3. Real-time PCR Analysis

Real time RT-PCR was carried out using an iCycler iQ5 detection system (Bio-Rad) with SYBR Green reagents (Applied Biosystems), as we previously described [62][179]. The PCR mixture (20 μL) contained 0.5 μM concentration of each primer, 4 μl of water, 10μL of SYBR Green mixture, and 5μL of cDNA template from previous step. The samples were placed in 96-well plates that were sealed with optical clear cap (Fisher) with the following reaction condition: initial PCR activation step (5 min at 95 ℃), and cycling steps (denaturation for 1 min at 94 ℃, annealing for 1 min at 59 ℃, extension for 1 min at 72 ℃; 38 cycles), final extension step (10 min at 72 ℃). Internal controls, HPRT or β-actin were amplified in separate wells. The sequences of primers are listed in the Table 2.3.

Table 2.3. Primers used for PCR assay

Gene	Forward Primer	Reverse Primer
h CXCR4	5'–CACTTCAGATAACTACACCG–3'	5'–ATCCAGACGCCAACATAGAC–3'
h CXCR7	5'–ATGGATCTGCATCTCTTCGACTAC–3'	5'–TCATTTGGTGCTCTGCTCCAAGG–3'
m CXCR7(1)	5'–TGGTCAGTCTCGTGCAGCAT–3'	5'–GCCAACATACCAGGAAGACC–3'
m CXCR7(2)	5'–ATGGATGTGCACTTGTTTGACTA–3'	5' –TCACTTGGTGTTCTGTTCCAGGG–3'
m β-actin	5'–ATGGATGACGATATCGCTGCG–3'	5'–CTAGAAGCACTTGCGGTGCAC–3'
m HPRT	5'–GGACTAATTATGGACAGGACTG–3'	5'–GCTCTTCAGTCTGATAAAATCTAC–3'
Genotype (1)	5'–CTGCCTCTCTTGGGAATGT–3'	5'–CTCTCTGGCCGTTCTCTC–3'
Genotype (2)	5'–GCCTTCTTGACGAGTTCTTC–3'	5'–CTCTCTGGCCGTTCTCTC–3'

2.4. Western Blotting

2.4.1. Solutions

The commercial sources of the buffers and chemicals used in this study for Western blotting are listed in Table 2.4.

Table 2.4. Buffers used for Western blot

Items	Catalog Number	Company
10x Tris Glycine Buffer	786-478	Biosciences
10x Tris/Glycine/SDS Buffer	161-0732	Bio-Rad
20x Tris-Buffered Saline and Tween 20	77500	USB Affymetrix
Western Lightning® Plus-ECL	NEL105001EA	PerkinElmer
Blotto (non-fat dry milk)	sc-2324	Santa Cruz Biotechnology
Albumin, Bovine Fraction V	9048-46-8	Research Products International Corp.

Milli-Q purified water was used to dilute the buffers. Electrophoresis buffer was diluted from 10x Tris/Glycine/SDS Buffer and the dilution contains 25mM Tris, 192mM Glycine and 0.1% (w/v) SDS at PH 8.3. Transfer buffer was diluted from 10x Tris Glycine buffer and 20% (v/v) methanol with a final concentration of 25 mM Tris, 192 mM Glycine at PH 8.3. Tris-Buffered Saline and Tween 20 (TBS-T) buffer was made from 10x TBS-T buffer and containing 500 mM Tris, 60 mM KCl, 2.8 mM NaCl, and 1.0% Tween-20. Blocking buffer was made with 5% non-fat dry milk in TBS-T buffer. Primary antibody was diluted in 5% bovine serum albumin (BSA) in TBST buffer.

2.4.2. Sampling

Cells were cultured in six well plates and serum-deprived for 10 h before stimulation with agonists at the indicated concentration. Then the supernatant was removed directly or by centrifuge and cells were solubilized in 300 µL Laemmli sample buffer (sigma-aldrich) and scratched with rubber policeman on ice followed by being heated in boiling water for 5 min.

2.4.3. Blotting

Precision plus protein dual color standard (Bio-Rad) was used as a reference to identify the approximate molecular weight. Samples were loaded and separated on 10% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) in a SDS-PAGE gel chamber (Bio-Rad) in electrophoresis buffer for 25 min with the voltage of 70 V, then the voltage was changed to 110 V for 60 min. After running the gel, assemble the stack in the order of two layers of absorbent paper, which was thoroughly soaked in transfer buffer; the SDS-PAGE gel; a wet polyvinylidene difluoride (PVDF) membrane (Thermo); two layers of absorbent paper, which was thoroughly soaked in transfer buffer. Gels were blotted using a semi-dry blotting apparatus (Bio-Rad) for 30 min with the voltage of 20 V. After transfer, the stack was carefully disassembled. The membrane was blocked for 1 h (room temperature, shaking) in Western blot blocking solution. The membrane was probed with the primary antibody overnight in 5% BSA in TBS-T buffer. The primary antibodies used for Western in this study are listed in Table 2.5.. Next day, the blots were washed in TBS-T buffer for four times 10 min each time. A horseradish-conjugated secondary antibody (Cell Signaling) was incubated for 1 h at room temperature (5 % dry milk in TBS-T buffer). Unbound antibodies were washed in TBS-T buffer four times for 10 min each time. The bound antibody was detected by incubating the blots in Western Lightning® Plus-ECL (PerkinElmer). The image was captured on a sensitive photographic film (research products international corp.), placed against the membrane, and visualized by medical film processor (Konica Minolta medical & graphic Inc.).

Table 2.5. The primary antibodies used for Western blotting

Items	Catalog Number	Clone	Company
anti-human CXCR7 mAb	MAB42273	11G8	R&D Systems
anti-human CXCR7 mAb	K0223-3	9C4	MBL
anti-human CXCR7 pAb	AF4227	-	R&D Systems
anti-human CXCR7 pAb	14840-1-AP	-	Proteintech
anti-mouse CXCR7 pAb	ACR-037	-	Alomone
P-p38 MAPK	4511	D3F9	Cell Signaling
P-SAPK/JNK	4668	8.1E+12	Cell Signaling
P-AKT	4060	D9E	Cell Signaling
P-p44/42 MAPK	4370	D13.14.4E	Cell Signaling
P-MEK1/2	9154	-	Cell Signaling
P-c-Raf	9421	-	Cell Signaling
β-tubulin	2128	9F3	Cell Signaling

2.4.4. Imaging Analysis

The images on the films were then scanned into the computer for presentation and analysis. The intensity of signals was acquired in the linear range of the digital images using specific densitometric software, Quantity One. Images were calibrated against the background and given in relative density units.

2.4.5. Stripping and Re-probing

Equal protein loading was verified by stripping off the original antibodies and re-probed with the primary antibodies. Blots were rinsed with TBS-T buffer and incubated for 20-30 min at room temperature in restore PLUS Western blot stripping buffer (Thermo). After which, the membrane

was extensively rinsed with TBS-T buffer three time for 1 min each time, and blocked for 60 min in 5% non-fat dry milk in TBS-T buffer. Subsequently, the blots were re-probed with desired primary antibodies as described previously [62][179].

2.5. Adenovirus Cre Gene Delivery

Cells isolated from mice bone marrow were collected and seeded in 6-well plates. After 3 days of differentiation, 15 µl of premade adenovirus Cre (ViGene) and control RFP adenovirus (ViGene) were added separately. Images with fluorescent signals in random fields were acquired and captured using an AMG EVOS® digital inverted multi-functional microscope (AMG) as we previously reported [181].

2.6. Mouse Blood Cell Counting

EDTA anti-coagulated mice periphery blood samples were used to obtain a complete blood count with blood collection tubes (VACUETTE). These samples were put on ice for processing. Then leukocytes, lymphocytes, neutrophils and monocytes were measured with an automated cell counter. For mice peripheral macrophages counting, mice were intraperitoneal injected with 2 ml of 3% thioglycolate for 72 h and then follow the same procedure as well.

2.7. Optical Microscopy Imaging

Single color fluorescent images were obtained by capturing the Calcein AM stained cells using an EVOS® digital inverted multi-functional microscope.

2.8. Cell Proliferation Measurement

Cell Counting Kit 8 (CCK-8, Bimake) was used for determining cell proliferation process. Both EA.hy 926 and Raw 264.7 cells were inoculated in 96-well plate for 48h with 100 µl of DMEM medium. Then, 10 µl of Cell Counting Kit 8 was added directly to cells in culture medium. OD values were measured using microplate reader at 450 nm every 0.5 h and up to 4h.

2.9. Plasmid Transfection

Vectors containing the human CXCR7 sequences were bought directly from GE Healthcare. Plasmid preparation was performed according to technical manual of "Precision LentiORF^{TM} Collection" (Dharmacon). One day prior to the transfection, 1321N1 cells were seeded in 1 ml of complete growth medium. At the time of transfection, cell density should be 50-70%. Then the plasmid transfections were carried out using Xfect reagent under conditions specified by the supplier (Clontech Laboratories).

2.10. Lentivirus Transduction

Vectors containing the human CXCR7 sequences were bought directly from GE Healthcare. Plasmid preparation was performed according to technical manual of "Precision LentiORF^{TM} Collection" (Dharmacon). These vectors and packaging vectors were then transfected into HEK293T cells. Supernatants containing lentiviruses were harvested 48h post-transfection. EA.hy 926 and Raw 264.7 cells were transfected with lentiviruses according to technical manual of "Drarmacon^{TM} Trans-Lentiviral Packaging Kits" (GE Healthcare).

Chapter 3. Results

3.1. Inhibitory Effect of TC14012 on ERK1/2 Pathway in Endothelial Cells

To determine ligand-biased signaling effects in human coronary artery endothelial cells, we have screened several CXCR7 endogenous (SDF-1, I-TAC) and exogenous (VUF11207, TC14012) ligands and tested their effects in human coronary artery endothelial cell line on ERK1/2 pathway. Western blot results showed one polypeptide TC10412 caused the inhibition effect on ERK1/2 pathway. Moving one step forward, dose-response and time-response experiments were performed to determine the maximum inhibition effect (Figure 5.1.A & B). These exciting data suggest that the ERK1/2 inhibition effect may be regulated through the certain receptor (hopefully CXCR7) in human coronary artery endothelial cells.

3.2. No Toxic Effect of TC14012 on Endothelial Cells

To determine whether the inhibition effect of TC14012 on ERK1/2 pathway was just a toxic effect or not? A cell viability kit called Resazurin was used to verify the toxicity of TC14012. Human coronary artery endothelial cells were seeded in a 96-well plate and pre-treated with Resazurin. Then those cells were treated with H_20 (negative control), TC14012 (30uM), H_20_2 (1uM, positive control) and PKC inhibitor (30uM, positive control) separately. We found that with time passed by, the fluorescence produced by cells which were pretreated with H_20_2 and PKC inhibitor, decreased dramatically (Figure 5.2.). On the contrary, TC14012 and H_20 stimulated cells remained in high fluorescence, which indicated that TC14012 had no toxic effects in those endothelial cells. The results also increase the possibility that the certain receptor (hopefully CXCR7) couples to inhibition of the ERK1/2 pathway in vascular endothelial cells.

3.3. The Effects of TC14012 on Different Agonists-Induced ERK1/2 Activation

Having proved that TC14012 is a non-toxic compound, we want to determine whether the stimulatory effects of SDF-1, UTP, VEGF and FBS on ERK1/2 pathway can be blocked by TC14012 or not. All these cells were pretreated with TC14012 for 40min, and then UTP, VEGF and FBS were added for 10min. We found that the stimulatory effects of SDF-1, UTP and VEGF on ERK1/2 pathway can be blocked by TC14012, but not for FBS (Figure 5.3.A & B & C). These data indicates that TC14012 may not only induce the inhibition effect through CXCR7 but also had a generalized inhibition on ERK1/2 pathway through other receptors.

3.4. TC14012 Inhibits the Upstream Kinases of ERK1/2 Pathway in Endothelial Cell

Next, in order to make sure the inhibition effect was happened on ERK1/2 kinase or its upstream kinases. The phosphorylation level of MEK and c-Raf were determined by Western blotting. The results showed that both MEK and c-Raf were blocked by TC14012 (Figure 5.4.), which suggests that ERK1/2 inhibition effect not happened exactly on ERK1/2 but on its upstream kinase, c-Raf.

3.5. Is TC14012 Induced ERK1/2 Inhibition Related to CXCR4 Blockage?

Since TC14012 has the dual identity, it not only acts as a CXCR7 agonist but also a CXCR4 antagonist. This brings one possibility that the inhibition effect on ERK1/2 was because of the blockage of CXCR4. To solve this problem, we screened a series of CXCR4 antagonists. The Western blotting results showed that only TC14012 caused inhibition effect on ERK1/2 pathway while other CXCR4 antagonists did not (Figure 5.5.A).

3.6. Did We Use High Enough Doses for All the CXCR4 Antagonists?

Some people may doubt that we did not use enough doses for these CXCR4 antagonists or these antagonists are just invalid. In order to exclude this situation, we chose anther cell line called THP-1 cells. This is a human monocytic cell line which only expresses CXCR4 but not CXCR7. We found that SDF-1 itself strongly activated ERK1/2 pathway while this inhibition effect can be blocked by all these CXCR4 antagonists except WZ811 (Figure 5.5.B). These data suggests the inhibition effect on ERK1/2 pathway caused by TC14012 is not regulated through CXCR4 blockage.

3.7. Evidence of CXCR7 Expression in Transfected 1321N1 Cells

In previous studies, the cell line HEK293 which they used to overexpress CXCR7 naturally had endogenous CXCR4 and CXCR7 expression, which brings difficulties in data interpretation [182]. Fortunately, after screening for more than 6 mammalian cell lines, we have found that a human 1321N1 astrocytoma cell line, which is completely devoid of CXCR4 and CXCR7 expression (Figure 5.6.). To assess whether CXCR7 has been successfully transfected into 1321N1 cell, Real-time PCR and Western blotting were performed. The results showed that after transfection, 1321N1 cell express high level of CXCR7 and there are no influences for the expression of CXCR4 (Figure 5.7.A & B & C).

3.8. Different Effects of CXCR7 Ligands on ERK1/2 Pathway in 1321N1 WT versus CXCR7-Transfected Cells

To verify the contribution of CXCR7, both wild type and CXCR7-transfected 1321N1 cells were stimulated with TC14012, SDF-1, I-TAC and VUF11207 at different time points. The Western blotting results showed that compare to other CXCR7 ligands, only TC14012 caused ERK1/2

inhibition effect in CXCR7-transfected but not in WT 1321N1 cells (Figure 5.8.A & B & C). These data indicated that CXCR7 independently couples to inhibition of the ERK1/2 pathway in the 1321N1 astrocytoma cell line.

3.9. Generation of CXCR7-floxed$^{+/+}$/LyzM-Cre$^{+/+}$ and CXCR7-floxed$^{+/+}$/LyzM-Cre$^{-/-}$ Mice

Having proved that CXCR7 independently couples to the inhibition of the ERK1/2 pathway with the stimulation of TC14012, we also want to know the role of CXCR7 in vivo. Since global knock out of CXCR7 caused postnatal death phenotype in mice [173], to overcome this problem, we used Cre-loxP system to obtain myeloid-specific deletion of CXCR7 in vivo. Both C57BL6J homozygous CXCR7-Flox$^{+/+}$/Cre$^{-/-}$ and Flox$^{-/-}$/LyzM-Cre$^{+/+}$ mice were bought from Jackson Lab and were crossed to obtain heterozygous Flox$^{+/-}$/LyzM-Cre$^{+/-}$ mice. Those mice were subsequently crossed and resulted in Flox$^{+/+}$/LyzM-Cre$^{+/-}$ and Flox$^{+/-}$/LyzM-Cre$^{+/+}$ mice. Those mice were continuously outcrossed to obtain homozygous Flox$^{+/+}$/LyzM-Cre$^{+/+}$ and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice identified by genotyping (Figure 5.9.A). Schematic description of Cre-loxP based conditional knock out of CXCR7 in mice. Cre was inserted after its LyzoM gene promoter. Two loxP sites were added before and after exon2 of mouse CXCR7 gene (Figure 5.9.B).

3.10. No Obvious Physical Abnormalities in Myeloid-specific CXCR7 Knock-out Mice

To determine whether there showed any physical or behavioral abnormalities in our myeloid-specific CXCR7 deletion mice. Both body weight and heart weight were measured from Flox$^{+/+}$/LyzM-Cre$^{+/+}$ and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice groups. Unlike the systemic knockouts, our new line was viable, fertile, and did not show obvious gross physical or behavioral abnormalities (Figure 5.10.A & B).

3.11. Number of Periphery Blood Leukocytes in WT and CXCR7 Specific Knock-out Mice

To better know about the physiological effect of CXCR7, periphery blood cells were isolated from $Flox^{+/+}$/LyzM-$Cre^{+/+}$ and $Flox^{+/+}$/LyzM-$Cre^{-/-}$ mice respectively. The number of four different kinds of cells in mice periphery blood was measured. There is significantly higher number of periphery blood monocytes in $Flox^{+/+}$/LyzM-$Cre^{-/-}$ mice compared with $Flox^{+/+}$/LyzM-$Cre^{-/-}$ mice (Figure 5.11.A), showing the unique role of CXCR7 in monocytosis. To our surprise, this case not only happened in monocytes but also in leukocytes, lymphocytes and neutrophils (Figure 5.11.B & C & D). These results reinforce the idea that CXCR7 plays a crucial role in recruiting mouse periphery blood cells.

3.12. The mRNA and Protein Expression of CXCR7 in WT and CXCR7 Specific Knock-out Mice

To determine whether deletion of CXCR7 happened in our CXCR7-$floxed^{+/+}$/LyzM-$Cre^{+/+}$ mice, mRNA level of CXCR7 from peritoneal macrophages (Figure 5.12.A) and bone marrow cells (Figure 5.12.B) stimulated with 20ng/ml M-CSF, differentiated to day 4 and day 5 were quantified by Real-time PCR both in $Flox^{+/+}$/LyzM-$Cre^{+/+}$ and $Flox^{+/+}$/LyzM-$Cre^{-/-}$ mice groups. Protein expression level of CXCR7 was determined by Western blotting (Figure 5.12.C). However, contrary to our expectation, the expression level of CXCR7 in $Flox^{+/+}$/LyzM-$Cre^{+/+}$ mice did not get down-regulated, but oppositely got up-regulated dramatically compared with $Flox^{+/+}$/LyzM-$Cre^{-/-}$ mice. Our finding suggests that this could be attributed to a potential compensatory mechanism and indicates that the gene editing tool Cre-loxP system may not always lead to partial deletion of certain genes as researchers expected.

3.13. Upregulation of CXCR7 mRNA is not Solely Attributed to the Disruption of LyzM Gene by Cre Insertion or the Inclusion of Floxed Sequence to CXCR7 Gene

Based on this unexpected upregulation of CXCR7 phenomenon and since both the Cre gene and the floxed sequence to CXCR7 gene are external factors in our mice model. Here we put up with a question, is the upregulation of CXCR7 mRNA solely attributed to the insertion of these two factors? To answer this question, Real-time PCR analysis of CXCR7 mRNA from peritoneal macrophages was performed in $Flox^{-/-}$/LyzM-$Cre^{+/+}$ and $Flox^{-/-}$/LyzM-$Cre^{-/-}$ mice groups (Figure 5.13.A). The same procedure was also used to determine mRNA level of CXCR7 from peritoneal macrophages (Figure 5.13.B) and bone marrow cells stimulated with 20ng/ml M-CSF, differentiated to day 5 (Figure 5.13.C). The results showed that comparing with $Flox^{-/-}$/LyzM-$Cre^{-/-}$ mice, $Flox^{-/-}$/LyzM-$Cre^{+/+}$ mice did not express higher level of CXCR7. Same procedure was also performed in $Flox^{+/+}$/LyzM-$Cre^{-/-}$ and $Flox^{-/-}$/LyzM-$Cre^{-/-}$ mice model and consistent with what happened in $Flox^{-/-}$/LyzM-$Cre^{+/+}$ and $Flox^{-/-}$/LyzM-$Cre^{-/-}$ mice before, $Flox^{+/+}$/LyzM-$Cre^{-/-}$ also express less CXCR7 comparing with $Flox^{-/-}$/LyzM-$Cre^{-/-}$ mice. Thus, we provide evidence indicating that the unexpected increasing of CXCR7 in $Flox^{+/+}$/LyzM-$Cre^{+/+}$ mice is not caused by Cre or floxed sequence insertion.

3.14. Adenovirus Delivery of the Cre Gene into CXCR7-floxed Macrophages in Vitro Mimicked CXCR7 Induction in Vivo

To confirm whether this CXCR7 upregulation phenomenon can be replicated in vitro or not, cells isolated from $Flox^{+/+}$/LyzM-$Cre^{-/-}$ mice bone marrow were seeded in 6-well plates and stimulated with 20ng/ml M-CSF. After 3 days of differentiation, adenovirus containing Cre gene and red fluorescence protein (RFP) was added. The detected red fluorescence confirmed that Cre gene was successfully transfected into these mouse cells (Figure 5.14.A). Total RNA were isolated from control and Cre-transfected group respectively, reverse transcribed and Real-time PCR amplified with CXCR7 primers (Figure 5.14.B). The results showed that CXCR7 mRNA expression level got increased compare with control group. Thus, these compelling evidence indicating that the paradoxical upregulation of macrophage CXCR7 in the myeloid-specific gene editing mice was replicable both in vitro and in vivo.

3.15. PCR Mapping Indicates Partial Deletion of Myeloid CXCR7 Gene via the Cre-loxP Approach

Debate may continue regarding whether the deletion of CXCR7 did happen in macrophages isolated from Flox$^{+/+}$/LyzM-Cre$^{+/+}$ mice or not. If the deletion did not happen, it is impossible to explain the upregulation of CXCR7 through a compensatory mechanism. To make this fuzzy region clear, a schematic layout describes the position of designed primers (Figure 5.15.A). Two pairs of primers were designed to check the inserted loxP sequence (F2, R1) and whether deletion of CXCR7 happened (F1,R1) in genomic DNA (Figure 5.15.B). Same procedure was also performed on genomic DNA from Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice delivered with Cre gene (Figure 5.15.C & D). If full deletion of CXCR7 happened, the gene segment of 370bp long between primers F1 and R1 should be detected and the gene segment of 700bp long between primers F2 and R1 should not be detected. If no deletion of CXCR7 happened, the gene segment of 370bp long between primers F1 and R1 should not be detected and the gene segment of 700bp long between primers F2 and R1 should be detected. If partial deletion of CXCR7 happened, both the gene segment of 370bp long between primers F1 and R1 and the gene segment of 700bp long between primers F2 and R1 should be detected. Since Real-time PCR analysis showed the 700bp-band was detected both in Flox$^{+/+}$/LyzM-Cre$^{+/+}$ mice and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice and 370bp-band was only detected in Flox$^{+/+}$/LyzM-Cre$^{+/+}$ mice and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice delivered Cre gene with adenovirus. It is conceivable that our Cre-loxP system works, partially delated CXCR7 and somehow leading to compensatory CXCR7 upregulation.

3.16. Injection of Thioglycolate Led to More Macrophages Infiltration into the Peritoneum in CXCR7-floxed$^{+/+}$/LyzM-Cre$^{+/+}$ Mice

To determine whether CXCR7 plays a crucial role in macrophage infiltration process or not, both Flox$^{+/+}$/LyzM-Cre$^{+/+}$and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice were injected with thioglycolate through tail vein for 72 h and the number of macrophages in mice peritoneum were measured respectively. The cell counting results revealed that with pre-injection of inflammatory reagent thioglycolate,

more macrophage infiltrated into the peritoneum in $Flox^{+/+}$/LyzM-$Cre^{+/+}$ mice compared with $Flox^{+/+}$/LyzM-$Cre^{-/-}$ mice (Figure 5.16.). Based on this phenomenon, further study the role of CXCR7 in macrophage biology will be imperative.

3.17. The Expression of CXCR7 in Wild Type Cells versus CXCR7-Transfected Cells

To further explore the role of CXCR7 in cell proliferation and differentiation process. We chose two different kinds of cell lines. They are: human immortalized endothelial cell named EA.hy 926 and murine macrophage named Raw 264.7. Both EA.hy 926 and Raw 264.7 express no CXCR4 and CXCR7 at mRNA level (Figure 5.17. A top). Western blotting results also confirmed that no expression of CXCR7 at protein level in EA.hy 926 and Raw 264.7 cells (Figure 5.17. B left). Then EA.hy 926 and Raw 264.7 cells were successfully transfected with lentiviruses containing the human CXCR7 sequences (Figure 5.17. A bottom & B right). This data indicates that both EA.hy 926 and Raw 264.7 cells are ideal cell line to overexpress CXCR7 and the independent role of CXCR7 in cell proliferation and differentiation can be determined.

3.18. Transfection of CXCR7 Inhibits Cell Proliferation both in EA.hy 926 and Raw 264.7 Cell Lines

To determine the role of CXCR7 in cell differentiation process, cell differentiation rate were measured both on wild type and CXCR7-transdected cells. To our surprise, those cells transfected with CXCR7 differentiated much slower comparing to wild type group (Fig. 5.18. A & B). These results indicated that CXCR7 does play an important role which is able to independently delay cell differentiation process both in EA.hy 926 and Raw 264.7 cells.

3.19. Transfection of CXCR7 Inhibits AKT and ERK Signaling Pathways both in EA.hy 926 and Raw 264.7 Cell Lines.

To unveil the mechanism why CXCR7 can independently inhibit cell differentiation rate in EA.hy 926 and Raw 264.7 cells, phosphorylation level of Akt, p38, ERK1/2, JNK and their loading control were determined by Western blotting. We found that basal level of Akt and ERK1/2 were inhibited after transfection of CXCR7 both in EA.hy 926 and Raw 264.7 cells whereas no influences on p38 and JNK pathways (Fig. 5.19. A & B). Since Akt and ERK1/2 is tightly related to cell proliferation and differentiation process, this may explain why transfection of CXCR7 can inhibit the differentiation process in EA.hy 926 and Raw 264.7 cells.

3.20. The Ligand-Biased Signaling Patterns in WT EA.hy 926 Cells versus CXCR7-Transfected EA.hy 926 Cells.

Most previous studies only focus on the interactions between SDF-1 and CXCR7, other ligands of CXCR7 have been ignored. Consider that there are many ligands which can bind to CXCR7 and the existence of biased signaling, we screened a series of CXCR7 ligands to stimulate our wild type EA.hy 926 cells and CXCR7-transfected EA.hy 926 cells. The results showed that TC14012 inhibited ERK pathway at 10 min in wild type cells and this inhibition effect was reversed in CXCR7-transfected cells (Fig. 5.20. A & B). Also, human I-TAC caused the stimulatory effect on ERK1/2 pathway at 10 min in wild type cells and this activation effect was blocked in CXCR7-transfected cells (Fig. 5.20. A & B). These results clearly indicate that CXCR7 is not just a scavenger receptor as proposed in the literatures. Instead, it can induce cell signaling, but in a ligand-dependent biased mechanism.

3.21. The Ligand-Biased Signaling Patterns in WT Raw 264.7 Cells versus CXCR7-Transfected Raw 264.7 Cells.

The same procedure was also performed in our wild type EA.hy 926 cells and CXCR7-transfected EA.hy 926 cells. The results showed that SDF-1 caused inhibition effect on AKT pathway in wild type cells but not in CXCR7-transfected cells. TC14012 and VUF11207

inhibited AKT pathway, but not in wild type cells (Fig. 5.21. A & B). TC14012 induced ERK activation and VUF11207 caused ERK inhibition in CXCR7-transfected cells, but not in wild type cells (Fig. 5.21. A & B). Combined with the data in EA.hy 926 cells, we can draw a conclusion that CXCR7 can independently induce cell signaling, not only in a ligand-dependent biased mechanism, but also in a cell-dependent biased mechanism.

Chapter 4. Discussion

Previously in our lab we successfully detected the expression of CXCR7 in macrophage-positive areas of mouse aortic atheroma and the induction of CXCR7 during monocyte-to-macrophage differentiation, leading to a switch of SDF-1 signaling profile and increased macrophage migration/phagocytosis [62]. However, most previous studies only focus on the interactions between SDF-1 and CXCR7 although there are a series of ligands which bind to CXCR7. Since biased signaling is a complex phenomenon and it exists to a large degree in the chemokine system, more CXCR7 ligands need to be investigated [49]. Biased signaling can occur in different forms, signaling can vary with the ligand [183], with the receptor [33] and with the tissue/cell [184]. Here for the first time we screened a serious of CXCR7 ligands and found that activation of CXCR7 by TC14012 inhibits ERK1/2 signaling pathway in HCAEC and in CXCR7-transfected 1321N1 cells. Furthermore, since global CXCR7-knock out mice showed postnatal lethal phenotype [173], we used Cre-loxP system to build up myeloid-specific CXCR7 knock out mice. To our big surprise, we found that CXCR7 is upregulated in macrophages of mice with partial-deletion of CXCR7 genes, possibly through a compensatory mechanism. In addition, our data indicate that CXCR7 expression inhibits ERK1/2 and AKT activations, leading to suppression of cell proliferation in EA.hy 926 and Raw 264.7 cells. Also, a series of CXCR7 endogenous and exogenous ligands were tested for their effects on multiple cellular signaling pathways, these results clearly indicate that CXCR7 is not just a scavenger receptor as proposed in the literatures; instead, it can induce cell signaling, but in a time- and ligand-dependent biased mechanism.

The chemokine SDF-1 plays a very important role in the biology of leukocytes and stem/progenitor cells. The activity of the SDF-1 signaling pathway is crucial in neural, vascular, and cardiac development and craniofacial organogenesis. Initially it was thought that CXCR4 is the only receptor for SDF-1. However, the discovery of CXCR7 which binds to SDF-1 with an ever higher affinity made this situation more complex [36]. Since most previous studies only focus on the interactions between SDF-1 and CXCR7, other ligands of CXCR7 have been

ignored. Although all these ligands can bind to CXCR7, they can trigger totally different outcome, which raises another issue-biased cell signaling. In our present study, by using different CXCR7 ligands, we found TC14012 induced inhibition effect on ERK1/2 pathway in HCAEC. Further we confirmed that this is not a generalized toxic effect by cell viability assay. Next the Western blotting data helped us know the inhibition effect in HCAEC was not happened exactly on ERK1/2 but instead happened from c-Raf. Although CXCR4 is not the focus of this study, we have to keep in mind that TC14012 is not only a CXCR7 agonist but also a CXCR4 antagonist. Since in HCAEC, both expression of CXCR4 and CXCR7 were detected by Real-time PCR and Western blotting (data not shown), there is a possibility that the TC14012-induced inhibition effect on ERK1/2 is not through CXCR7 activation but through CXCR4 inactivation. To solve this problem, we used a series of CXCR4 antagonists in HCAEC and observed that only TC14012 caused ERK1/2 inhibition while other CXCR4 antagonists did not. Debate continues whether we used enough dosage of these CXCR4 antagonists to block CXCR4 in HCAEC or not. In this case, a human monocytic cell line called THP-1 cell, which express CXCR4 but not CXCR7, was chosen. Western blotting results showed that through using the same dosage, all of these CXCR4 antagonists except WZ811 were able to inhibit ERK1/2 pathway. To further confirm whether TC14012-induced inhibition effect is regulated through CXCR7 or not, we screened a series of cell lines and found one human astrocytoma cell line called 1321N1 cell. In previous studies, the cell line used for CXCR7 transfection study is HEK293 cell [182]. However, based on our data, this cell line naturally expresses both CXCR4 and CXCR7, which complicates data interpretation. Fortunately, 1321N1 cell line naturally expresses no CXCR4 or CXCR7, which indicates that this cell line is a better model for doing CXCR7 transfection study. After successfully transfected CXCR7 into 1321N1 cells, we screened a series of CXCR7 ligands and tested their effects in WT and CXCR7-transfected 1321N1 cells. We found that TC14012 only caused ERK1/2 inhibition in CXCR7-transfected cells but not in WT cells at the indicated time points. Thus, we provide string evidence indicating that TC14012-induced ERK1/2 inhibition effect is regulated through CXCR7.

Global knock of CXCR7 caused postnatal death phenotype in mice, which brings difficulties in studying the roles of CXCR7 in vivo [173]. These mice showed enlarged enlarged semilunar valves, occasional ventriculap septal defect (VSD) and overriding aorta, which indicates important roles of CXCR7 in cardiovascular system. To overcome this difficulty, we used Cre-

loxP system to obtain myeloid-specific knock out CXCR7 mice. This new line was viable, fertile, and did not show obvious gross physical or behavioral abnormalities. In our present study, we expect to see higher number of periphery blood monocytes in CXCR7-floxed$^{+/+}$/LyzM-Cre$^{+/+}$ mice compared with CXCR7-floxed$^{+/+}$/LyzM-Cre$^{-/-}$ mice, which can indicate a role of CXCR7 in monocytosis. However, to our surprise, the upregulated cell number not only happened in monocytes, but also in leukocytes, lymphocytes and neutrophils. Next we got shocked because CXCR7 mRNA and protein expression were not decreased but instead, they were dramatically upregulated in CXCR7-floxed$^{+/+}$/LyzM-Cre$^{+/+}$ mice compared with littermate CXCR7-floxed$^{+/+}$/LyzM-Cre$^{-/-}$ mice, both in bone marrow-derived macrophages and in peritoneal macrophages. We further demonstrated that upregulation of CXCR7 mRNA is not solely attributed to the disruption of LyzM gene by Cre insertion or the inclusion of floxed sequence to CXCR7 gene. Interestingly, this CXCR7 induction phenomenon in vivo can be mimicked in vitro through adenovirus delivery of Cre gene into CXCR7-floxed macrophages. Till now, there still remains a big question: did CXCR7 deletion truly happen in our CXCR7-floxed$^{+/+}$/LyzM-Cre$^{+/+}$ mice? If the deletion did not happen, all the previous data will meaningless. To answer this question, specific pairs of primers were designed and a series Real-time PCR were performed. PCR mapping indicated partial deletion of myeloid CXCR7 gene via the Cre-LoxP approach. Thus, we made one hypothesis that the induction of CXCR7 is through a potential compensatory mechanism. In addition, we observed injection of thioglycolate led to more macrophage infiltration into the peritoneum in CXCR7-floxed$^{+/+}$/LyzM-Cre$^{+/+}$ mice. It is imperative to further study the role of CXCR7 in macrophage biology.

Here we also show for the first time that transfection of CXCR7 into EA hy.926 and Raw 264.7 cells caused a much slower proliferation capacity. Furthermore, we have demonstrated that CXCR7 expression significantly suppressed cellular basal phosphorylation of AKT and ERK1/2, with no impact on JNK and p38. Both AKT and ERK1/2 are vital pathways in cell proliferation, we reveal a previously unknown function of CXCR7 in control of myeloid cell proliferation in vitro and in vivo and this may also help explain why global knock-out CXCR7 mice showed early-death phenotype with cardiac hypertrophy. Moreover, we screened a series of CXCR7 ligands and found that TC14012 showed inhibition effect on ERK1/2 pathway in wild type EA hy.296 cells, but not in CXCR7-transfected EA hy.296 cells. Also, human I-TAC induced a stimulatory effect on ERK1/2 pathway in wild type EA hy.296 cells, but not in CXCR7-

transfected EA hy.296 cells. In addition, we observed SDF-1 inhibited AKT pathway in Raw 264.7 wild type cells but not in Raw 264.7 CXCR7-transfected cells. TC14012 and VUF11207 inhibited AKT pathway in Raw 264.7 CXCR7-transfected cells, but not in Raw 264.7 wild type cells. TC14012 activated ERK1/2 and VUF11207 inhibited ERK1/2 in Raw 264.7 CXCR7-transfected cells, but not in Raw 264.7 wild type cells. To sum up, further studies about CXCR7 biased signaling are needed.

In summary, we report evidence that activation of CXCR7 by TC14012 inhibits ERK1/2 signaling pathway in HCAEC and in CXCR7-transfected 1321N1 cells. Moreover, CXCR7 is upregulated in macrophages of mice with partial-deletion of CXCR7 genes, possibly through a compensatory mechanism. Also, CXCR7 expression inhibits ERK1/2 and AKT activations, leading to suppression of cell proliferation in EA.hy 926 and Raw 264.7 cells. These findings indicate that CXCR7 is not just a scavenger receptor, but a receptor which can induce cell signaling.

For future studies, using endothelial specific knock out mice will help us better understand the role of CXCR7 in cardiovascular system.

Chapter 5. Figures and Legends

Figure 5.1. TC14012 induced ERK1/2 inhibition in endothelial cell.

Cellular phosphorylation and total levels of ERK in HCAEC were detected by standard Western blotting after the cells were stimulated by TC14012 for 40 min at the indicated concentrations (A) or after the cells were stimulated by TC14012 with 2×10^-5 M at the indicated time points (B).

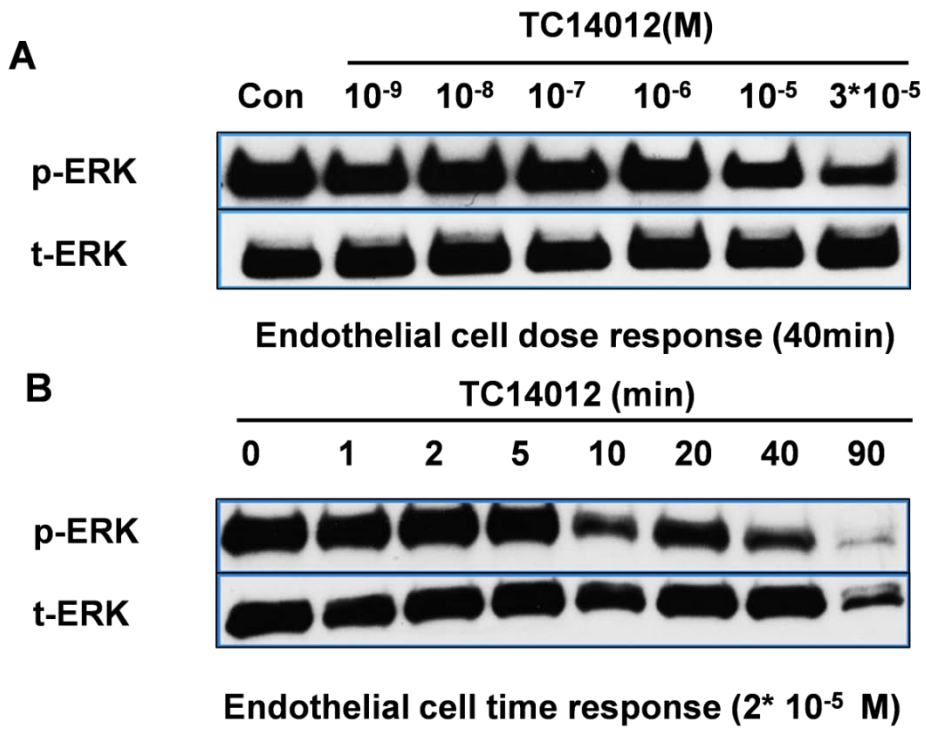


Figure 5.2. The inhibitory effect on ERK1/2 pathway induced by TC14012 is not a toxic effect.

Human coronary artery endothelial cells were seeded in 96-well plates at the same cell density. After 12 h starvation those cells were pre-treated with TC14012 (3×10^{-5} M), H_20_2 (10^{-3} M), and PKC inhibitor (3×10^{-5} M) for 5 min and then resazurin was added directly to cultured cells in serum-supplemented medium to reach a final concentration of 10%. The Relative Fluorescence Unit of cells was detected after 4h-stimulation. N.S. stands for statistic non-signaficance. (n=4)

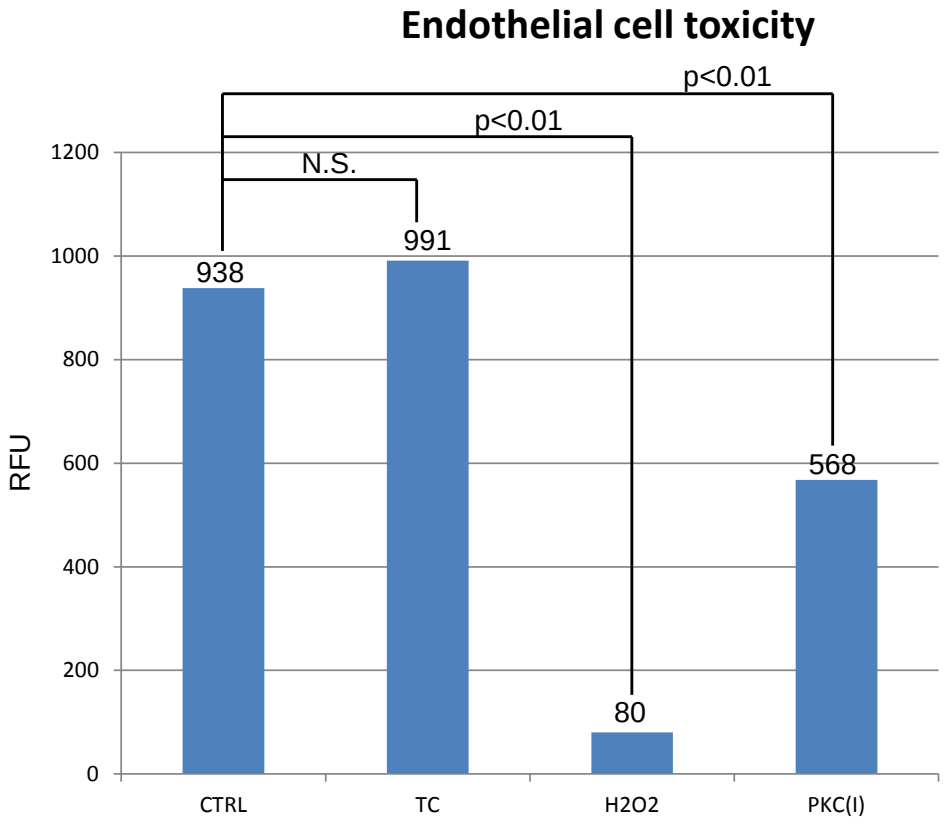


Figure 5.3. The stimulatory effects of SDF-1, UTP and VEGF on ERK1/2 pathway can be blocked by TC14012 in endothelial cell.

Cells were all seeded in 6-well plates. All Cells were pretreated with TC14012 for 30 min before stimulation. Then FBS, UTP, SDF-1, VEGF were added for 10 min stimulation. p-ERK and total ERK were detected by standard Western blotting. **A**. Control, TC14012 (2×10^{-5} M), 10%FBS, 10%FBS+ TC14012 (2×10^{-5} M), UTP (10^{-4}M), UTP (10^{-4}M) + TC14012 (2×10^{-5} M). **B**. Control, TC14012 (2×10^{-5} M), SDF-1 (100ng/ml), SDF-1 (100ng/ml) + TC14012 (2×10^{-5} M), UTP (10^{-5}M), UTP (10^{-5}M) + TC14012 (2×10^{-5} M). C. Control, TC14012 (2×10^{-5} M), VEGF (3ng/ml), VEGF (3ng/ml) + TC14012 (2×10^{-5} M), UTP (3×10^{-6}M), UTP (3×10^{-6}M) + TC14012 (2×10^{-5} M).

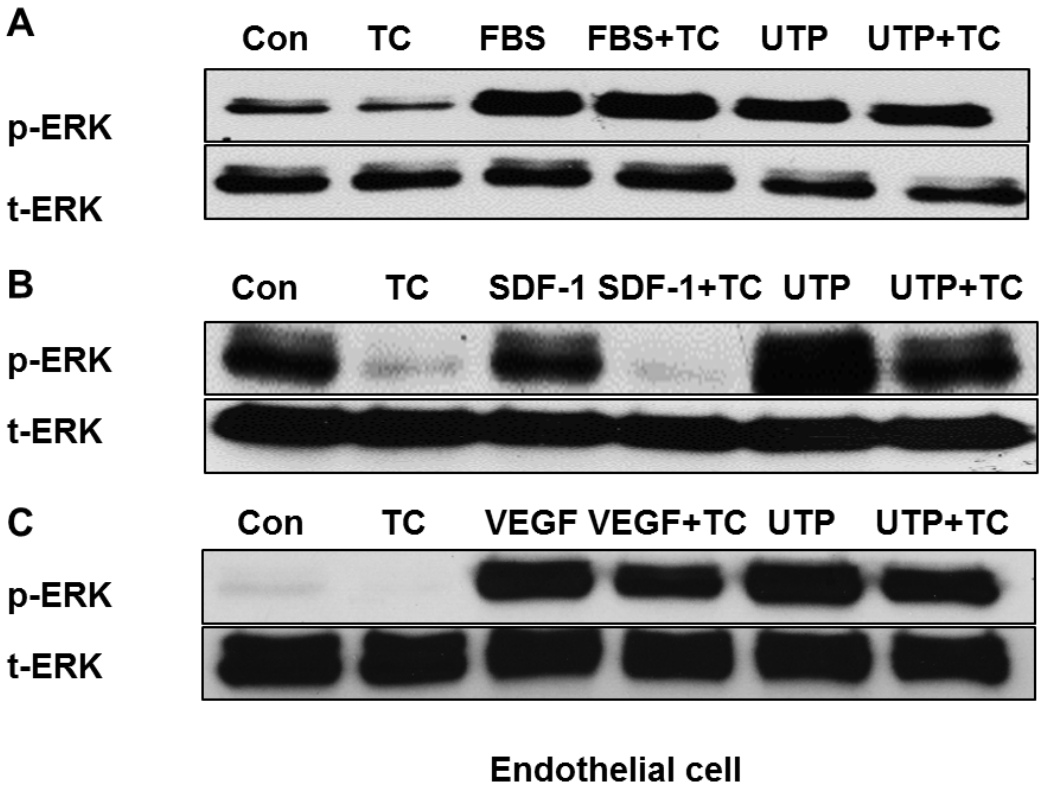


Figure 5.4. The upstream kinases of ERK1/2 can be blocked by TC14012 in endothelial cell.

Cells were seeded in 6-well plates and starved overnight. Then, cellular phosphorylation levels of MEK, c-Raf were detected by standard Western blotting in human coronary artery endothelial cells. β-Tubulin was used as loading control, cells were stimulated by different reagents for 40 min. Control, TC14012 (20 uM), SDF-1 (100 ng/ml), SDF-1 (100 ng/ml) + TC14012 (20 uM).

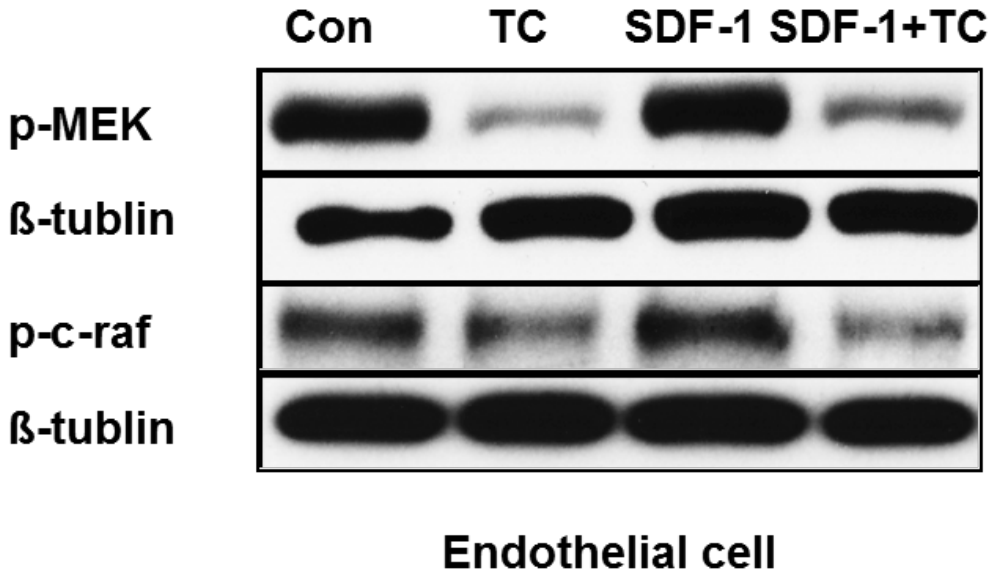


Figure 5.5. The effect of TC14012 and other CXCR4 antagonists on ERK1/2 pathway in endothelial cells and THP-1 cells.

Cells were all seeded in 6-well plates, for HCAEC, cells were starved overnight; for THP-1 cells, they were starved for 4 h. All Cells were pretreated with CXCR4 antagonists for 30 min before stimulation. Then SDF-1 was added for 10 min stimulation. p-ERK and total ERK were detected by standard Western blotting in human coronary artery endothelial cells and THP-1 cells. Control, TC14012 (20uM), AMD3100 (10uM), AMD3645 (10uM), IT1t (1uM) and WZ811 (10uM) (A). Control, SDF-1(100ng/ml), SDF-1+AMD3100 (10 uM), SDF-1+AMD3645 (10 uM), SDF-1+IT1t (10uM), SDF-1+WZ811 (10uM), SDF-1+TC14012 (20uM) (B).

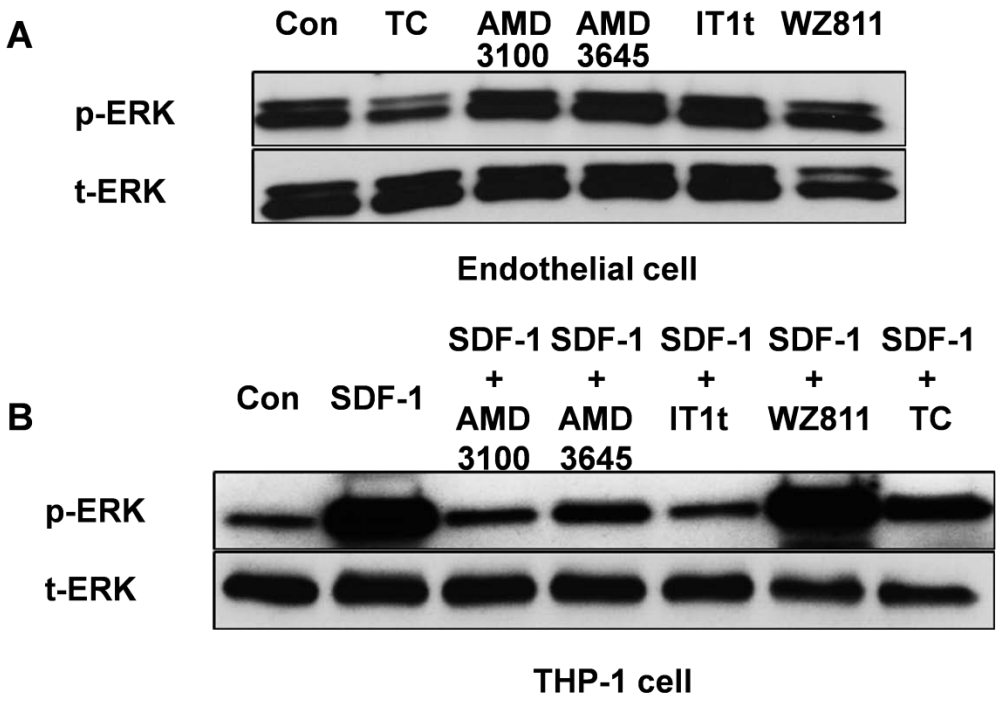


Figure 5.6. Evidence of CXCR4 and CXCR7 expression in HEK293 and 1321N1 cell.

Both expression level of CXCR4 and CXCR7 were detected by Real-time PCR in HEK293 and 1321N1cells (RT+: with reverse transcriptase; RT-: without reverse transcriptase).

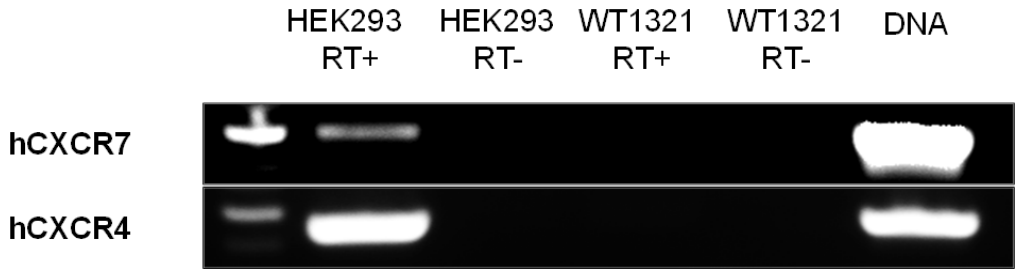
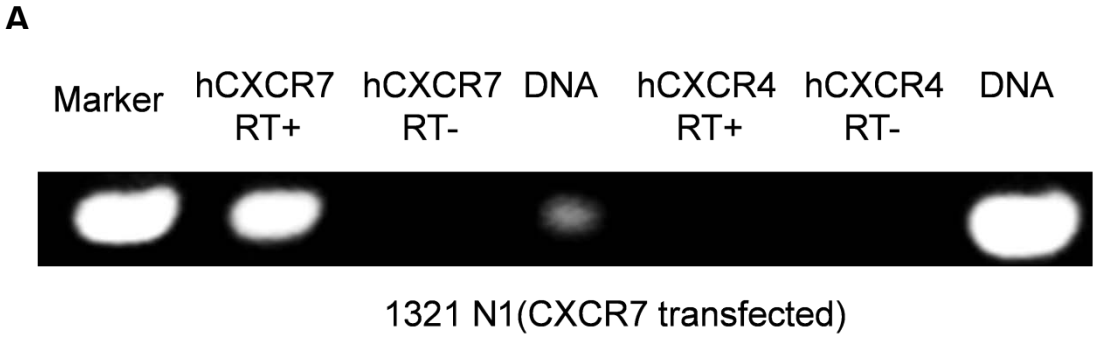
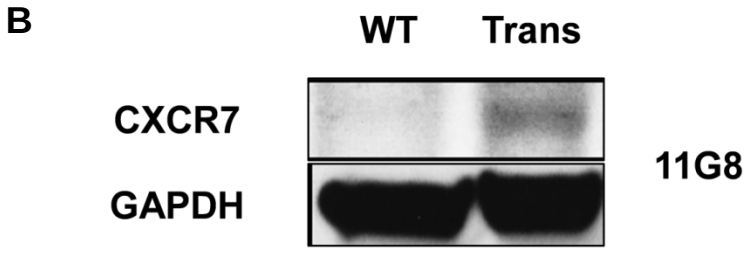


Figure 5.7. The expression of CXCR4 and CXCR7 in WT and CXCR7-transfected 1321N1 cells.

The mRNA expression level of CXCR4 and CXCR7 were determined by Real-time PCR (A) and cellular CXCR7 protein was detected by standard Western blotting using two different mouse anti-human CXCR7 monoclonal antibodies: clone 11G8 and clone 9C4 (B & C).





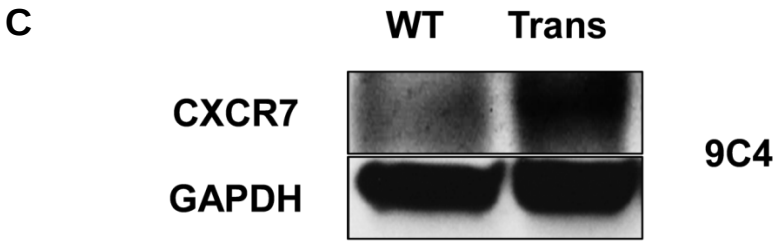


Figure 5.8. ERK1/2 was inhibited by TC14012 in CXCR7-transfected 1321N1 cells but not in WT 1321N1 cells.

p-ERK, total ERK and β-tublin were detected by standard Western blotting. Control, TC14012 (2×10^{-5} M), SDF-1(100ng/ml), I-TAC (300ng/ml) and VUF11207 (10^{-5} M). Cells were stimulated at the indicated time points: 10min (A), 20min (B) and 40min (C).

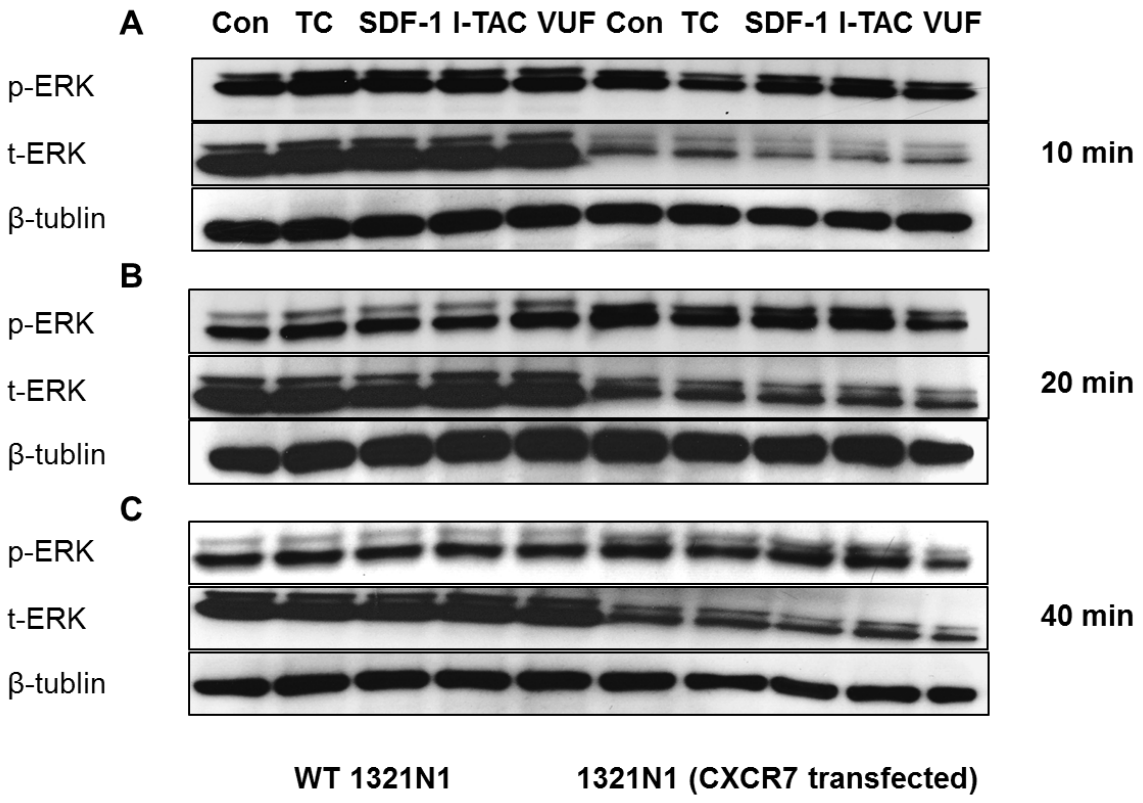


Figure 5.9. Generation of myeloid-specific deletion of CXCR7 and wild type mice.

Both C57BL6J homozygous CXCR7-Flox$^{+/+}$/Cre$^{-/-}$ and Flox$^{-/-}$/LyzM-Cre$^{+/+}$ mice were bought from Jackson Lab. The breeding process to obtain homozygous Flox$^{+/+}$/LyzM-Cre$^{+/+}$ and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice was laid out (A) and a schematic description of Cre-loxP based myeloid cell specific knock out of CXCR7 in mice was shown (B).

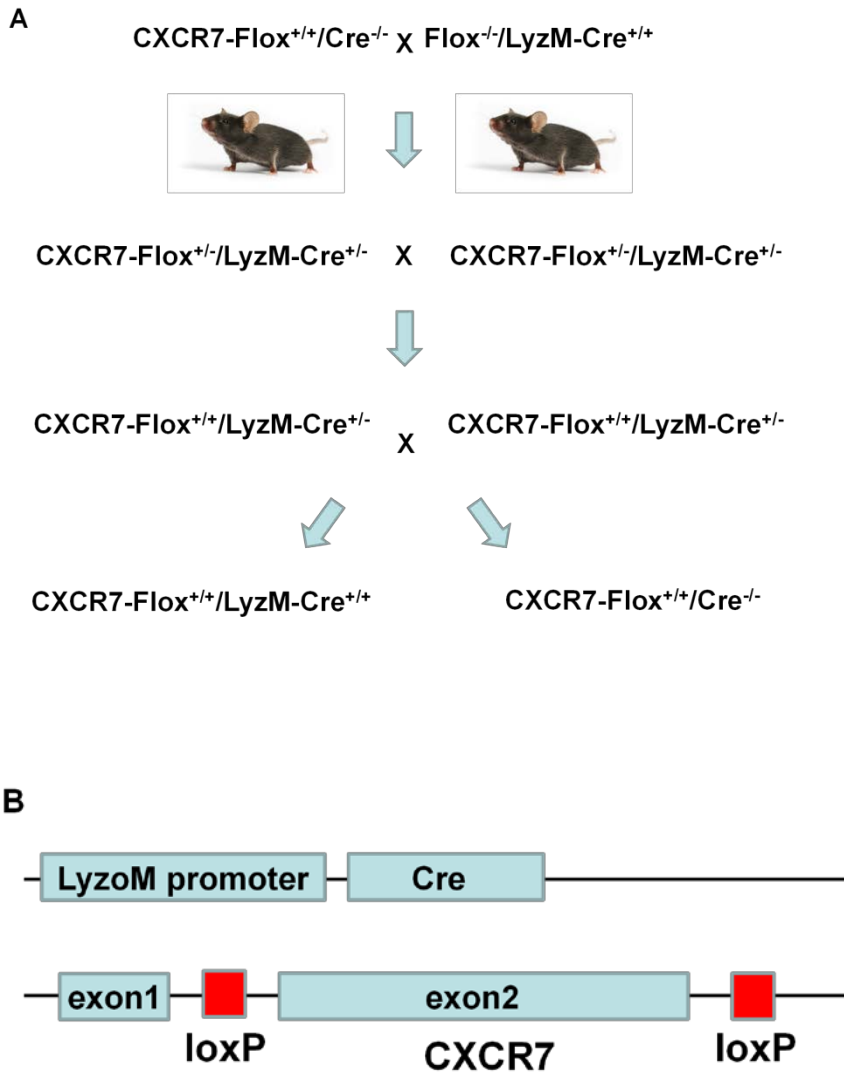


Figure 5.10. Myeloid-specific CXCR7 deletion did not show obvious physical abnormalities.

To investigate whether myeloid specific knock-out CXCR7 will cause any physical abnormalities in mice models or not, both body (A) and heart weight (B) were measured both in $Flox^{+/+}$/LyzM-$Cre^{+/+}$ and $Flox^{+/+}$/LyzM-$Cre^{-/-}$ mice groups.

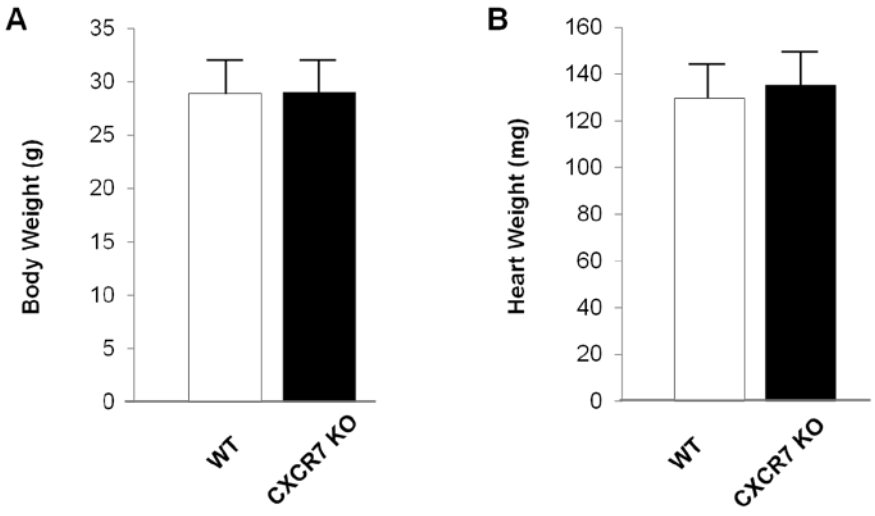


Figure 5.11. Measurement of mice periphery blood leukocytes.

Periphery blood cells were isolated from Flox$^{+/+}$/LyzM-Cre$^{+/+}$ and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice respectively and the number of leukocytes (A), lymphocytes (B), neutrophils (C) and monocytes (D) were counted with an automated cell counter. (n=7, * p<0.05).

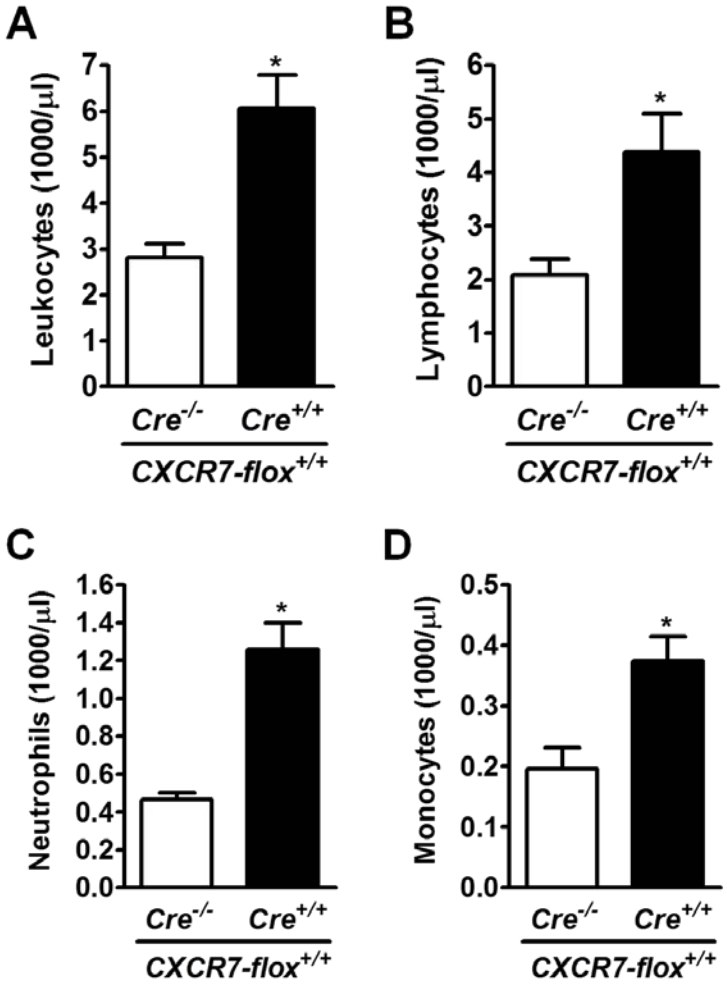


Figure 5.12. CXCR7 mRNA and protein expression in Flox$^{+/+}$/LyzM-Cre$^{+/+}$ mice versus Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice.

The mRNA expression of CXCR7 was determined by Real-time PCR both in mice peritoneal macrophages (A) and bone marrow cells (B). Protein expression of CXCR7 was determined by standard Western blotting (C).

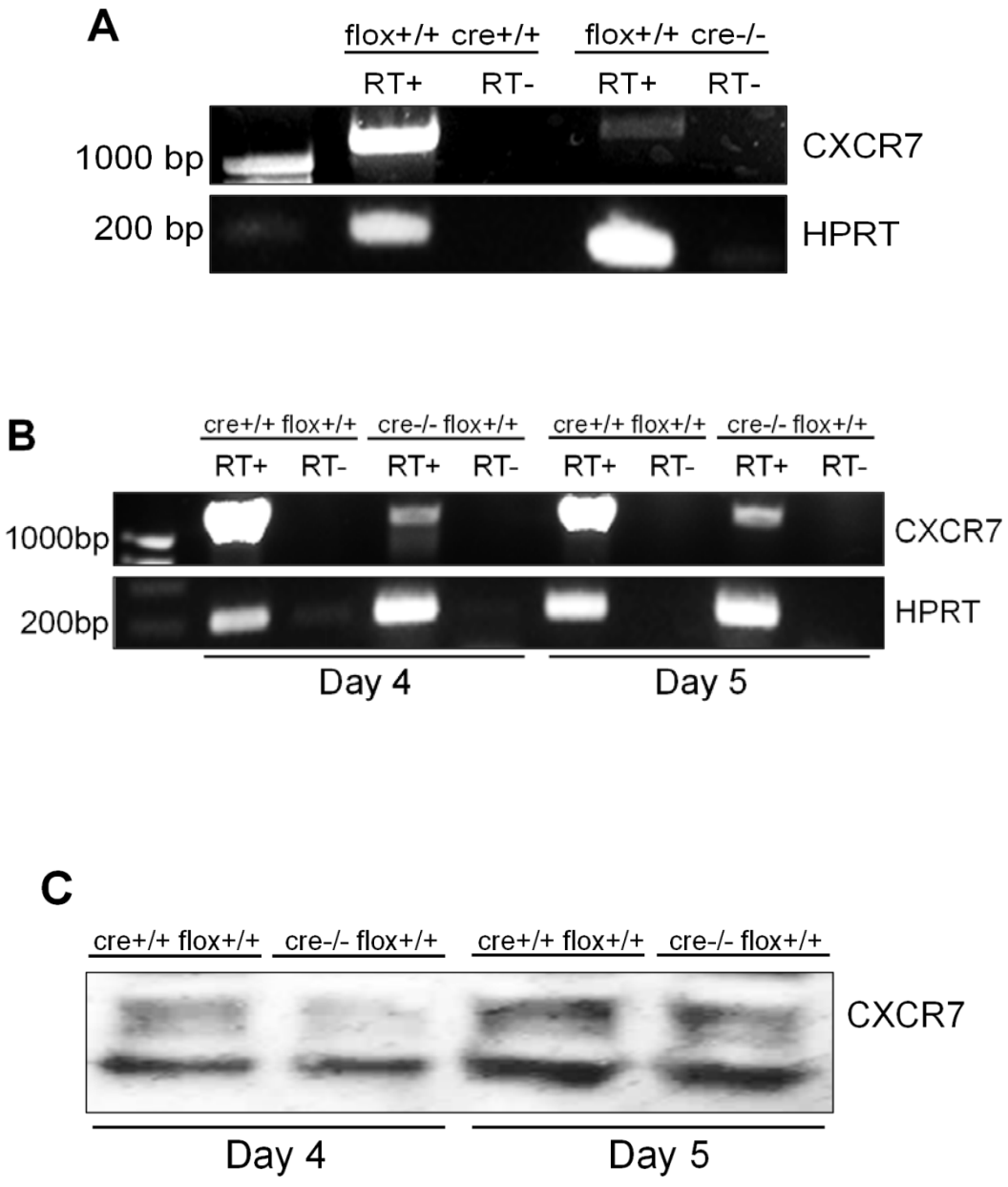


Figure 5.13. The effect of Cre insertion or Floxed sequence inclusion on upregulation of CXCR7.

The mRNA expression of CXCR7 from peritoneal macrophages was quantified by Real-time PCR both in $Flox^{-/-}$/LyzM-$Cre^{+/+}$ and $Flox^{-/-}$/LyzM-$Cre^{-/-}$ mice group (A). The same procedure was performed to determine mRNA level of CXCR7 from peritoneal macrophages (B) and bone marrow cells (C) in $Flox^{+/+}$/LyzM-$Cre^{-/-}$ and $Flox^{-/-}$/LyzM-$Cre^{-/-}$ mice group.

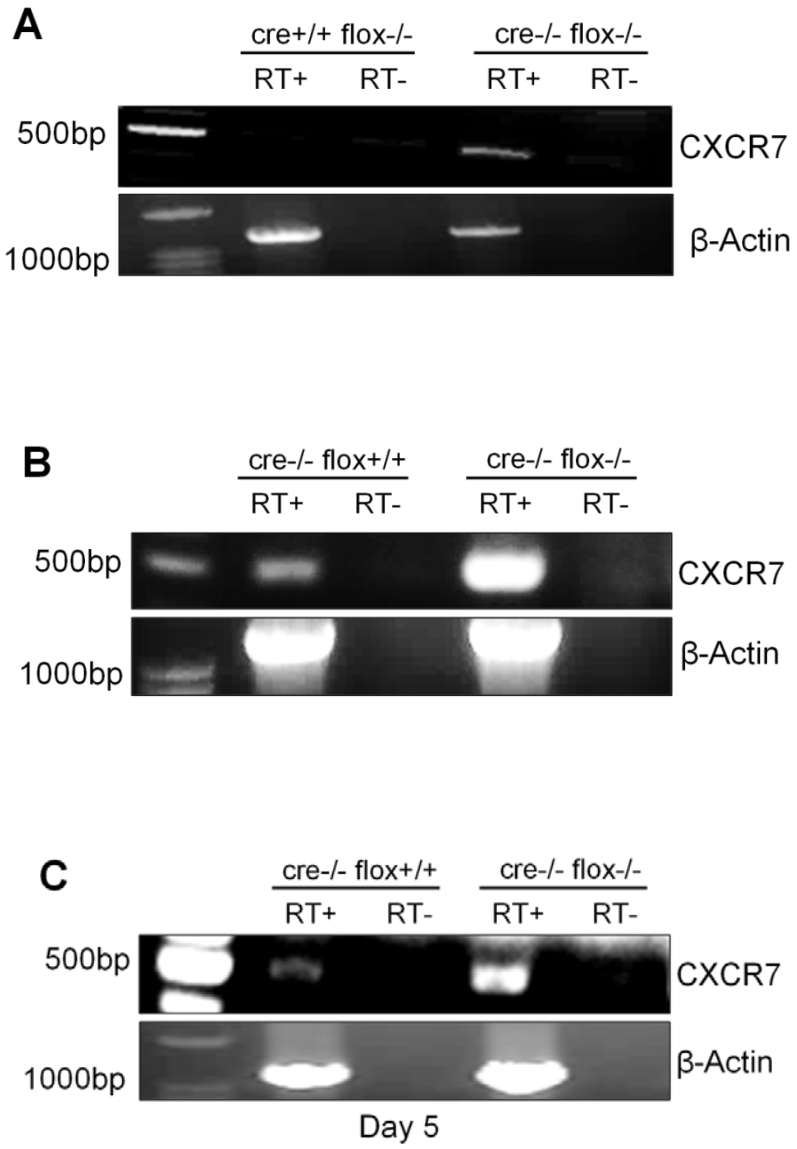
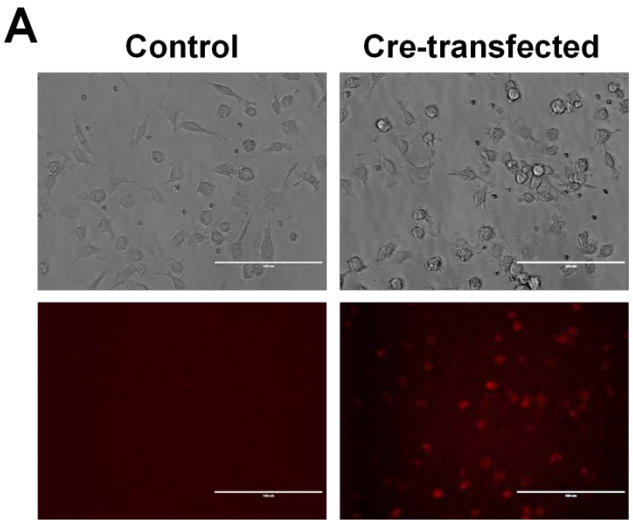


Figure 5.14. In vitro expression of CXCR7 with adenovirus delivery of the Cre gene into macrophages.

Cells isolated from mouse bone marrow were collected and seeded in 6-well plates. After 72 h of differentiation, 15 µl of premade adenovirus Cre (ViGene) and control RFP adenovirus (ViGene) were added separately. Images with fluorescent signals in random fields were acquired and captured using an AMG EVOS® digital inverted multi-functional microscope (AMG) (A). The expression level of CXCR7 from bone marrow cells was quantified by Real-time PCR (B).



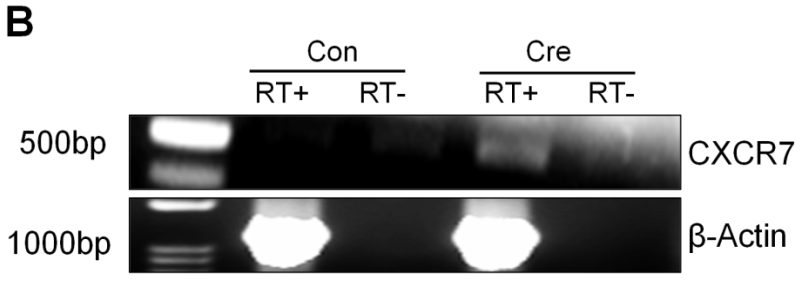
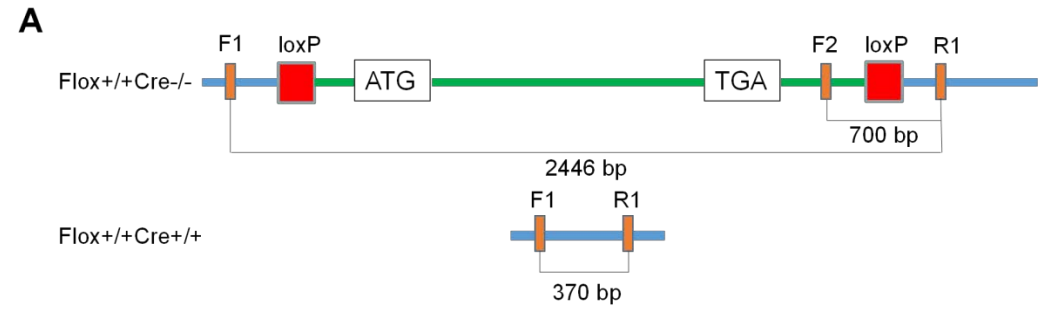
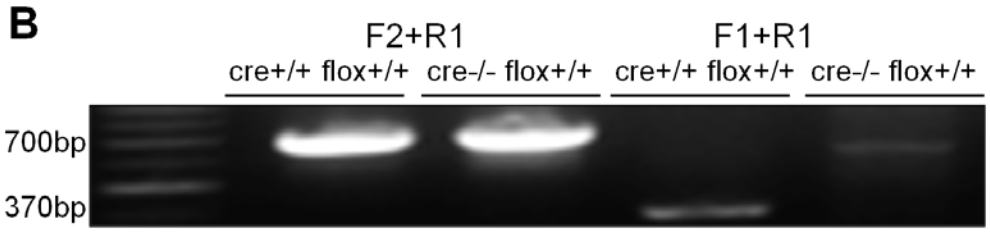
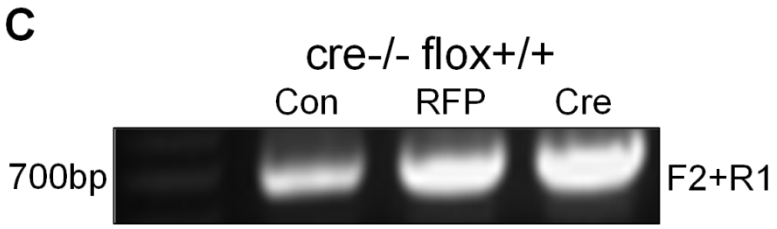


Figure 5.15. PCR mapping indicates partial deletion of CXCR7 in mice model.

Genomic DNA were isolated from Flox$^{+/+}$/LyzM-Cre$^{+/+}$ mice and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ separately. Specific pairs of primers were designed and a schematic description of these primers is shown (A). The certain 370bp-band and 700bp-band expression were detected by Real-time PCR (B). For Cre gene delivery through adenovirus in Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice, same procedure was performed (C & D).







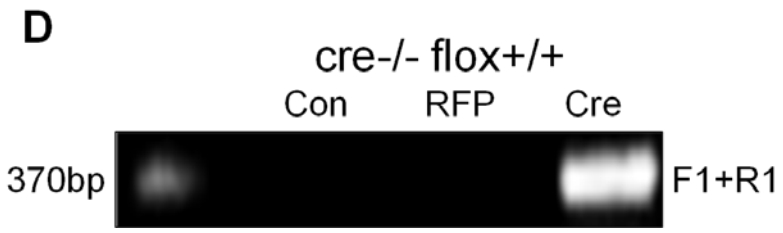

D
cre-/- flox+/+
Con
RFP
Cre
370bp
F1+R1

Figure 5.16. Different numbers of macrophages infiltrated into the peritoneum in Flox$^{+/+}$/LyzM-Cre$^{+/+}$ mice and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice.

Both Flox$^{+/+}$/LyzM-Cre$^{+/+}$ and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ mice were intraperitoneal injected with 2 ml of thioglycolate and waited till 72 h. Then macrophages were isolated from Flox$^{+/+}$/LyzM-Cre$^{+/+}$ mice and Flox$^{+/+}$/LyzM-Cre$^{-/-}$ groups and the numbers of these macrophages were counted separately. (n=5, * p<0.05)

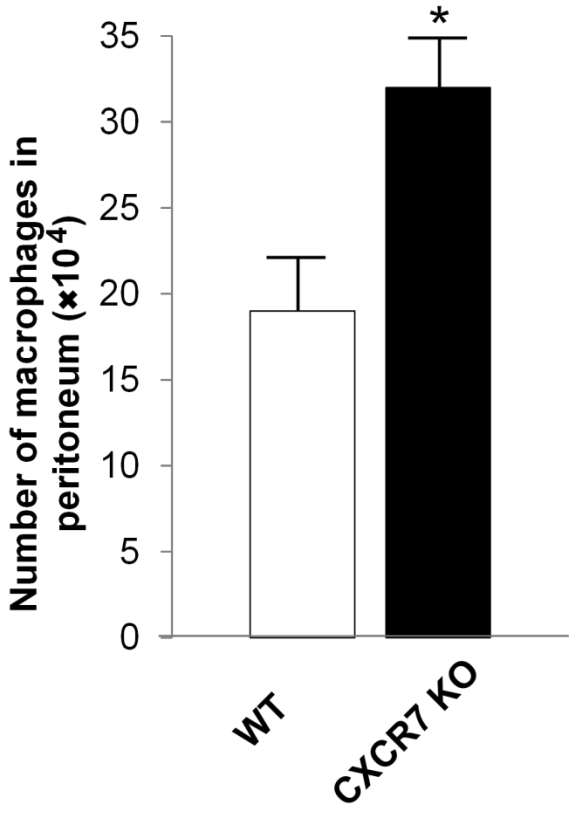


Figure 5.17. Expression of CXCR4 and CXCR7 in wild type and CXCR7-transfected cells.

The mRNA expression level of CXCR4 and CXCR7 were quantified by Real-time PCR both in wild type and CXCR7-transfected cells (A). The protein expression level of CXCR7 was determined by Western blotting both in wild type and CXCR7-transfected cells (B).

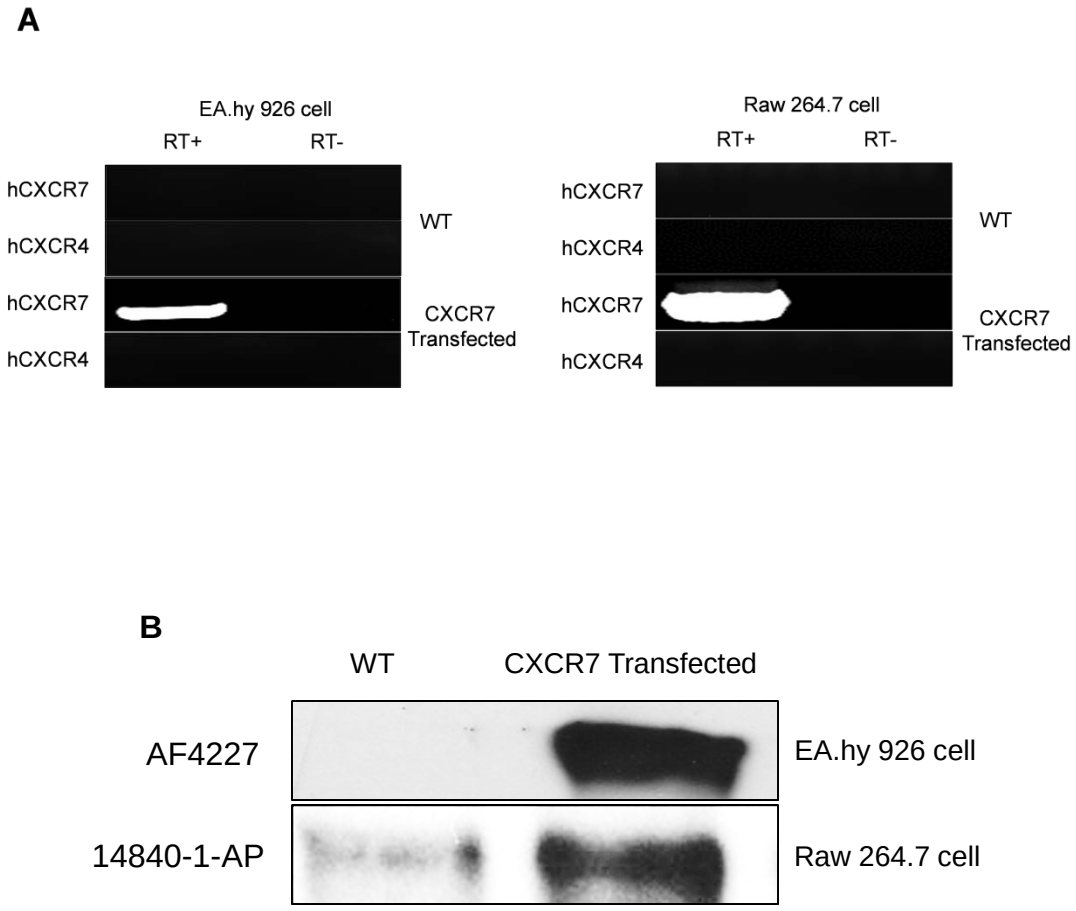


Figure 5.18. CXCR7 inhibited cell proliferation.

Cell proliferation rate was determined by cell counting kit in wild type and CXCR7-transfected cells. OD values were measured by microplate reader at 450 nm every 0.5 h and up to 4h (A & B). (n=4, * p<0.05)

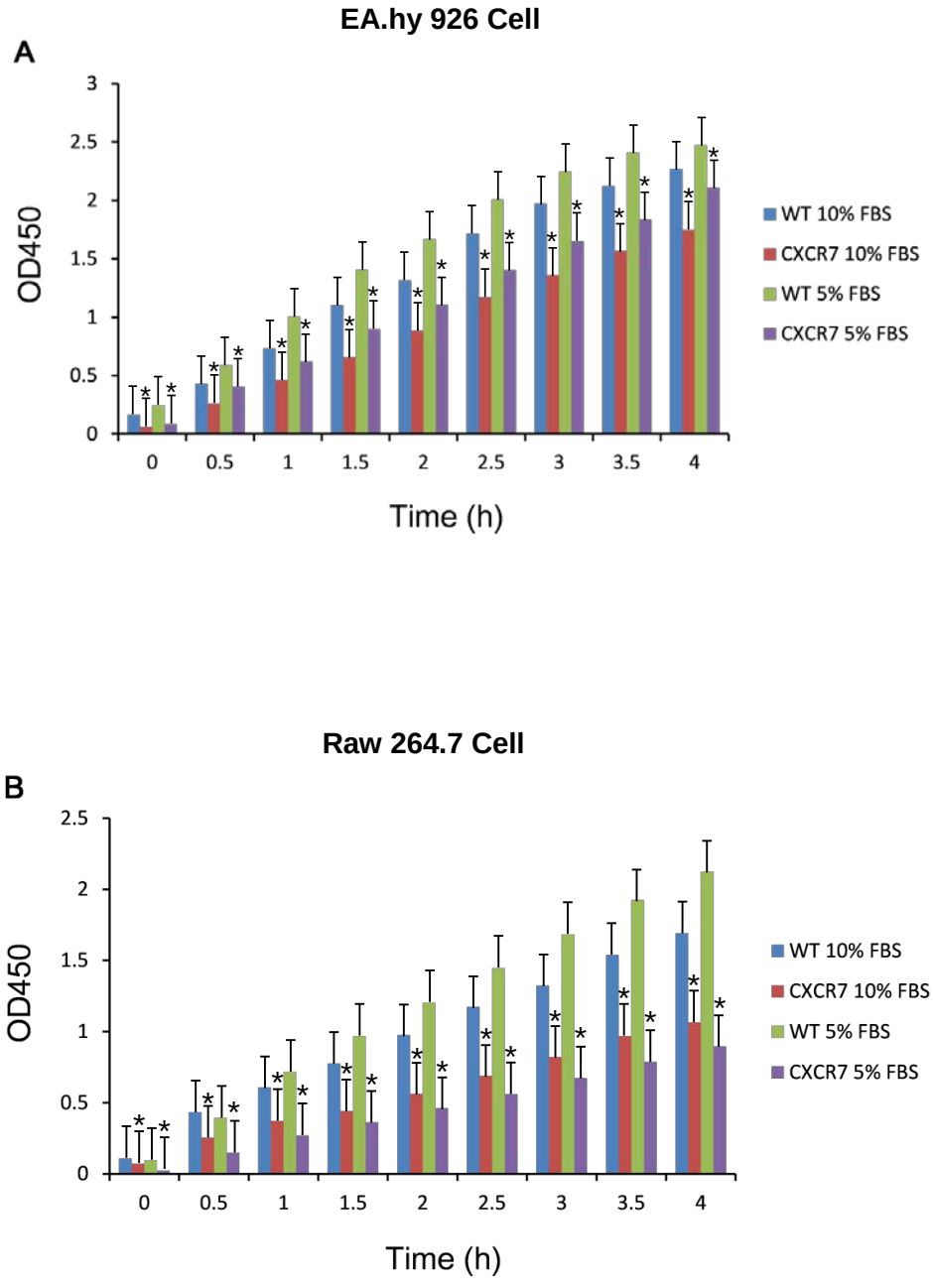
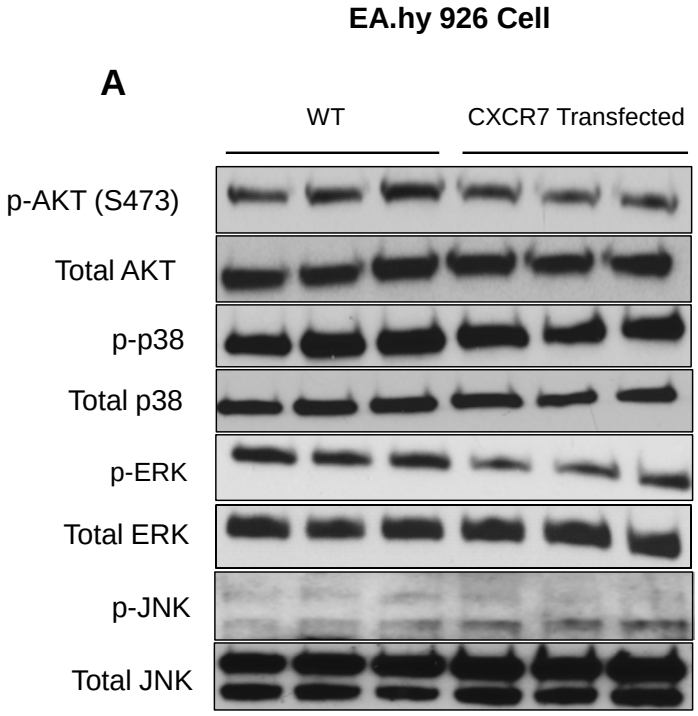


Figure 5.19. CXCR7 induced AKT and ERK1/2 inhibition.

Cells were seeded in 6-well plates and starved overnight without any treatment of reagents. Cellular phosphorylation levels of AKT, p38, ERK1/2, JNK and their total kinase levels were determined by Western blotting in wild type and CXCR7-transfected cells (A & B).



Raw 264.7 Cell

B

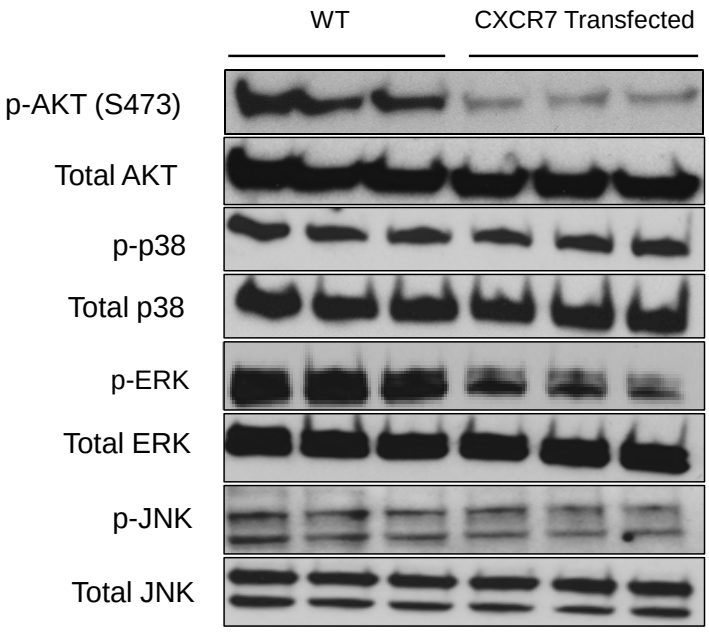
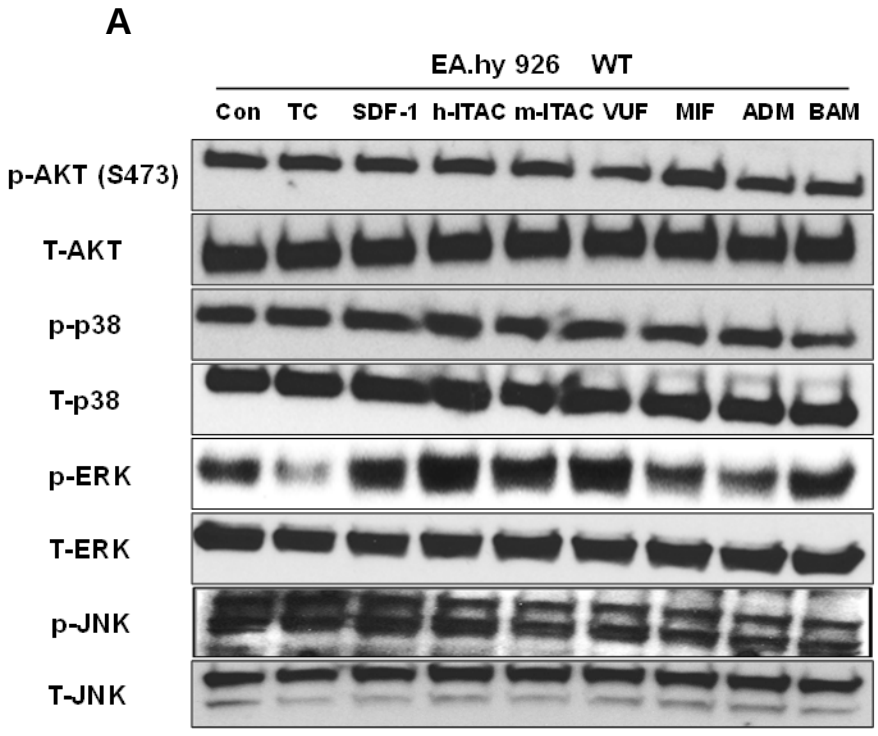


Figure 5.20. The effects of different CXCR7 ligands on EA hy.926 cells.

Both WT and CXCR7-transfected cells were seeded in 6-well plates at the same density. After overnight starvation, cells were stimulated with the following CXCR7 ligands for 10 min: TC14012 (20 μM), SDF-1 (100 ng/ml), h-ITAC (300 ng/ml), m-ITAC (300 ng/ml), VUF11207 (10^-5 M), MIF (100 ng/ml), ADM (10^-7 M), BAM22 (3×10^-6 M). Then Cellular phosphorylation levels of AKT, p38, ERK1/2, JNK and their total kinase levels were determined by Western blotting.



B

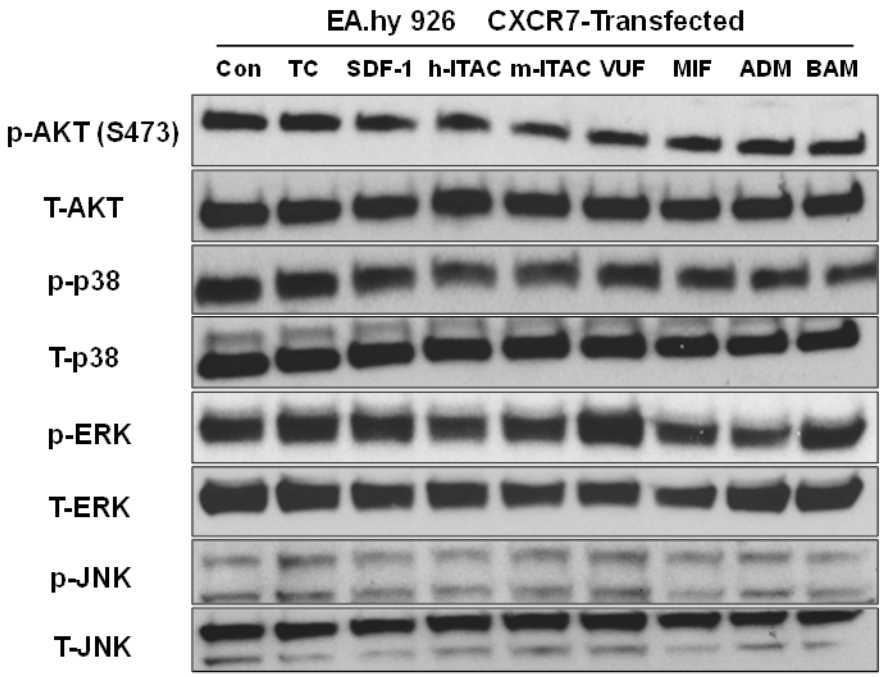
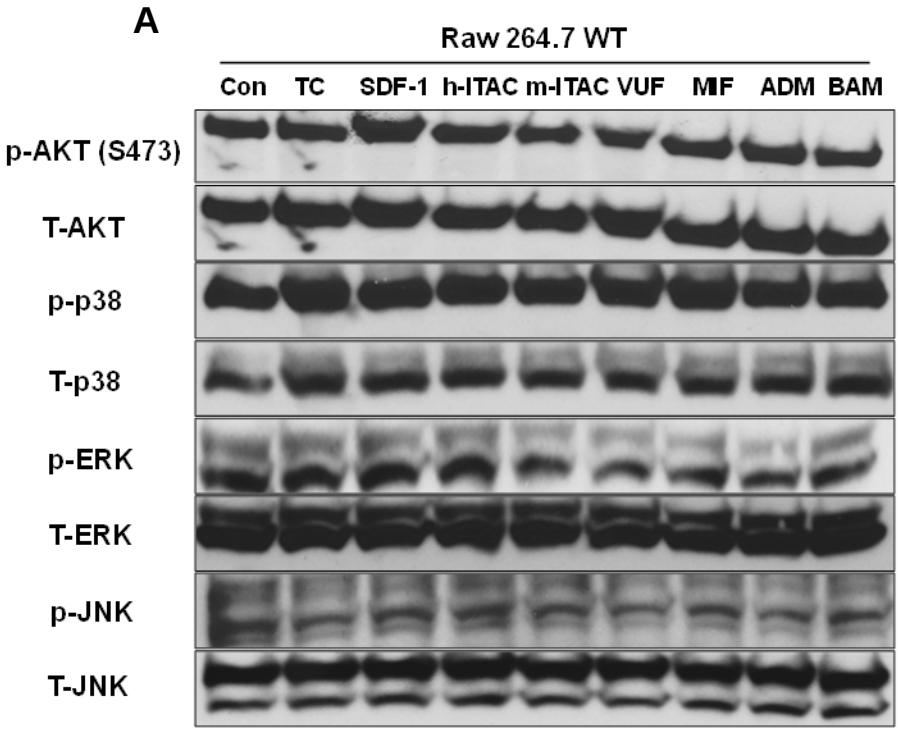
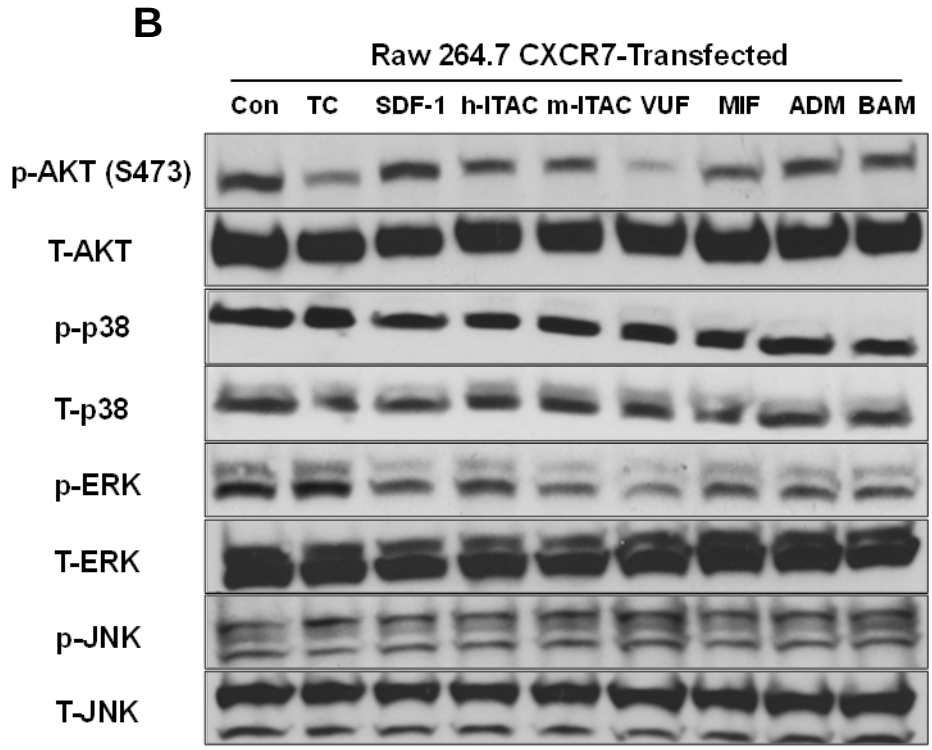

EA.hy 926 CXCR7-Transfected
Con
TC
SDF-1
h-ITAC
m-ITAC
VUF
MIF
ADM
BAM
p-AKT (S473)
T-AKT
p-p38
T-p38
p-ERK
T-ERK
p-JNK
T-JNK

Figure 5.21. The effects of different CXCR7 ligands on Raw 264.7 cells.

Both WT and CXCR7-transfected cells were seeded in 6-well plates at the same density. After overnight starvation, cells were stimulated with the following CXCR7 ligands for 10 min: TC14012 (20 μM), SDF-1 (100 ng/ml), h-ITAC (300 ng/ml), m-ITAC (300 ng/ml), VUF11207 (10^-5 M), MIF (100 ng/ml), ADM (10^-7 M), BAM22 (3×10^-6 M). Then Cellular phosphorylation levels of AKT, p38, ERK1/2, JNK and their total kinase levels were determined by Western blotting.




B
Raw 264.7 CXCR7-Transfected
Con
TC
SDF-1
h-ITAC
m-ITAC
VUF
MIF
ADM
BAM
p-AKT (S473)
T-AKT
p-p38
T-p38
p-ERK
T-ERK
p-JNK
T-JNK

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