Role of CXCR7 in Human Coronary Artery Smooth Muscle Restenosis

by

Thamer Alqurashi

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Approved by

Jianzhong Shen, Chair, Associate Professor, Drug Discovery and Development
Vishnu Suppiramanniam, Professor, Drug Discovery and Development
Murali Dhanasekaran, Professor, Drug Discovery and Development
Peter Panizzi, Associate Professor, Drug Discovery and Development
Chengming Wang, Professor of Veterinary Pathobiology

Abstract

Objective—Whether CXCR7 is a signaling or non-signaling scavenger receptor is still in debate. Our previous study showed that during monocyte-to-macrophage differentiation, CXCR7 mediates positive signaling to the p38 and JNK, but not the ERK1/2 signaling pathways. Here, we studied the expression and signaling function of CXCR7 in human coronary artery smooth muscle cells (HCASMC).

Methods and Results—Real-time RT-PCR analysis detected medium level of CXCR7 mRNA expression in HCASMC, which was significantly upregulated by TNFα stimulation. Ligand screening assay found that among all the commercially available CXCR7 ligands, TC14012 was unexpectedly found to inhibit the phosphorylation of ERK1/2 in a time- and dose-dependent manner. This new ERK1/2-inhibitory effect of TC14012 was not due to cell toxicity, and it was not mimicked by CXCR4-selective antagonists, including AMD3100, AMD3645 and IT1t. Since TC14012 is a CXCR4 antagonist and CXCR7 agonist, our data suggest a potential role of CXCR7 in the negative signaling to the ERK1/2 pathway induced by TC14012 in HCASMC. This was supported by the fact that when CXCR4 was blocked by TC14012, SDF-1 switched signaling property from ERK1/2-stimulatory into ERK1/2-inhibitory effect. In addition, we found that TC14012 also suppressed the basal and SDF-1-induced phosphorylation of MEK1, a direct upstream kinase for ERK1/2. Furthermore, we found that TC14012 dose-dependently inhibited HCASMC proliferation induced by 5% FBS, consistent with the well-known cellular function of EKR1/2 signaling pathway.

Conclusions—Selective activation of CXCR7 by TC14012 mediates a novel negative signaling to the ERK1/2 MAPK pathway, leading to a suppression of HCASMC proliferation. The finding highlights that CXCR7 may be a new drug target in combating stenosis or re-stenosis coronary artery diseases.

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CVDs	Cardiovascular diseases
ACR	Angiotensin-Converting Enzyme
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
MMPs	Matrix Metalloproteinases
FFP	Farnesyl Pyrophosphate
GGPP	Geranylgeranyl Diphosphate
PCI	Percutaneous Coronary Intervention
BMS	Bare Metal Stent
DES	Drug Eluting Stent
SMCs	Smooth Muscle Cells
PDGF	Platelet-Derived Growth Factor
MAPK	Mitogen-Activated Protein Kinase
ERK	Extracellular Signal-Regulated Kinase
GPCRs	G protein-Coupled Receptors
CXCR7	C-X-C Chemokine Receptor type 7
CGRP1	Calcitonin Gene-Related Peptide
VIP	Vasoactive Intestinal Peptide
SDF1	Stomal Cell-Derived Factor 1

CXCL11	C-X-C Motif Chemokine 11
PBSF	Pre-B-Cell Growth Stimulatory Factor
PIP2	Phosphatidylinositol 4,5-bisphosphate
DAG	Diacylglycerol
IP3	Inositol 1,4,5-trisphosphate
PI3K	Phosphoinositide 3-Kinase
I-TAC	IFN-inducible T cell α-chemoattractant
GBM	Glioblastomas
EPCs	Endothelial Cell Progenitors
HCASMCs	Human Coronary Artery Smooth Muscle Cells
ATCC	American Type Culture Collection
FBS	Fetal Bovine Serum
TBS-T	Tris-Buffered Saline and Tween 20
BSA	Bovine Serum Albumin
PVDF	Polyvinylidene Difluoride
CCK-8	Cell Counting Kit-8

Chapter 1. Introduction

1.1 Cardiovascular diseases (CVDs):

CVDs are the leading cause of death worldwide with 17.3 million deaths per year. This number is expected to grow to more than 23.6 million by 2030 (Association, 2016). They include all heart and blood vessel malfunctions. Heart diseases include cardiomyopathy, hypertension, heart failure, pulmonary heart disease, congenital heart disease, rheumatic heart disease, and valvular heart disease. For diseases that involve blood vessel malfunctions include coronary and peripheral artery diseases, cerebrovascular diseases, renal artery stenosis, and aortic aneurism. Risk factors of cardiovascular diseases include gender, age, lack of physical activity, smoke, obesity, high fat diet, alcohol, hypertension, diabetes, and family inheritance (Institut of Medicine, 2010; Kannel & McGee, 1979; Suls & Bunde, 2005).

1.2 Atherosclerosis:

Atherosclerosis is a term that is derived from the Greek word hardening paste. Atherosclerosis is narrowing of the artery due to the accumulation of lipids, such as cholesterol and triglyceride. The complications of atherosclerosis is considered the main contributor of CVDs (Libby, 2002; Swirski & Nahrendorf, 2013). These complications are coupled with stenotic or non-stenotic occlusions as shown in figure 1. The stenotic occlusion is associated with less compensatory expansion of the artery, thus narrowing of the lumen. As a result, inadequate blood flow occurs to the supplied organs, showing symptoms of stable angina, which can be clearly detected by perfusion scan. On the other hand, non-stenotic is more common than stenotic occlusions. They are associated with compensatory artery expansion with larger lipid core and thinner fibrous cap, which make

it more susceptible for rupture. Non-stenotic occlusion is mainly asymptomatic. for many years. Once the lesion is ruptured, thrombogenic materials will be exposed to the circulated blood, resulting in the formation of a blood clot and cause acute coronary events, such as unstable angina, myocardial infarction, and stroke (Libby, 2002; Nakazawa et al., 2011). Many studies revealed that non-stenotic occlusion atherosclerosis is the main cause of myocardial infarction.

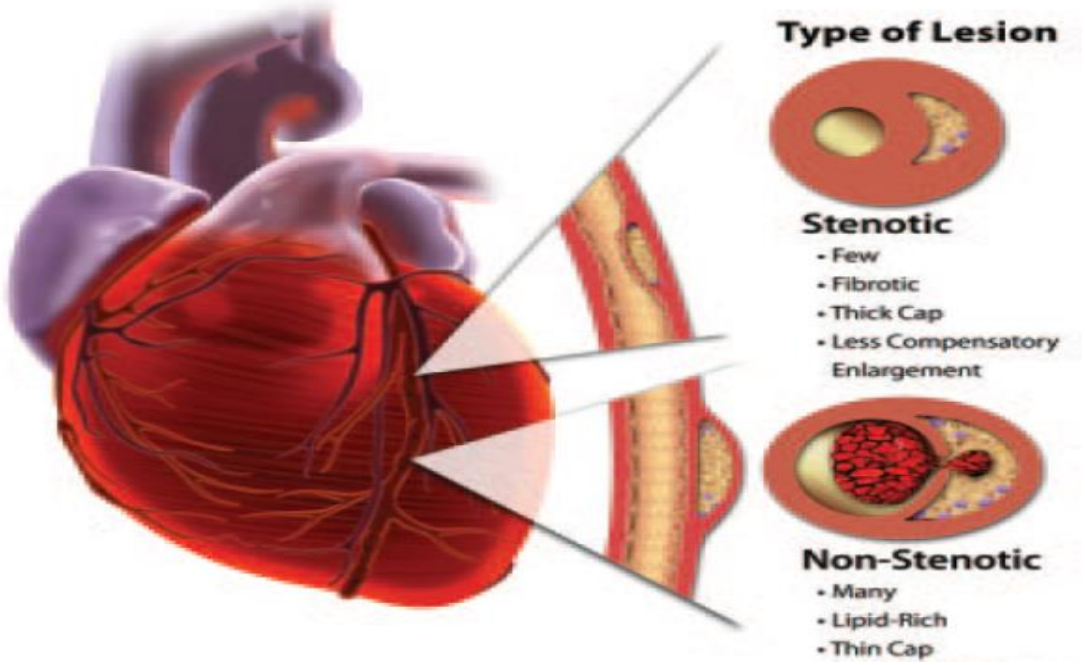

Figure 1. Pattern of atherosclerosis lesion types (Bassett et al., 2012). This diagram describes two morphologically different lesions in human coronary atherosclerosis, stenotic and nonstenotic lesions. Stenotic lesions are characterized by having smaller lipid cores, more fibrosis, calcification, thick fibrous caps, and less compensatory enlargement. On the other hand, nonstenotic lesions are more common and characterized by having large lipid cores and thin fibrous caps, which are prone to rupture.

1.3 Treatment of atherosclerosis:

Preventing is a crucial step to curing the disease. Controlling the risk factors will prevent or delay the incidence of atherosclerosis and its complications. The non-pharmacological approach is usually the first choice of treatment. This approach can be achieved by following certain actions, including healthy diet, physical activity, weight loss, and quitting smoking. In certain limit, conventional medications and surgical approaches are highly recommended. The pharmacological approach can be achieved via preventing platelets` aggregation in the narrowed artery by using anti-platelets medications, such as aspirin. Beta blockers, angiotensin-converting enzyme (ACR) inhibitors, calcium channel blockers, and diuretics are in some cases recommended to decrease heart rate and thus reduce the risk of heart attack and arrythmia. Currently, the most common prescribed medication that has been used to prevent and treat atherosclerosis is a group of medications known as statins.

Statins lower the cholesterol synthesis by inhibiting the conversion of acetoacetyle-CoA and acetyl-CoA to Mevalonate by inhibiting the activity of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase enzyme. This enzyme is the first committed step in the process of mevalonate pathway in which the production of the endogenous cholesterol biosynthesis is taking place as illustrated in figure 2.

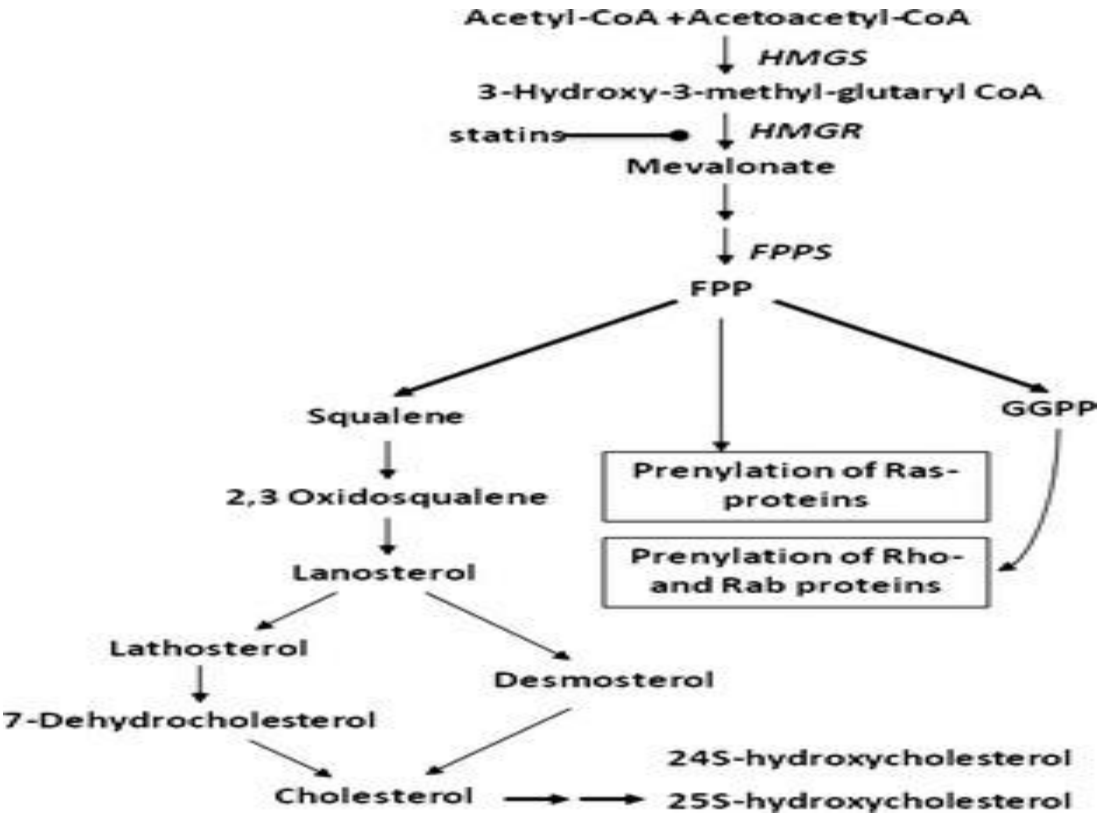

Figure 2: Diagram of the endogenous cholesterol biosynthesis. (Wood, Li, Müller, & Eckert, 2014). This diagram shows the cholesterol biosynthesis pathway, including major enzymes and intermediate compounds. The pathway starts by the conversion of Acetyl-CoA and acetoacetyl-CoA to 3hydroxy-3-methylglutaryl-CoA (HMG-CoA), catalyzed by 3-hydroxy-3-methylglutaryl CoA synthase (HMGS). HMG-CoA is then converted to mevalonate by the action of both HMG-CoA reductase (HMGR) and the cofactor NADPH. This step is targeted by statins, which are a substrate for HMG-CoA reductase. Then, mevalonate is converted to farnesyl pyrophosphate (FPP) by farnesyl pyrophosphate synthase (FPPS). FPP synthesis is the key of forming of geranylgeranyl pyrophosphate (GGPP), cholesterol, ubiquinone, and dolichol. Both FPP and GGPP are essential for allowing GTP-binding proteins such as Rho, Rac, and Ras to be implanted

in membranes by a process called prenylation. FPP is also the precursor of squalene, which is converted to cholesterol after 19 reactions.

The structure of statins is similar to HMG-CoA, allowing statins to compete with HMG-CoA to the binding site of the reductase enzyme but with higher affinity. This competition will significantly decrease the production of mevalonate and thus the level of the endogenous cholesterol biosynthesis. Statins have earned great success by lowering the incidence of acute coronary syndromes in patients with high blood cholesterol level. The benefits of statins are not limited to lowering cholesterol level. Statins can also attenuate the expression of cell adhesion molecules in leukocytes and endothelial cells, resulting in inhibition of leukocytes-endothelial adhesion. In addition, statins inhibit chemokines and matrix metalloproteinases (MMPs) production, which interfere with the migration of leukocytes. Furthermore, statins can lower the intensity of oxidative stress and inflammation by modifying signaling pathways and transcription factors involved in both conditions (Ii & Losordo, 2007; Libby & Aikawa, 2003; Winjns et al., 2010). It was shown by Toumisto that HMG-CoA reductase enzyme was overexpressed in macrophages, which are another important cells that are involved in the pathophysiology of atherosclerosis. This finding suggests that statins may add a potential benefit in atherosclerosis via acting on macrophages (Tuomisto et al., 2003).

Since statins inhibit the earliest step in the mevalonate pathway, they also inhibit several intermediates of the pathway, such as farnesyl pyrophosphate (FFP) and geranylgeranyl diphosphate (GGPP) as seen in figure 2 (Pella, Rybar, & Mechirova, 2005). Both of these proteins play an important role in protein prenylation, which is a post translational

modification via adding an isoprene unit to many proteins involved in several signaling pathways, such as the small GTP-binding proteins, Ras, Rac1, and Rho.

Statins` beneficial effect is not limited to the hepatic cells and is also extended to vascular cells, such as endothelial cells, and smooth muscle cells. This partially explains statins` effect on cardiovascular system independent of their cholesterol lowering effect as seen in figure 3.

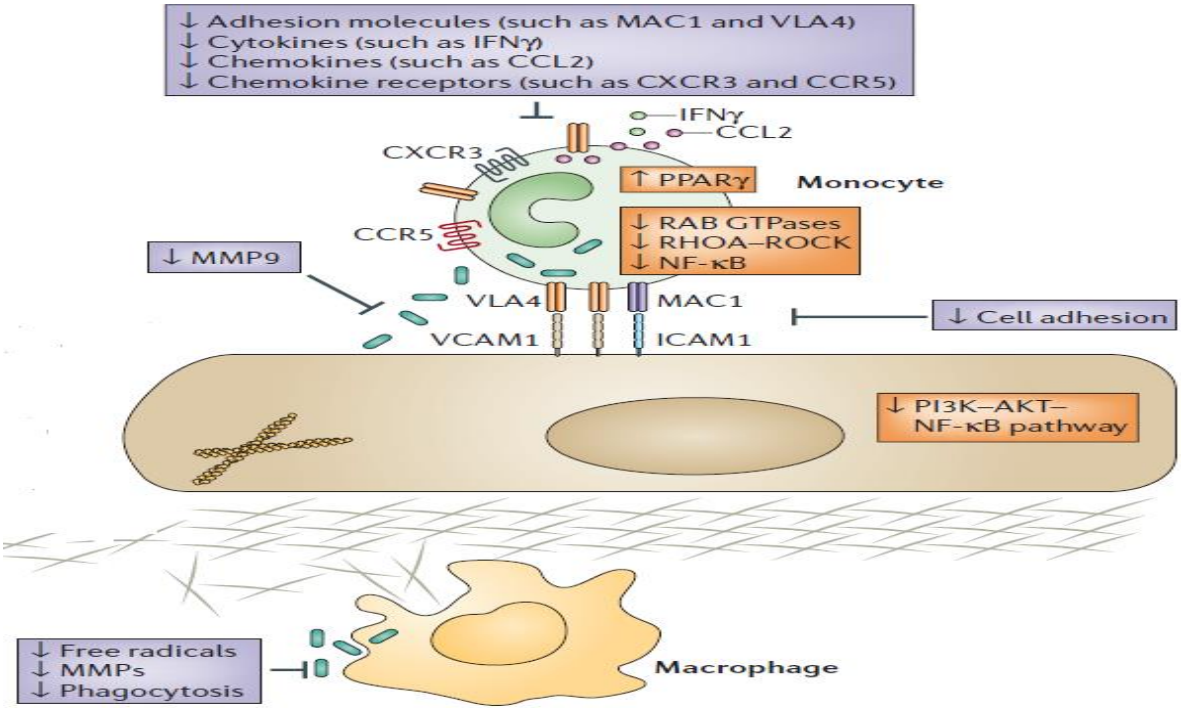

Figure 3. The broad effects of statins on monocytes and macrophages. (Greenwood, Steinman, & Zamvil, 2006). The expression of cell adhesion molecule was reduced in endothelial cells by statins. This effect results in less leukocytes-endothelial adhesion and leukocytes infiltration. Furthermore, statins block the secretion of both chemokine and matrix metalloproteinase (MMP), resulting in less leukocyte migration. Statins also block the prenylation of Rho and other small GTPases by inhibiting the formation of both FFT and GGPP in the endothelium, resulting in further attenuation of

adhesion molecule signaling pathways. This may also result in stabilization of the endothelial cell-cell junction. The effect of statins on the cytoskeleton alters leukocyte motility and directional migration in response to chemotactic gradients.

However, two thirds of cardiovascular patients gain no benefits from statins (hu, Cheung, & Tomlinson, 2012). It was reported during 2011–2012 that prescription cholesterol-lowering medication was used by 27.9% of adults aged 40 and over as seen in figure 4 (Gu, Paulose-Ram, Burt, & Kit, 2014). Despite the massive use of pharmacological approaches, overall morbidity and mortality from cardiovascular disease is rapidly increasing to be the primary cause of death on this whole planet.

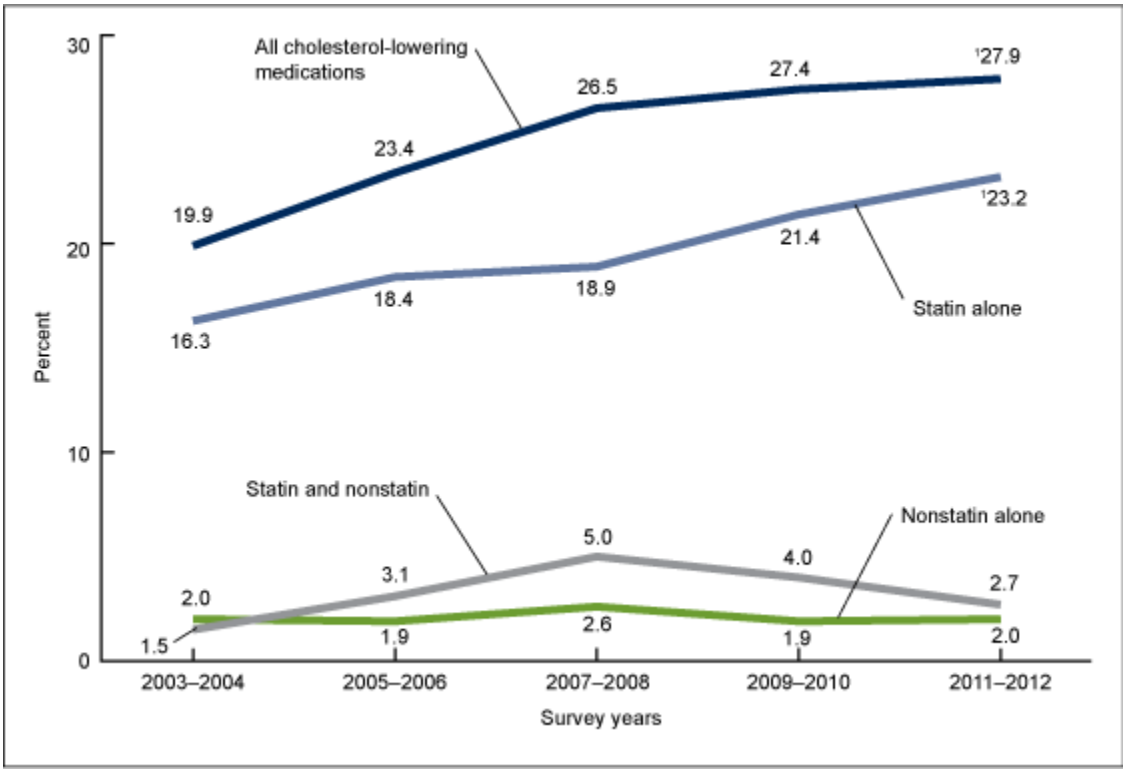

Figure 4. Use of statins between 2003–2012 in USA for adults aged 40 and over (Gu et al., 2014). This data reported that during 2011–2012 that prescription cholesterol-lowering medication was used by 27.9% of adults aged 40 and over. This number is expected to be higher in the following years and still overall morbidity and mortality from

cardiovascular disease is rapidly increasing to be the primary cause of death on this whole planet.

The primary reason for the failure of statins and other medications used for the treatment of atherosclerosis is that all of them are aimed to relieve symptoms and reduce the impact of risk factors, but not treating the root cause of the disease. This is because the past belief of atherosclerosis complications was due to stenotic type and therefore, the current treatment is focused on the reduction of the severity of stenosis. Recently, however, non-stenotic atherosclerosis started gaining attention and is believed to be the major provoker for the most acute coronary syndrome (Libby, 2001, 2002). Therefore, the new treatment approach`s goal should focus on stabilizing the rupture- prone non-stenotic lesion, rather than symptoms reduction or revascularization.

In late atherosclerosis stage, surgical approach is the first choice of treatment. The common surgical procedure is percutaneous coronary intervention (PCI), also known as coronary angioplasty. It is a procedure where a small catheter is inserted into an artery, usually the groin artery. Then, the catheter is threaded to the coronary arteries. Another catheter with a balloon at its tip (a balloon catheter) is inserted in the coronary artery and placed in the blockage. Then, the balloon is expanded to push the plaque against the artery wall to reopen the artery, relieving the blockage and improving blood flow. After that both catheters are withdrawn (O'Shaughnessy et al., 2003). In many procedures, a stent, a tiny metal or plastic tube, is placed in the affected artery following the angioplasty. When the balloon is inflated to remove the plaque, the stent expands and attaches to the artery wall (A. Kastrati et al., 2005). The stent supports the inner artery wall and reduces

the chance of the artery becoming narrower or re-blocked. This procedure is called stenting.

To inhibits platelet aggregation and formation of blood clots, dual antiplatelet drugs are used before and after stenting. A 325 mg of oral aspirin is given to all patients. In addition, a 300 mg loading dose of oral clopidogrel is given before the procedure and then 75 mg daily for three months (O'Shaughnessy et al., 2003). During the procedure, it is important to administer bolus doses of intravenous heparin. In some cases intravenous glycoprotein IIb/IIIa inhibitors are recommended (O'Shaughnessy et al., 2003).

To evaluate the efficacy of the first generation of stent replacement, which is bare-metal stent (BMS), ten thousand patients were evaluated and results showed that more than 30% of patients developed angiographic restenosis or in-stent restenosis (Cassese et al., 2014). In-stent restenosis is determined by coronary angiography as 50% or more re-narrowing of the vessel diameter. A new generation of stent, drug-eluting stent (DES), has been developed to decrease the incidence rate of in-stent restenosis. DESs are now used in more than 500,000 patients annually in the United States alone (Benjamin et al., 2017). The available DES platforms are made of stainless steel, which represent the early generation, and cobalt–chrome or platinum–chrome, which represent the new generations. Cobalt-chrome stents are reported to have thinner sturt thickness and thus are least associated with restenosis (Pache et al., 2003). The main advantage of DESs is to provide controlled local release, with the help of biocompatible and biodegradable polymers, of antiproliferative, immunosuppressant agents, or combination of both. These agents are highly lipophilic to be easily distributed through cell membranes around the wall. The most common drugs coated over stent of the early generation of DES are

paclitaxel and sirolimus, while the new generation involves everolimus or zotarolimus. However, in-stent restenosis incidence was still as high as 15% with the early generation of DES and 12% with the newer generation (Benjamin et al., 2017; Raungaard et al., 2015). However, BMSs are still widely used due to the unaffordable price of DES. Therefore, in-stent restenosis is still a major problem and coronary artery disease is still the leading cause of death worldwide.

1.4 The pathophysiology of in-stent restenosis:

The primary cause of in-stent restenosis is the proliferation and migration of smooth muscle cells (SMCs), resulting in neointimal formation around the stent as seen in figure 6. This neointimal formation phenomenon starts within weeks to months due to the mechanical injury caused by stent placement procedure. Stenting strips out endothelial cells that forms the first layer, lining the inner surface of coronary artery, in a process called endothelium denudation as seen in figure 5 (C Indolfi et al., 2002; Ciro Indolfi, Curcio, & Chiariello, 2003; Uchida et al., 2010). Endothelial cells function as a barrier preventing SMCs from the blood-circulated growth factors and cytokines (Platelet-derived growth factor (PDGF), interleukin-1, interleukin-6, and tumor necrosis factor), and provide inhibitory ligands, such as nitric oxide and heparin sulfate proteoglycan. In addition, endothelial cells play a crucial role in maintaining vascular health and provide anti-inflammatory and anticoagulant properties. Thus, endothelium denudation exposes SMCs to the growth factors, resulting in pushing vascular SMCs to switch from non-proliferative phase (G0) and enter gap phase (G1), preparing for new divisions (C Indolfi et al., 2002). While this response is necessary to conceal the stent within the vessel wall, over proliferation and migration of SMCs from the tunica media to the intima re-narrows

the artery (Uchida et al., 2010). It is worth to note that neointimal formation was negligible in stent free angioplasty, suggesting that stenting-inducing arterial injury is the trigger of SMCs proliferation (C Indolfi et al., 2002). In addition to SMCs proliferation, in-stent thrombosis, which is characterized by fibrin and platelet deposition, is also a major problem mainly associated with BMS and early generation of DES (Joner et al., 2006; Räber et al., 2012). However, the use of new generation of stent platforms, cobalt-copper and platinum-copper, and dual antiplatelet drugs, such as aspirin and clopidogrel, significantly reduces the incidence of in-stent thrombosis.

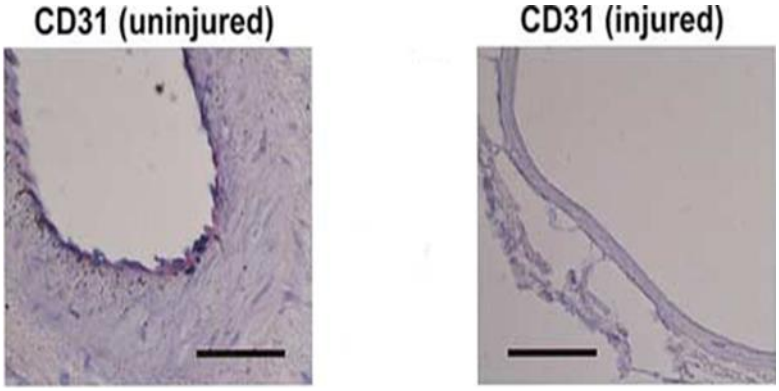

Figure 5: analysis using anti-CD31 to detect the presence of endothelium to compare among uninjured (left), injured (right) (Sata et al., 2000). Anti-CD31 immunostaining showed that the endothelium was completely stripped out after stenting, allowing the circulated growth factors to reach the vascular SMCs and trigger their proliferation.

There are several studies that were able to create an animal model that mimic the stenting mechanical injury (Ciro Indolfi, Esposito, et al., 2000; Sata et al., 2000). The animal model is created by inserting a straight spring wire catheter into the femoral artery through a small muscular incision. Then, the catheter is left in place for one minute to strip the endothelium layer and to inflame the artery. The muscular incision is then tied, and the

blood flow of the femoral artery is restored after the catheter is removed. After 5-7 days of stenting, neointimal formation was detected around the stent. At day 14, the neointimal formation was significantly increased as seen in figure 6 (Ciro Indolfi, Esposito, et al., 2000; Sata et al., 2000).

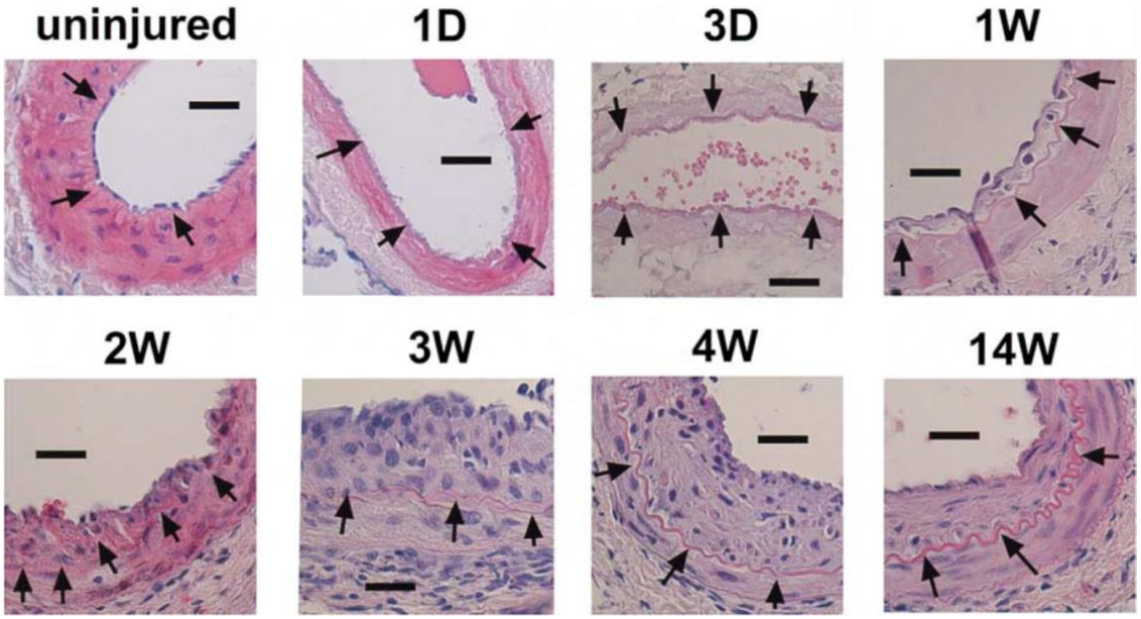

Figure 6: Rapid Onset of Neointima in the Mouse Artery (Sata et al., 2000). This diagram shows the effect of Stenting on the formation of neointimal hyperplasia at different time points. At one and three days after stenting, the media contained only a few of HCASMCs. However, after one week, thin neointimal formation started to be observed. The neointimal hyperplasia continued to grow for 3 or 4 weeks after which lesion formation did not advance further. Arrows indicate internal elastic lamina.

The molecular mechanism pathways associated with the formation of neointima after stenting, mitogen-activated protein kinase (MAPK) signaling pathways, have been studied in-vivo. MAPK signaling pathways are one way for cells to communicate by generating a signal from a receptor on the surface to the DNA in the nucleus of the cell. Among these pathways, the MAPK/ extracellular signal-regulated kinase (ERK) pathway,

also known as the Ras-Raf-MEK-ERK pathway, is the primary pathway for cell proliferation. Thus, it has been studied for its role in the formation of neointima after stenting. Once the surface receptor, tyrosine kinase receptor, is activated, it triggers a series of tyrosine phosphorylations, resulting in activating and connecting the receptor to ras. The activated ras, binds to raf, which in turn, activates MEK1. This cascade will activate transcription factors that are responsible for the proliferation of the cell. The function of ras protein, the key protein transducer of MAPK pathway, was investigated *in-vivo* after stenting. Ras protein was inactivated by treating the animal with N17 H *ras,* the mutant version of ras that dominantly inhibits the activation of ras protein (Ciro Indolfi et al., 1995). A significant reduction (55%) of neointimal formation was observed after blocking ras activation, confirming the important role of ras protein in vascular SMCs. Similar reduction was observed on neointimal formation after blocking MEK1 by mutant gene inhibitor (C. Indolfi et al., 1997).

Another signaling pathway, cAMP/PKA, was found to play an important role in the proliferation of vascular SMCs. Protein kinase A activity is dependent on the level of cAMP and it has several other functions in the cell, including glycogen, sugar, and lipid metabolism regulation. To investigate cAMP/PKA signaling pathway in vascular SMCs, the cells were treated *in-vitro* with a prominent cAMP activator, 8-Br-cAMP, and the growth of the cells were evaluated in different time points. It was found that activation of cAMP/PKA pathway significantly reduced the proliferation of the vascular SMCs. Similar result was obtained after investigating cAMP/PKA pathway *in-vivo*. On the carotid arteries, 8-Br-cAMP was locally delivered by using Pluronic gel. After 14 days of treatment, significant reduction on the neointima formation (54%) was observed in the

treated group (Ciro Indolfi et al., 1997). Similar result was obtained after systemic administration of 8-Br-cAMP (Ciro Indolfi, Di Lorenzo, et al., 2000). Both in-vivo and in-vitro studies on the role of cAMP/PKA pathway in the proliferation of vascular SMCs signify the importance of targeting cAMP/PKA pathway as a potential therapeutic approach of in-stent restenosis.

Formation of the neointima needs vascular smooth muscle cells to migrate from the tunica media to the intima in addition to their proliferation. The migration of vascular smooth muscle cells start by reforming of the cytoskeleton, changing the expression of the adhesion molecules between the matrix and vascular SMCs and activating the motor proteins. All these processes start from the actin polymerization, forming vascular SMCs lamellipodia toward the stimulus (Gerthoffer, 2007). Just behind the leading edge, focal contact forms to enhance the adhesion of the cell membrane to the matrix. On the other hand, the focal contact in the tailing side of the cells is degraded allowing vascular SMCs to detach (Gerthoffer, 2007).

Proliferation and migration of vascular SMCs are stimulated when a stimulus, called chemokine, binds to different kinds of receptors on the surface of the cells. One of these kinds of receptors is called G protein-coupled receptors (GPCRs), which are widely spread through out the body.

1.5 Chemokines and their receptors:

Chemokines are a family of small cytokines, which are small proteins ranging from 8 to 12 kDa (Soulika & Pleasure, 2014). Chemokines consist of 50 small peptides that have an important role in regulating the process of cell trafficking (Bromley, Mempel, & Luster, 2008). They are widely expressed throughout the body and their roles are extended to other physiological and pathological conditions such as angiogenesis, hematopoiesis,

atherosclerosis, and cancer (Romagnani, Lasagni, Annunziato, Serio, & Romagnani, 2004).

Chemokine receptors are GPCRs containing 7 transmembrane domains. They are classified based on the conserved cysteine residues from N-terminal of their structure into four subfamilies: (CXC, CC, CX3C, and C). They can also be classified based on their function into homeostatic and inflammatory, or both.

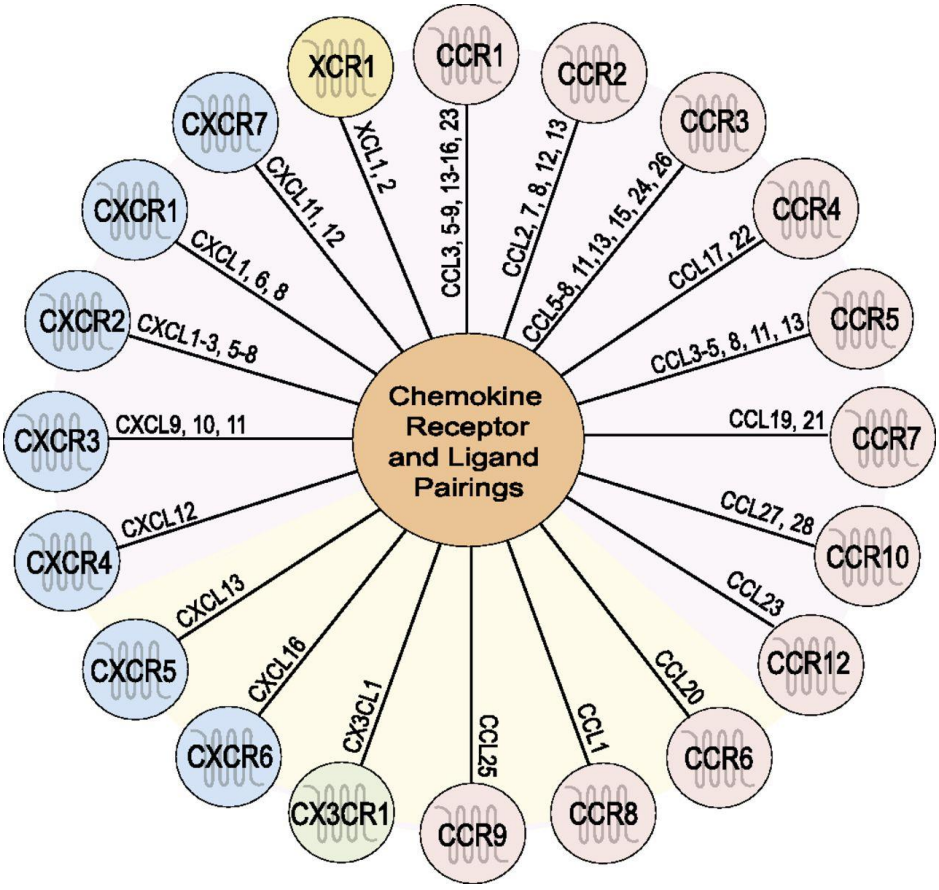

Figure7. Chemokine receptors and their ligands (White, Iqbal, & Greaves, 2013). This diagram demonstrates the pairings of different chemokine receptors and their ligands. The common chemokine receptors (C, CC, CXC, and CX3C) are represented around the outer ring of the wheel, with their chemokine ligands shown along the wheel

spokes. Receptors with a single known ligand are shown in the area of the circle shaded yellow.

1.6 Introduction of C-X-C chemokine receptor type 7 (CXCR7):

CXCR7 is a typical seven-transmembrane GPCR, which is the most diverse receptor family in eukaryotes. CXCR7 neither binds to G protein, nor exerts chemotaxis (Balabanian et al., 2005; Burns et al., 2006; Infantino, Moepps, & Thelen, 2006a). CXCR7 was originally cloned from a dog thyroid and named RDC-1 (Libert et al., 1989). The gene sequence of CXCR7 in humans, dogs, mice, and rats remain highly conserved. The location of the human CXCR7 gene is on the 2q37.3 region, whereas in mice it is located on the 55.6 cM region in the chromosome (Heesen et al., 1998). CXCR7 encodes two exons, but only one of them functions as a translated coding for CXCR7 (Broberg et al., 2002).

The molecular function of CXCR7 is still under debate; It was originally, considered as a receptor for both calcitonin gene-related peptide (CGRP1) and vasoactive intestinal peptide (VIP). However, studies have not been able to prove this finding (Cook et al., 1992; McLatchie et al., 1998). After that, it was proposed to be a decoy receptor, acting as a scavenger for both stromal cell-derived factor 1 (SDF1) and C-X-C motif chemokine 11 (CXCL11). As a result, CXCR7 promotes their internalization and degradation (Boldajipour et al., 2008; Luker, Steele, Mihalko, Ray, & Luker, 2010; Naumann et al., 2010). Therefore, CXCR7 was thought to reduce the activity of CXCR4, a receptor that shares ligand specificity with CXCR7 (Burns et al., 2006). However, it should be noted that this negative impact of CXCR7 on CXCR4 was only observed on cell lines that over-

expressed both receptors *in-vitro*. It is still unknown if the same is true in cell lines that naturally express both receptors are.

Recently, CXCR7 was deorphanized after it was found to bind to different ligands, such as SDF-1 and I-TAC. CXCR7 activates intracellular signaling pathways, either by direct modulation, through β-arrestin-dependent pathway (Singh et al., 2013) or after heterodimerization with CXCR4 *in-vitro*. However, none of these are conclusive.

Interestingly, CXCR7, unlike other GPCRs, neither binds to G protein nor activates calcium signaling (Graham, Locati, Mantovani, Rot, & Thelen, 2012). The N-terminal of the second intracellular loop is important for the calcium signaling, where most chemokine receptors share a conserved motif DRYLAIV. However, the CXCR7 sequence is changed to DRYLSIT (A to S and V to T). It is believed that CXCR7 is unable to induce Gi and calcium signaling as a result of this structural change (Graham et al., 2012).

These new findings of CXCR7 places many previous described SDF-1 functions attributed to CXCR4 in question. It was found that murine fetal liver cells from CXCR4 knockdown mice and several human cancer cell lines that lack CXCR4 may still bind SDF-1, suggesting the presence of another SDF-1-binding receptor on the cell surface (Burns et al., 2006). In addition, there is no *in-vivo* evidence available yet showing that the endogenous CXCR7 heterodimerizes with CXCR4. Therefore, it is thought that CXCR7 may signal through CXCR4-independently *in-vivo* (Balabanian et al., 2005; Burns et al., 2006).

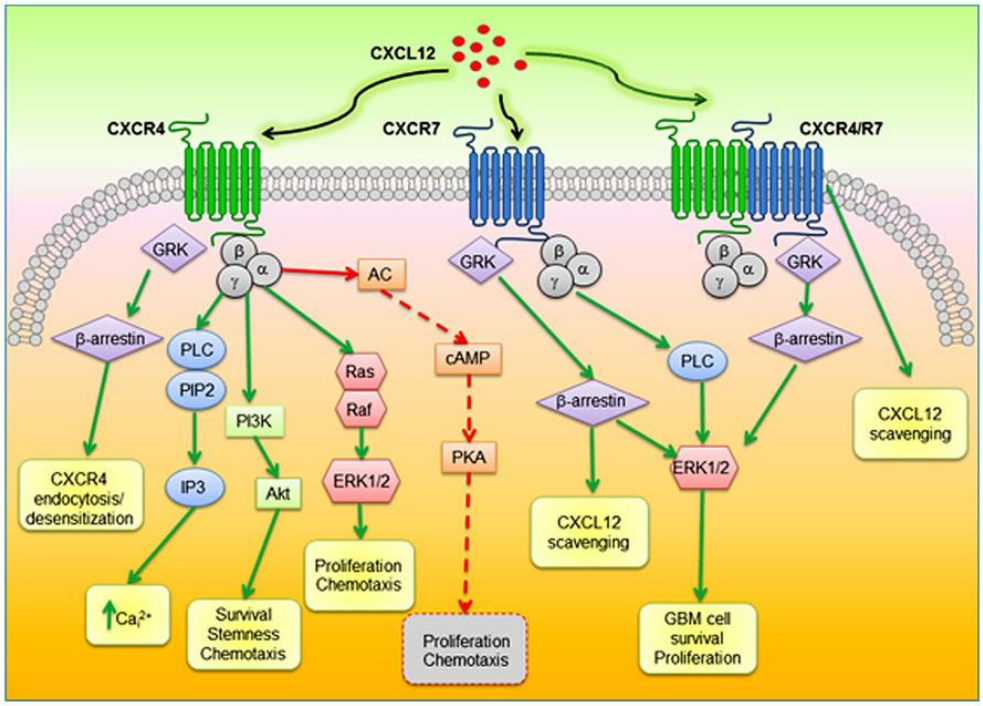

Figure 8. Current understanding of CXCR7 signaling pathways (Wurth, Bajetto, Harrison, Barbieri, & Florio, 2014). CXCR7 was initially proposed to be a decoy receptor, acting as a CXCL12 (and CXCL11) scavenger and able to promote ligand internalization and degradation, to reduce CXCR4 activity. The current vision is that CXCR7 can activate intracellular signaling pathways, either by direct modulation, through β-arrestin-dependent pathway (Singhetal.,2013) or after heterodimerization with CXCR4 in vitro (co-transfecting both receptors in vitro). However, there is no evidence showing that the endogenous CXCR7 heterodimerizes with CXCR4 in-vivo. Therefore, CXCR7 may signal negatively through CXCR4-independent way in vivo.

After the de-orphanization of CXCR7 as a new high affinity receptor for SDF-1, great effort has been made to re-define the cellular functions of SDF-1/CXCR4 axis. Systemic CXCR7 knockout mice are generated to understand its *in-vivo* role. However, the systemic knockout of CXCR7 are prenatally lethal due to the stenotic valve defects

(Gerrits et al., 2008). Mutation and promoter deletion studies suggest that NF- kB pathway is the primary pathway for CXCR7 expression (Tarnowski et al., 2010). In addition, the presence of the CXCR7 protein on the cell surface doesn`t reflect its high mRNA expression in several tissues, including heart, brain, spleen, and liver. This finding suggests that CXCR7 might be regulated in a posttranslational manner (Burns et al., 2006).

1.7 Ligands of CXCR7:

- **SDF1 or CXCL12:**

SDF-1 is a member of the CXC chemokine subfamily and initially characterized as a soluble pre-B-cell growth stimulatory factor (PBSF), which promotes proliferation of progenitor bone marrow B cells (Nagasawa, Kikutani, & Kishimoto, 1994; Tashiro et al., 1993). There are seven SDF-1 isoforms that have been identified. SDF-1α is the dominant isoform and found in almost all tissues in human (Juarez, Bendall, & Bradstock, 2004; Yu et al., 2006). It regulates many important biological and pathological processes, including motility of stem cells, angiogenesis, and tumor development (Peled et al., 1999). SDF-1 is well known for its role in stem and progenitor cell trafficking. In addition, it is a potent chemotactic inducer for several immune cells, such as T-lymphocytes and monocytes, dendritic cells, and hematopoietic progenitor cells (Wanshu, Liu, Ellison, & Shen, 2013a). SDF-1 was highly expressed in certain pathological conditions, such as ischemia, inflammation, hypoxia, cancer, and autoimmune diseases (Karin, 2010; Li & Ransohoff, 2009). SDF-1 was also identified as an HIV-entry cofactor after binding to its receptor CXCR4 (Bleul et al., 1996; Feng, Broder, Kennedy, & Berger, 1996). Later on, SDF-1 was

identified as homing inducer of progenitor leukocytes into the bone marrow and plays an important role in adaptive immune system (Ishii et al., 1999; Nanki & Lipsky, 2000).

It has been known that CXCR4 is the exclusive receptor by which SDF-1 exerts its function. SDF-1 changes the three-dimensional conformation after binding to CXCR4. As a result, G proteins are dissociated into α- and βγ-subunits and activated though GTP-GDP exchange, resulting in several cell signaling pathway activations (Bajetto, Bonavia, Barbero, Florio, & Schettini, 2001). Through αi subunits, SDF-1 inhibits cAMP formation while through αq, it induces PLC-β. PLC- β, in turn, catalyzes the hydrolysis of Phosphatidylinositol 4,5-bisphosphate (PIP2), producing both diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which are second messengers to induce several cell responses. IP3 binds to its receptor, which is a calcium channel located in the endoplasmic reticulum, to release Ca^{2+} and activates protein kinase C. Through CXCR4, SDF-1 can also activate phosphoinositide 3-kinase (PI3K)/AKT, and MAPK to regulate cell survival, chemotaxis, and proliferation. In addition, SDF-1 was found to activate JNK and p38 to control cell survival (Lin et al., 2014; Pan et al., 2013; Subramaniam et al., 2013).

However, the efficacy and specificity of SDF-1 were questioned after the deorphanization of CXCR7 (Kalatskaya et al., 2009a). SDF-1 binds to CXCR7 with 10 times higher affinity than to CXCR4 (Balabanian et al., 2005). It was found that SDF-1 induced CXCR4 and CXCR7 homo- or heterodimers, depending on the expression level of both receptors *in-vitro* (Levoye, Balabanian, Baleux, Bachelerie, & Lagane, 2009). In this research it was found that CXCR7 expression, at both protein and mRNA levels, was highly induced in the macrophage positive area of aortic atheroma of ApoE-null mice, however none was

detected in healthy arteries. As a result, SDF-1 signaling switches from activating the pro-survival pathway (Akt, ERK1/2) to the pro-inflammatory pathway (p38, JNK), resulting in an increase in macrophage phagocytosis activity (Wanshu et al., 2013a).

There are two identified forms of SDF-1, monomeric and dimeric forms. Both exert different effects and potency. For instance, in human breast cancer, the dimeric form was found to be more potent than the monomeric in recruiting β-arrestin 2 (Ray et al., 2012). In addition, the dimeric exerted higher chemoattractive activity in the same cells. However, in human colon carcinoma cell line, dimeric form feebly induced β-arrestin 2 recruitment and chemotaxis whereas monomeric form potently induced calcium mobilization, β-arrestin 2 recruitment, and cell migration (Drury et al., 2011). These differences are inconclusive and need further investigation.

- **IFN-inducible T cell α-chemoattractant (I-TAC) or CXCL11:**

CXCL11 (I-TAC) is another small chemokine molecule that targets activated T cells. Its gene expression is highly induced by IFN-γ and IFN-β and at lesser extent IFN-α in response to certain pathological conditions, such as infection or cancer. Genes of many members of the CXC chemokine family are located on human chromosome 4, including I-TAC. It is vastly expressed in the pancreas, peripheral blood leukocytes, thymus, liver, spleen, lung and with lesser extent in prostate, placenta, and intestine (Cole et al., 1998). I-TAC binds to two receptors, CXCR4 and CXCR3. There are two variants of CXCR3, CXCR3-A and CXCR3-B. I-TAC induces proliferation signals when binding to either CXCR3-A or CXCR7. On the other hand, when binding to CXCR3-B, I-TAC inhibits cell growth signaling (Lasagni et al., 2003). Either high expression of CXCR3-A or downregulation of inhibitory signal via CXCR3-B promotes the migration and invasion of

prostate cancer cells (Wu, Dhir, & Wells, 2012). Parallel to SDF-1, I-TAC neither induces $Ca^{2}+$ signaling, nor activates ERK or Akt pathways (Proost et al., 2007). It was found that I-TAC recruits β-arrestin-2 when binding to CXCR7 (Rajagopal et al., 2010).

- **VUF11207 and VUF11403:**

Both ligands were synthesized from a styrene amide scaffold and evaluated with pKi values ranging from 5.3 to 8.1. Both are CXCR7 agonists with high affinity, recruiting β-arrestin-2 (Wijtmans et al., 2012). Thus, these two ligands are very important in order to be considered in any CXCR7 studies.

- **AMD300:**

AMD300 is a classical CXCR4 antagonist, having an antiretroviral effect. It strongly inhibits the effect of X4-tropic HIV replication *in-vitro* (De Clercq, 2005). It has been proved by the FDA in the immunodeficiency (SCID)-Hu Thy/Liv mouse model *in-vivo* (Datema et al., 1996). In addition, AMD300 and its derivatives are strong candidates for cancer treatment. This is because AMD300 causes a huge release of hematopoietic stem cells into the peripheral blood by antagonizing CXCR4 (De Clercq, 2005).

By using the BRET assay, AMD300 was found to bind to another receptor, CXCR7. Interestingly, AMD300 enhances SDF-1 binding to CXCR7. Furthermore, AMD300 alone is able to recruit β-arrestin2 to CXCR7, but inhibits recruiting of CXCR4. Therefore, AMD300 is considered as a CXCR7 agonist (Kalatskaya et al., 2009a).

- **TC14012:**

TC14012 is a peptidomimetic that is derived from its parent compound TC140 to be more serum stable and less toxic. It is characterized to be as a CXCR4 inverse agonist (Tamamura et al., 1998; Trent et al., 2003), but CXCR7 agonist (Tamamura et al., 2001).

TC14012 is able to recruit β-arrestin2 to CXCR7 with higher potency than AMD300 (EC50 od 350nM for TC14012 vs 140μM for AMD300) and only one log weaker than SDF-1 (Gravel et al., 2010). Thus, TC can be an ideal tool in the study of the molecular function of CXCR7.

- **CCX771**

CCX771 is the only known human CXCR7 agonist with an IC50 of 4.1 nM and it has not been available in the market yet. It was reported that CCX771 was highly selective toward CXCR7 with no effect on SDF-1 binding to CXCR4 in NC-37 tumor cells (B. A. Zabel et al., 2009). In addition, CCX771 was found to inhibit tumor growth, lung metastasis and tumor angiogenesis through CXCR7 *in-vivo* (Yamada et al., 2015). This ligand could be a very important tool in order to study the function of CXCR7 and its relationship with SDF-1.

1.8 CXCR7`s functions in biological vs pathological conditions:

- **CXCR7 function in biological condition:**

In biological condition, CXCR7 was found to be expressed less than in pathological inflammation and cancer development, suggesting an important role for CXCR7 in these pathological conditions. Thus, it becomes a potential focus for many researchers. The biological role of CXC must be discussed to be able to understand its role in pathological conditions, which is still controversial.

CXCR7 signaling pathways regulate many biological conditions, such as cell adhesion and migration, cell cycle, amino acid metabolism, and ligase activity (Yoshida, Nomura, & Teramoto, 2009). Large amounts of evidence indicate that CXCR7/CXCR4 heterodimerization regulate CXCL12-induced chemotaxis in lymphocytes (Levoye et al.,

2009). CXCR7`s role in migration was also found in embryogenic morphogenesis after the binding of SDF-1 (Aman & Piotrowski, 2008; Friedl & Gilmour, 2009). Silencing of CXCR7 indicate that CXCR7-induced migration is regulated by Wnt/β-catenin signaling (Aman & Piotrowski, 2008). CXCR7 has also been found to be involved in corticogensis by inducing the interneurons migration to form the cortical plate (Sánchez-Alcañiz et al., 2011). CXCR7 expression might be necessary for homing HSCs to their niches by sustaining the capacity for cell migration. It was recently reported that CXCR7 enhances B cell retention in the bone marrow (Wang et al., 2012) and facilitates the homing of progenitor cells to their niches (Shiozawa et al., 2011). The expression of CXCR7 in blood-derived switch memory B cells correlated with their ability to differentiate into plasma cells (Infantino, Moepps, & Thelen, 2006b).

In embryogenesis, CXCR7 was found to be expressed in the liver at stages E11–E13 but that expression declined at E15– E17 (Burns et al., 2006). The expression of CXCR7 induced the growth of the liver bud as it was vascularized and developed to fetal hematopoietic organ. After knocking out CXCR7 in mice, results supported the potential role for CXCR7 in the vascular system.

These data suggest an important role of CXCR7 in vasculogenesis and angiogenesis. More than 95% of perinatal deaths that occur within 24 hrs are due to severe cardiac defects in Cxcr7-deficient mice. The lethal phenotype is most likely due to a defective expression of CXCR7 on endothelial cells (Sierro et al., 2007). Gene screening of the CXCR7 knockout mice revealed a change in the expression of important factors in the formation of valve, vessel protection or endothelial cell growth and survival (Sierro et al.,

2007). In zebrafish, the knockdown of CXCR7 showed an important role of CXCR7 in vasculogenesis and angiogenesis (Miao et al., 2007).

On the other hand, studies provided evidence that CXCR7 expression was induced during inflammation, and tumor development, mediating cell survival, adhesion, and tumor growth, suggesting an important role for CXCR7 in different disease conditions (Yan et al., 2012). However, very few researches have been conducted for elucidating the role of CXCR7 in cardiovascular diseases, and virtually nothing is known for its role in HCASMCs.

- **CXCR7 function in pathological conditions:**

  a. CXCR7 and Cancer:

The impact of expression of both CXCR4 and CXCR7 receptors to SDF-1 in cancer are tissue dependent. For instance, in breast cancer cell line MDA-MB-231, the SDF-1 chemotaxis`s effect was increased after transfecting the cells with CXCR7 (Décaillot et al., 2011). The same increased effect was observed in rat mammary adenocarcinoma cell line MTLn3 under the condition of upregulated CXCR4 expression (Hernandez et al., 2011). On the other hand, CXCR7 was shown to decrease the chemotaxis effects of SDF-1 in other cell lines, such as human neuroblastoma cell lines (Liberman et al., 2012). In addition, CXCR7 interfered with SDF-1-CXCR4 interaction and thus deregulated their metastasis promotion.

In breast cancer derived from an immune-deficient mouse model, CXCR7 prevented tumor cell invasion and spontaneous lung metastasis formation (Hernandez, Magalhaes, Coniglio, Condeelis, & Segall, 2011). Also, the interaction between CXCL12 and CXCR7 did influence CXCR4-mediated transendothelial migration of human tumor cells (B. A.

Zabel et al., 2009; Brian A. Zabel, Lewén, Berahovich, Jaén, & Schall, 2011). On the contrary, the activation of CXCR7 was able to promote metastasis in the breast cancer model (Miao et al., 2007). The role of CXCR7 in tumor migration is still not conclusive and further studies on its role with CXCR4 are needed.

Large body of evidence shows the importance of CXCR7 as a potential target for preventing cancer cells spreading, their metastasis, and angiogenesis. In an *In vivo* study, CXCR7 antagonism was shown to inhibit the growth of breast and lung cancer in mice (Miao et al., 2007). However, another study found that CCX771, a synthetic CXCR7 antagonist, was able to inhibit trans-endothelial migration compared to a CXCR4 antagonist, AMD3100 (Brian A. Zabel et al., 2011). Also, CCX771 was found to recruit b-arrestin to CXCR7 in the lymphoblast leukemia model (B. A. Zabel et al., 2009). These findings raise a question mark on whether CCX771 is an agonist or antagonist to CXCR7. On the other hand, CXCL12 increases human lymphoma cells' migration via binding both CXCR7 and CXCR4, suggesting that CXCR7 might be a potential target for cancer treatment (Brian A. Zabel et al., 2011).

In addition, a recent in vivo study conducted in NOD/SCID mice indicated that CXCR7 contributes to homing of both acute myeloid leukemia and normal CD34C progenitor cells to the bone marrow and spleen (Melo, Ferro, Duarte, & Olalla Saad, 2018); however more mechanistic information is needed to fully understand the role of CXCR7 in HSCs. Although the ability of CXCR7 to regulate the BMSC niche is still under 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 (To, Levesque, & Herbert, 2011).

Due to the current absence of a structural model for CXCR7, limited number of ligands for CXCR7 have been reported (Kalatskaya et al., 2009b; Montpas et al., 2015; Wijtmans et al., 2012). On the other hand, more information is provided for CXCR4 using advanced approaches, such as virtual screening and GPCR homology modeling, which can be used for CXCR7 ligand identification (Yoshikawa et al., 2013; Yoshikawa, Kobayashi, Oishi, Fujii, & Furuya, 2012). Therefore, market available molecules, which can block CXCR4 and CXCR7 simultaneously, represent an ideal pharmacological approach because both receptors are involved in cancer malignancy (Duda et al., 2011). However, it is important to note that these molecules act differently when they bind to both receptors. If they are antagonist to one of them, they show partial agonist activity to another. For instance, AMD3100 is a CXCR4 antagonist, which may also act as a CXCR7 partial agonist (Kalatskaya et al., 2009b). There was another finding that makes the issue more complex that CXCR7 agonists were found to downregulate the expression level of CXCR4, selectively via activating b-arrestin (Uto-Konomi et al., 2013). These intricate biological findings may be due to tissue specific biased signaling. Thus, the study of ligands, which can interact with both CXCR4 and CXCR7 must be examined in specific tissues, considering their properties as agonist or antagonist.

There are a couple of methods that have been developed, targeting both receptors, CXCR4 and CXCR7 to fight cancer. Compounds, derived from the family of chalcones were synthesized to have high affinity binding with CXCL12 and thus preventing CXCL12 from interacting with both CXCR4 and CXCR7. These compounds were shown to inhibit inflammatory responses in eosinophils (Hachet-Haas et al., 2008). Another method is the development of an RNA oligonucleotide named NOX-A12, which can bind and neutralize

CXCL12 with high affinity (Liang et al., 2007). NOX-A12 has been clinically approved Because of its high antitumor activity for the treatment of leukemia and multiple myeloma. In addition, NOX-A12 was shown to be effective in inhibiting or delaying recurrences of glioblastomas (also called GBM) following irradiation (Liu et al., 2014).

b. CXCR7 and Cardiovascular Diseases:

Large body of evidence shows that CXCR7 has an important role in the developing heart and is associated with many cardiovascular diseases. Heart hypertrophy is the phenotype that appeared after knocking out CXCR7, which is characterized by increasing the thickness of pulmonary, and aortic valves (Gerrits et al., 2008; Sierro et al., 2007). It is believed that the heart hypertrophy is caused after the disruption of endothelial migration in the absence of CXCR7 gene. This phenomenon was reproduced in endothelial cell specific CXCR7 knockout mice, suggesting that CXCR7 has a major role in endothelial cells in developing the heart (Sierro et al., 2007). The mechanism of this phenomenon is due to the finding that CXCR7 functions as a scavenger receptor for CXCL12 in heart valves, preventing the binding of CXCL12 to its receptor CXCR4 and thus, resulting in heart hyperplasia (Naumann et al., 2010). However, it is still possible that CXCR7 may independently ,from CXCR4, transduce cell signaling in these endothelial cells and maintain their survival and promote adhering to endothelial cell progenitors (EPCs) (Yan et al., 2012). It is also noteworthy to mention that CXCR7 can promote the migration of smooth muscle cells through the activation of β- arrestin. These data suggest that CXCR7 may independently signal to promote different cells responses depending on the type of tissues, known as tissue biased.

The relationship between CXCR7 and CXCR4 seem to play a major roles in the cardiovascular system. *In-vitro* heterodimerization of both receptors was indicated in the formation of angiogenesis. Deletion of either CXCR4 or CXCR7 results in ventricular septum defects, indicating the importance of both receptors as a potential targets for certain cardiovascular diseases (Sierro et al., 2007; Zou, Kottman, Kuroda, Taniuchi, & Littman, 1998). However, there is a desperate need to investigate the heterodimerization phenomenon *in-vivo* since such heterodimers having enhanced responses to CXCL12 was observed only in HEK293 cells with overexpressed CXCR4 and CXCR7 (Sierro et al., 2007).

The critical role of CXCL12 in the cardiovascular system, as mentioned previously, makes itself and its receptors an important element of cardiovascular disease research. Recently, a lot of research has been conducted on the CXCL12-CXCR4 axis in different cardiovascular diseases, such as myocardial infarction and heart ischemia. However, very limited studies mentioned the contribution of CXCR7 to any cardiovascular diseases. One of the limited studies available discovered that CXCR7 may protect cardiac cells after binding to CXCL12-b (B. A. Zabel et al., 2009), indicating that CXCR7 signaling may able to take part in the process of myocardial infarction regeneration (Sierro et al., 2007; Yan et al., 2012). This indication was confirmed when CXCR7 knockout endothelial cells was found unable to cause vascular homeostasis and cardiac remodeling after myocardial infarction (Hao et al., 2017). The angiogenic function of EPCs is enhanced by CXCR7 via Akt/GSK-3b/Fyn-mediated Nrf2 activation in diabetic limb ischemia (Dai et al., 2017).

1.9 Biased signaling:

Biased signaling or functional selectivity has been a widely accepted phenomenon among researchers. It is a mechanism by which a receptor preferentially activates one signaling pathway over other available ones. There are three types of biased signaling: ligand, receptor, and tissue or cell bias. Ligand bias is a condition where different ligands bind to the same receptor, but activate different pathways, each of which is unique to one of the ligands. On the other hand, receptor bias is when one ligand binds to different receptors, but induces different pathways, each of which is unique to one of the receptors. Lastly, tissue bias is accorded when different pathways are activated in different tissues even though the same ligand-receptor complex is activated in both tissues (Steen, Larsen, Thiele, & Rosenkilde, 2014).

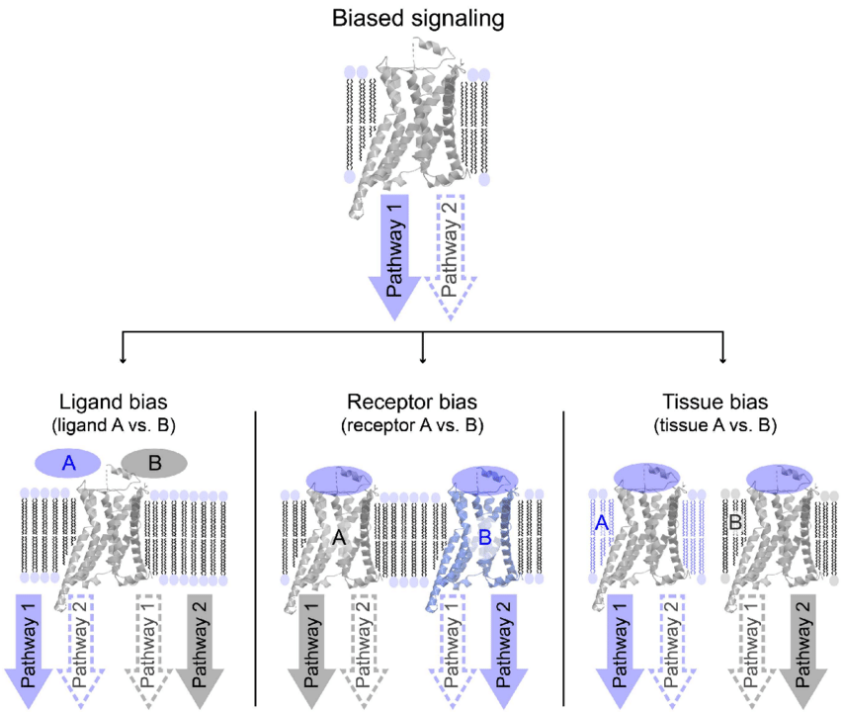

Figure 9. Overview of different types of biased signaling (Steen et al., 2014). Biased signaling describes a well-accepted phenomena where a receptor prefers to induce one

signaling pathway over other possible ones. There are three types of biased signals, ligand bias, receptor bias, and tissue bias. The left figure depicts ligand bias, which is used to differentiate between two ligands acting on the same receptor. Ligand A preferably induces pathway 1, whereas ligand B induces pathway 2. In receptor bias, in the center, is used to differentiate between two different receptors bind to the same ligand and activates two different pathways. In tissue bias, two different tissues are activated by the same ligand-receptor complex. In tissue A, pathway 1 is favorably induced, whereas pathway 2 is more likely to be activated in tissue B.

1.10 Hypothesis and Specific Aim:

1.10.1 Hypothesis:

It is hypothesized that activation of CXCR7 may provide therapeutic opportunity for coronary restenosis by attenuating the proliferation of the Human coronary artery smooth muscle cells (HCASMCs) through the inhibition of the ERK1/2 Pathway.

1.10.2 Specific Aims:

a. **Define the negative role of CXCR7 in control of ERK1/2 signaling pathway in VSMCs and thus being able to attenuate the cells proliferation**. After treating VSMCs with CXCR7 agonists, the cells` lysate will be collected to detect the expression level of the proliferation mediator protein, ERK, using the western blot technique. It is expected that ERK phosphorylation will be decreased. Moreover, proliferation and migration assays will be conducted to further prove the efficacy of CXCR7 agonists in prevention of VSMCs accumulation.

b. **Determine the role of smooth muscle cells` CXCR7 in vascular injury-induced atherosclerosis.** The aim will be applied by creating a specific CXCR7 knock out mouse model with mechanically-induced atherosclerosis. It is expected that the absence of CXCR7 will allow the migration and proliferation and thus accumulation of VSMCs in the injured region. Conversely, it is also expected that the activation of CXCR7, using CXCR7 agonists, will prevent restenosis and thus revascularization.

Chapter 2. Reagents and Methods.

2.1 Reagents:

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

Table 1: Chemicals:

Reagents	Catalog Number	Company
TC14012	4300	Tocris
Resazurin	AR002	R & D
H2O2	BDH3540−2	BDH
Rottlerin	557370	Calbiochem
UTP	06625	Sigma
VEGF	V7259	Sigma
FBS	1500−500	Seradigm
WZ811	3951	Tocris
Polybrene	TR-1003-G	Specialty Media
Human SDF-1α	300−28A	Peprotech
AMD3100	3299	Tocris
AMD3645	4179	Tocris
IT1t	4569	Tocris
Human I-TAC	300−46	Peprotech
VUF11207	4780	Tocris
Human MIF	300−69	Perpotech
ADM	22−2−10	American Peptide
BAM 22P	1650	Tocris

Blasticidin	J61883	Alfa Aesar
Carbenicillin	BP2648	Fisher Scientific

2.2. Cell Culture

2.2.1. Cell Line and Culture

All cells used in this project including HCASMC, THP-1, 1321N1, Raw 264.7 and EA.hy926 were purchased from the 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) and medium 231 (gibco) for the HCASMCs. For other cells, the culture media used was 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% CO2.

2.2.2. Passing

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*105 cells/ml. For HCASMC and EA.hy926 cells, they were maintained in 75 cm2 flask, detached by trypsin (HyClone) and then centrifuged at ~1100 rpm for 5 mins and subsequently re-suspended in a concentration of ~ 2-4*105 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) the following day. To resuscitate these frozen cells, the cryovials were rapidly removed into a 37 °C water bath and the vials were gently swirled till the medium started to thaw. Then the cells suspension was diluted with pre-warmed growth medium and centrifuged at ~1100 rpm for 5 min. After the centrifugation, the cells were re-suspended in complete growth medium into the appropriate culture vessel at the appropriate culture environment.

2.2.3. Starvation

For THP-1 cells, they were starved for 4h in RPMI-1640 without FBS. For HCASMC, 1321N1, Raw 264.7 and EA.hy 926 cells, they were starved overnight in DMEM (medium 231 from gibco for the HCASMC) without FBS. Cells were pretreated with inhibitors or antagonists for 30 min before stimulation.

2.2.4. Cell Viability

To measure the cell viability and cytotoxicity, Resazurin dye, a non-toxic, water soluble, redox-sensitive dye, was used. The dye changes the medium from blue/non-fluorescent state to a pink/highly-fluorescent state via reduction by viable cells. 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).

2.2.5. Cell Activation

To determine TC14012 best concentration for inhibiting ERK1/2 in HCASMCs, dose response curve was conducted as the following: cells were stimulated for 40 min at the

indicated concentrations: 100 nM, 1 uM, 10 uM, 20 uM, and 30 uM. Then, cells were stimulated with 20 uM (best indicated concentration) 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, FBS on ERK1/2 pathway can be blocked by TC14012 in HCASMCs, cells were pretreated with TC14012 (20uM) for 30 min. Then cells were separately stimulated with 10% FBS, and SDF-1 (200ng/ml). To determine whether the upstream kinases of ERK1/2 can be blocked by TC14012 in HCASMCs, cells were pretreated with TC14012 (20 uM) for 40 min. Then cells were stimulated with SDF-1 (200ng/ml). To determine the effect of TC14012 and other CXCR4 antagonists on ERK1/2 pathway in HCASMCs, cells were stimulated with TC14012 (20uM), AMD3100 (10uM), AMD3645 (10uM), IT1t (1uM) and WZ811 (10uM) for 40 min. To determine the effect of TC14012 and other CXCR4 antagonists on ERK1/2 pathway in THP-1 cell, they were pretreated with AMD3100 (10uM), AMD3645 (10uM), IT1t (1uM) and WZ811 (10uM) for 30 min and then SDF-1(100ng/ml) was added for 10 min.

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, HCASMC cells, 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.

Table 2. Reaction composition for cDNA synthesis.

Component	**Volume/reaction**
10xTaq RT buffer	5 µL
25mM MgCl2	11 µL
10mM dNTP	10 µL
oligo dT	2.5 µL
Rnase inhibitor	1 µL
Reverse Transcriptase or RNase free H2O	1.25 µL
RNA	variable
RNase free H2O	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 was previously described (Ding, Ma, Littmann, Camp, & Shen, 2011; Wanshu, Liu, Ellison, & Shen, 2013b). 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 conditions: 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), and final extension step (10 min at 72 ℃). Internal controls, GAPDH or β-actin were amplified in separate wells. The sequences of primers are listed in the Table 3.

Table 3. Primers used for PCR assay

Gene	Forward Primer	Reverse Primer
h CXCR4	5'–CACTTCAGATAACTACACCG–3'	5'–ATGGATCTGCATCTCTTCGACTAC–3'
h CXCR7	5'–ATCCAGACGCCAACATAGAC–3'	5'–TCATTTGGTGCTCTGCTCCAAGG–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 4.

Table 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 for each experiment 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 an 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 50 min. After running the gel, the stack was assembled in the order of two layers of absorbent paper, which was thoroughly soaked in transfer buffer. The stack consisted of the SDS-PAGE gel, a wet polyvinylidene difluoride (PVDF) membrane (Thermo), and 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 blot 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 5. The primary antibodies used for western blot.

Items	Catalog Number	Clone	Company
anti-human CXCR7 mAb	MAB42273	11G8	R&D Systems
mAb anti-human CXCR7	K0223-3	9C4	MBL
pAb anti-human CXCR7	AF4227	-	R&D Systems
pAb anti-human CXCR7	14840-1-AP	-	Proteintech
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	9427	-	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 (Ding et al., 2011; Wanshu et al., 2013b).

2.5. Plasmid Transfection

Vectors containing the human CXCR7 sequences were bought directly from GE Healthcare. Plasmid preparation was performed according to technical manual of “Precision LentiORFTM Collection” (Dharmacon). One day prior to the transfection, HCASMCs 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).

2.6. Lentivirus Transfection

Vectors containing the human CXCR7 sequences were bought directly from GE Healthcare. Plasmid preparation was performed according to technical manual of “Precision LentiORFTM Collection” (Dharmacon). These vectors and packaging vectors were then transfected into HEK293T cells. Supernatants containing lentiviruses were harvested for 48h post-transfection. HCASMCs cells were transfected with lentiviruses according to technical manual of “DrarmaconTM Trans-Lentiviral Packaging Kits” (GE Healthcare).

2.7. Proliferation Assay

Cell Counting Kit-8 (CCK-8) was used to measure the proliferation rate of HCASMCs. CCK-8 is a colorimetric assay uses water-soluble tetrazolium salt WST-8, which is reduced by dehydrogenases of viable cells to form a water-soluble, orange-colored product (formazan). After seeding cells in 96-well plate, cells were allowed to grow for 2-3 days. Then, 10% of the CCK-8 was added to each well and incubated in 37°C for 0.5 to 4 hrs., until color becomes orange. Microplate reader was used to measure the absorbance CCK-8 at 450 nm.

3. Results:

3.1: Evidence of CXCR7 Expression in HCASMCs.

To measure the expression level of CXCR7 in HCASMCs, its mRNA level was analyzed by RT-PCR. RT-PCR result showed that CXCR7 was expressed in moderate amount and was further increased after 60 mins of treatment with TNF-α. This data indicates that CXCR7 is upregulated in an inflammatory environment, suggesting an important role of CXCR7 in inflammation conditions, such as coronary restenosis.

3.2 a&b: Differential Effect of CXCR7 Agonists on ERK1/2 Phosphorylation in HCASMCs.

To find a CXCR7 agonist that decreases the phosphorylation level of ERK pathway, which is a well-known proliferation pathway, multiple CXCR7 agonists were screened. Endogenous agonists, such as SDF-1 and I-TAC, and exogenous, such as TC14012, and VUF-1 were screened. Western blot result showed that TC-14012 was the only compound that inhibited ERK phosphorylation in both situations, with and without TNF-α. Thus, TC14012, as seen in figure 10, could be a potential compound used to inhibit neointimal formation.

3.3: Time Dependent Effect of TC14012 on ERK1/2 Phosphorylation in HCASMCs.

HCASMCs were stimulated with TC 20uM in different time points, 3, 10, 20, 40, 60, 80, 180 mins, and ERK phosphorylation were analyzed by western blot. Each point was compared with its control for its effect on ERK phosphorylation. We found that TC started its inhibition after 20 mins of stimulation. Based on this experiment, we apply TC stimulation for 20 mins for the rest of our research.

3.4: TC Dose Dependent Effect on ERK1/2 Phosphorylation in HCASMCs

To determine TC14012`s best concentration for inhibiting ERK1/2 in HCASMCs, dose response curve was conducted as the following: cells were stimulated for 40 min at the indicated concentrations: 100 nM, 1 uM, 10 uM and 20 uM 30 uM. Results showed that the significant inhibition of ERK1/2 phosphorylation started at 20uM of TC14012.

3.5: Differential Effect of TC14012 on SDF-1 and FBS-Induced c-Raf/MEK1/ERK1/2 Phosphorylation in HCASMCs.

To investigate TC`s ability to antagonize the proliferative effect of SDF-1 and FBS, HCASMCs were pre-treated with TC for 20 mins and then treated with SDF-1 or FBS for 10 mins. ERK phosphorylation level was analyzed using western blot. Results showed that TC was able to antagonize SDF-1-induced ERK phosphorylation and inhibited the upstream activator of ERK, MEK1 and c-Raf.

3.6: Effect of TC14012 on AKT, JNK, P38, and MEK4 Phosphorylation in HCASMC.

After HCASMCs were pre-treated with TC for 20 mins, and then treated with SDF-1 and FBS (10%) for 10 mins, phosphorylation levels of JNK, AKT, P38, and MEK-4 were analyzed using western blot. Results showed that TC could neither activate nor inhibit the phosphorylation levels of JNK, AKT, P38, and MEK4. However, TC plus SDF-1 worked together to make synergistic inhibition to AKT and JNK. These data suggest that TC is an ERK signaling biased ligand, however, TC works synergistically with SDF-1 probably through CXCR4 to inhibit JNK and AKT pathways.

3.7: Expression level of mRNA of CXCR4 in HCASMCs.

Since many studies found a strong relationship between CXCR4 and CXCR7, such as heterodimerization and sharing same endogenous ligand, SDF-1, investigating any

possible role for CXCR4 in TC`s effect is important. The expression level of mRNA CXCR4 was measured in HCASMCs using real-time PCR. It was shown that CXCR4 was highly expressed, suggesting that CXCR4 may have a role in TC`s effect on ERK phosphorylation.

3.8: Investigation of any possible role of CXCR4 on TC-inhibited ERK phosphorylation.

This experiment was conducted to identify whether CXCR4 was involved in TC-inhibited ERK phosphorylation. AMD compounds were used as CXCR4 antagonists to find whether the same inhibition of TC1401 effect can be achieved. (**A**) TC was the only compound that reduced SDF-1-induced ERK phosphorylation in HCASMCs, suggesting that TC inhibition might selectively be mediated by CXCR7. (**B**) Other CXCR4 antagonists were used and found that all of them were not functioning in HCASMCs, suggesting that TC might be functioning through CXCR7, not CXCR4. (**C**) To evaluate the effectiveness of these CXCR4 antagonists, THP-1 cells, a human monocytic cell line that only express CXCR4, was used. As expected, these antagonists were in fact effective in antagonizing SDF-1-induced ERK phosphorylation, proving that TC-inhibited ERK phosphorylation might be mediated through CXCR7.

3.9: Knocking down CXCR7 in HCASMCs and validating it by real-time PCR and western blot.

To study the role of CXCR7 in TC-inhibited ERK phosphorylation, CXCR7 gene need to be knocked down. shRNA technology was used to silence the CXCR7 gene by causing destruction of CXCR7`s mRNA. After transfecting HCASMCs with shRNA, both mRNA and protein levels of CXCR7 were measured by RT-PCR and western blot respectively.

Both levels indicated that CXCR7 expression in knocked down group were significantly lower than its expression in the wild type group.

3.10: TC14012-inhibited ERK phosphorylation in wild type vs CXCR7-shRNA in HCASMCs.

After knocking down CXCR7 in HCASMCs, both wild type and CXCR7 knocked down cells were treated with TC for 20 mins. Then, ERK phosphorylation was measured and analyzed in both groups. As expected, TC was able to inhibit ERK phosphorylation in the wild type while it lost its ability in the knocked down group to inhibit ERK phosphorylation. This finding indicates that CXCR7 was required for TC`s ability to inhibit ERK phosphorylation in HCASMCs.

3.11: TC14012`s effect on HCASMCs` proliferation.

To study the functional significance of TC, TC`s effect on HCASMC proliferation were evaluated. HCASMCs were pre-treated with different doses of TC, 1, 10, and 20 μM before treating them with 5% BCS for 48hrs. It was found that TC was able to antagonize BCS-induced proliferation, suggesting that TC could be a potential candidate for preventing intimal hyperplasia and thus coronary stenosis or restenosis.

3.12: **Cytotoxicity comparison between TC and current antiproliferative agents used in stent coating in HCASMCs.**

Cells were seeded in 96-well plate, starved over-night, and then treated with TC 20μM, Paclitaxel 20μM, or Rapamycin 20μM for 24 hrs. We found that TC was the only compound that had no cytotoxic effect on HCASMCs, which are important in the regulation of vessel dilatation and contraction, and blood flow. Therefore, TC can prevent HCASMCs proliferation while maintaining the cells function and integrity.

3.13: **Cytotoxicity comparison between TC and current antiproliferative agents used in stent coating in vascular endothelial cells.**

Cells were seeded in 96-well plate, starved over-night, and then treated with TC 20µM, Paclitaxel 20µM, or Rapamycin 20µM for 24 hrs. Similar to TC`s effect on HCASMCs, TC was the only compound that has no cytotoxic effect on endothelial cells, which is essential in preventing intimal hyperplasia.

4. Figures:

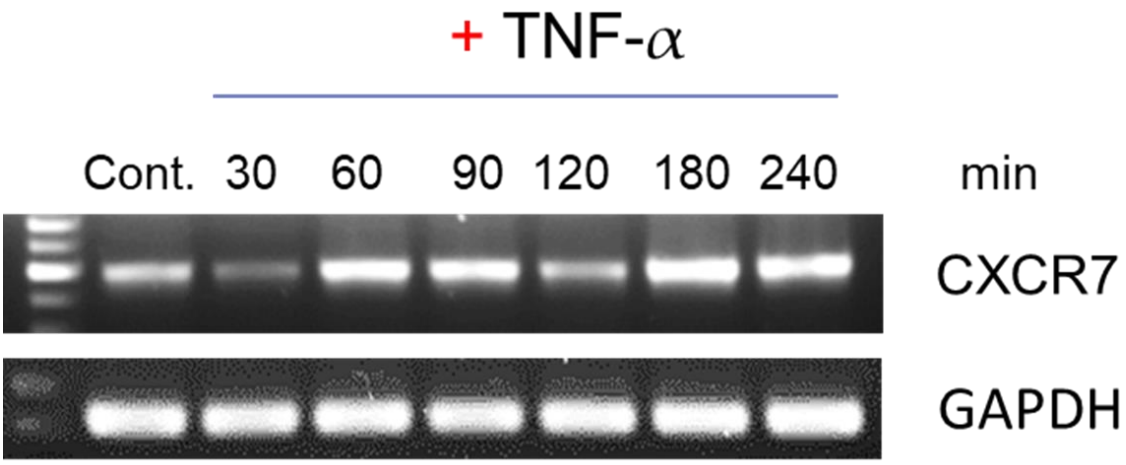

Figure 4.1: Evidence of CXCR7 Expression in HCASMCs. The gene expression of CXCR7 was evaluated by measuring CXCR7`s mRNA level in HCASMCs using RT-PCR. The figure shows that CXCR7 gene expression is expressed in the basal level and it is further expressed after 60 mins treating the cells with TNF-α, which is a pro-inflammatory mediator. This data suggests that CXCR7 might play an important role in inflammatory conditions.

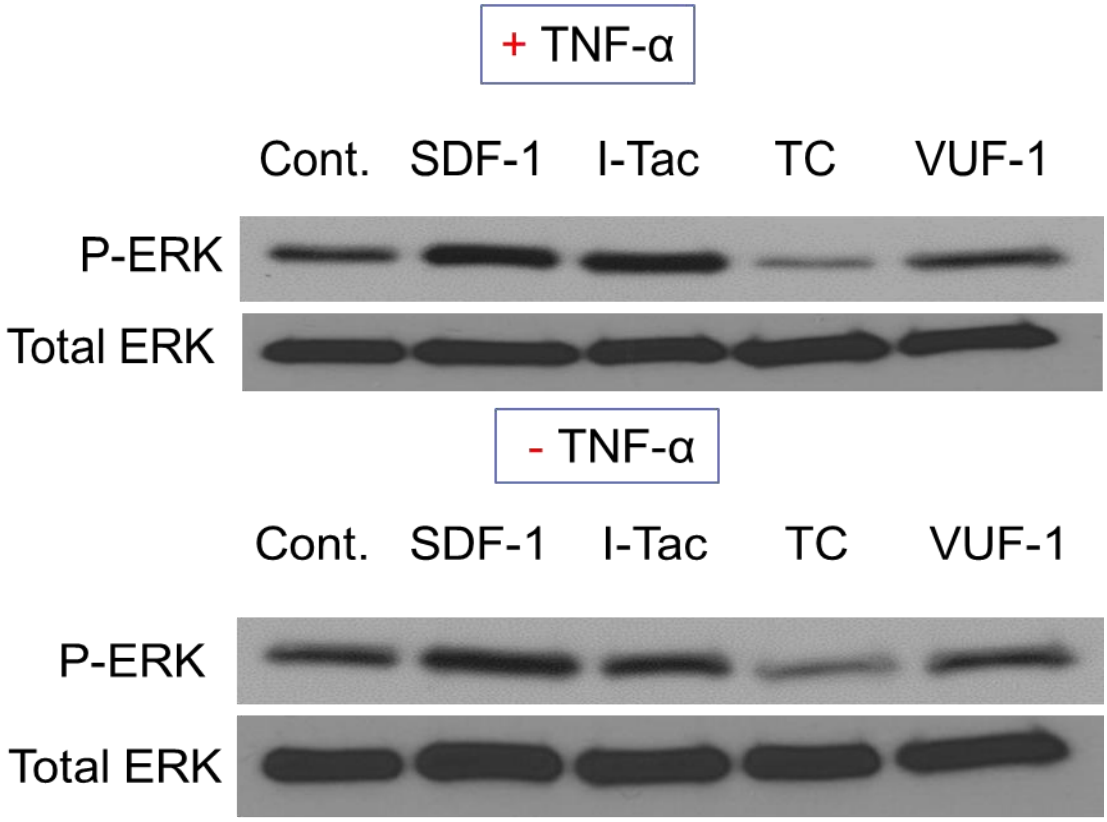

Figure 4.2 a&b: Differential Effect of CXCR7 Agonists on ERK1/2 Phosphorylation in HCASMCs. Different CXCR7 agonists were screened and analyzed in HCASMCs by measuring the phosphorylation level of ERK protein using Western Blot. It was found that TC14012 was the only CXCR7 agonist that decreased the phosphorylation level of ERK protein. This data suggests that TC14012 could be a potential drug that may prevent the proliferation of VSMCs.

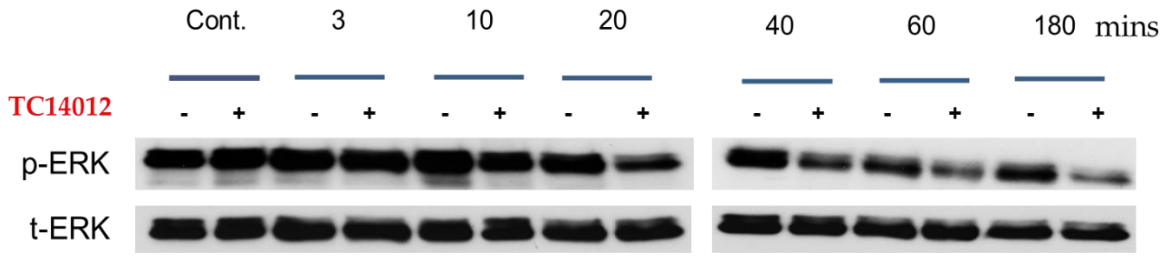

Figure 4.3: Time Dependent Effect of TC14012 on ERK1/2 Phosphorylation in HCASMCs. HCASMCs were treated with 20uM TC14012 and measured the ERK phosphorylation level in different time points, 0, 3, 10, 20, 40, 60, 180 minutes using Western Blot. After 20 mins of treatment, TC14012 started its inhibitory effect on ERK protein.

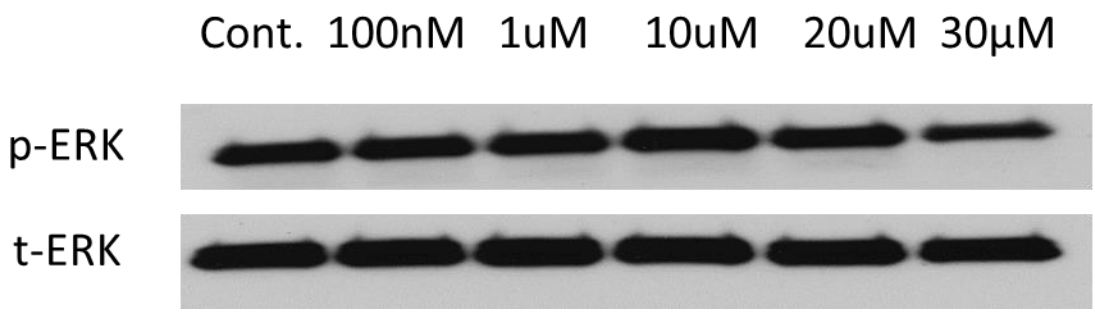

Figure 4.4: TC14012 Dose Dependent Effect on ERK1/2 Phosphorylation in HCASMCs. To find the lowest concentration of TC14012 that is able to decrease the phosphorylation level of ERK protein, HCASMCs were treated with different concentrations of TC14012**,** 100nM, 1uM, 10uM, 20uM, 30uM. The figure shows that 20uM induceed significant inhibition.

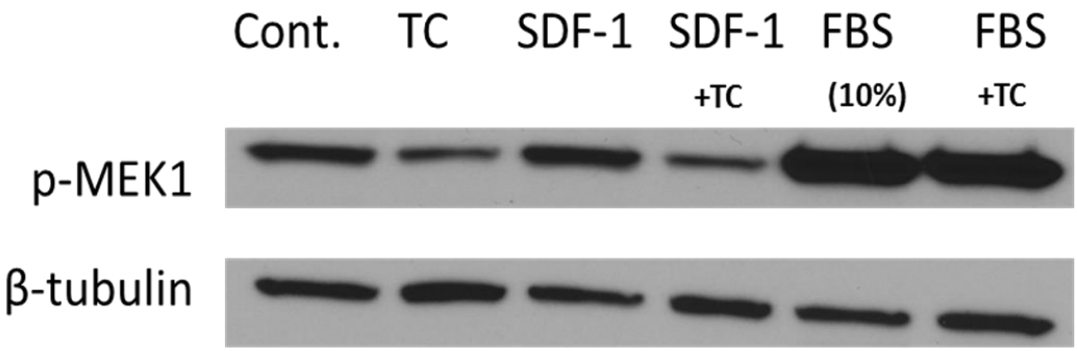

Figure 4.5: Differential Effect of TC14012 on SDF-1 and FBS-Induced MEK1/ERK1/2 Phosphorylation in HCASMCs. The phosphorylation level of the upstream activator of ERK protein, MEK1, was also analyzed using Western Blot. TC14012 was found to decrease both the basal level and SDF-1 induced MEK1 phosphorylation.

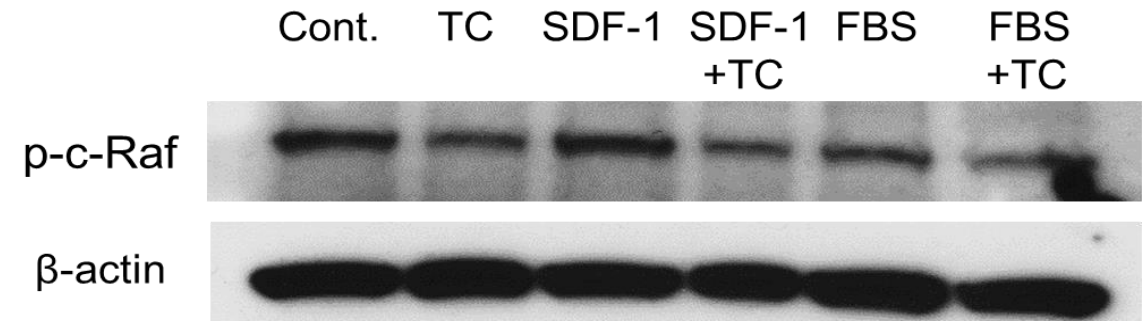

Figure 4.6: Differential Effect of TC14012 on SDF-1 and FBS-Induced c-Raf Phosphorylation in HCASMCs. The phosphorylation level of the other upstream activator of ERK protein, c-Raf, was also analyzed using Western Blot. TC14012 was found to decrease both the basal level and SDF-1 induced c-Raf phosphorylation.

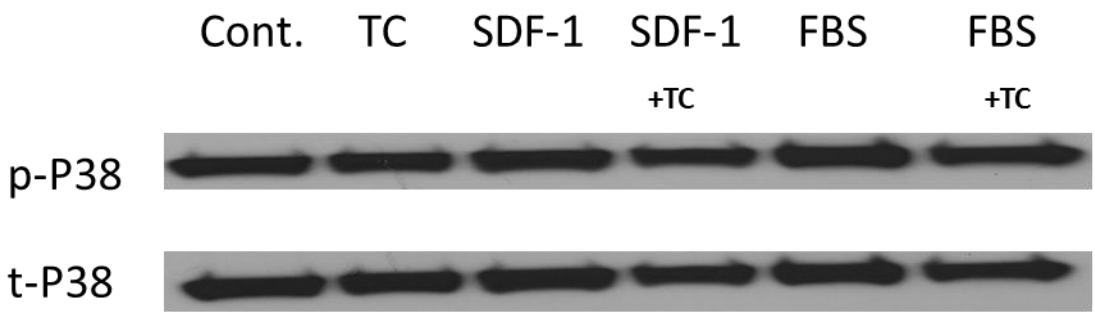

Figure 4.7: Effect of TC14012 on P38 Phosphorylation in HCASMC. Other pathways were analyzed to investigate the bias signaling characteristic of TC. This figure shows the phosphorylation level of P38 in HCASMCs. TC14012 neither activated nor inhibited the basal and SDF-1 induced phosphorylation level of P38 protein.

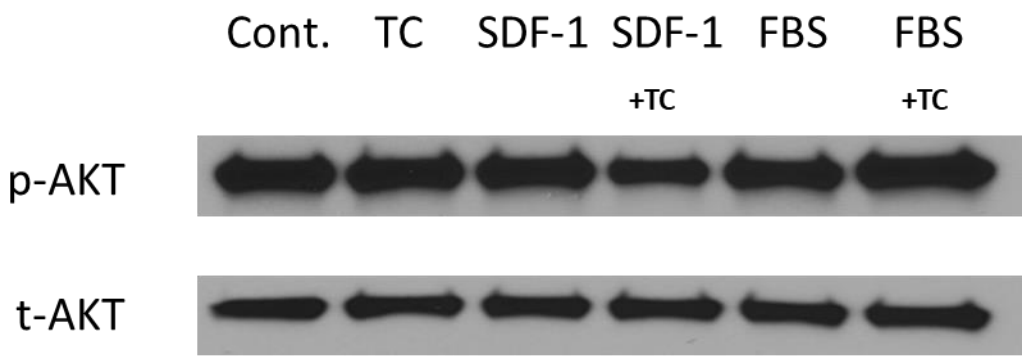

Figure 4.8: Effect of TC14012 on AKT Phosphorylation in HCASMC. another pathway, AKT, was analyzed in HCASMCs to investigate the bias signaling characteristic of TC. TC14012 neither activated nor inhibited the basal and SDF-1 induced phosphorylation level of AKT protein.

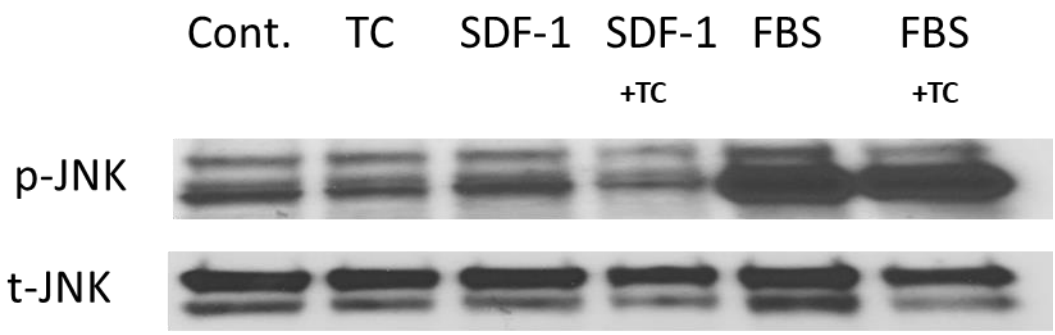

Figure 4.9: Effect of TC14012 on JNK, Phosphorylation in HCASMC. Other pathways were analyzed to investigate the bias signaling characteristic of TC14012. This figure shows the phosphorylation level of JNK in HCASMCs. TC14012 neither activated nor inhibited the basal and SDF-1 induced phosphorylation level of JNK protein.

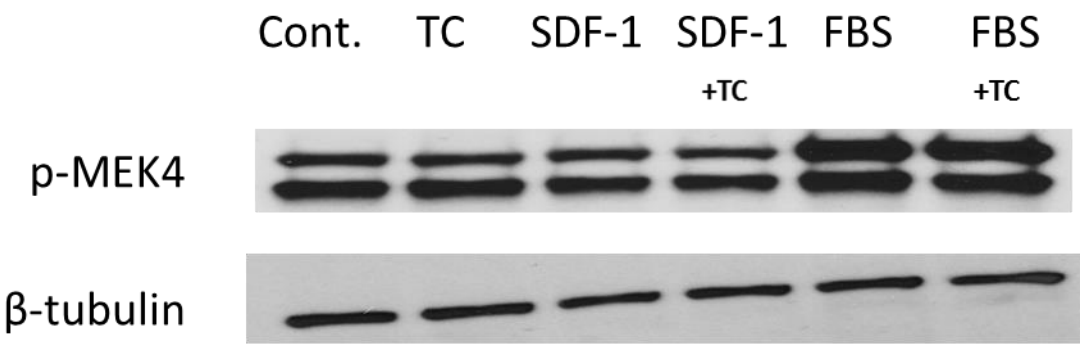

Figure 4.10: Effect of TC14012 on the upstream activator of JNK and P38, MEK4 Phosphorylation in HCASMC. The upstream activator of both JNK and P38 was analyzed in HCASMCs to investigate the bias signaling characteristic of TC. TC14012 neither activated nor inhibited the basal and SDF-1 induced phosphorylation levels of MEK4 protein. This data suggest that TC is a ligand bias, which functions specifically through the inhibition of ERK pathway.

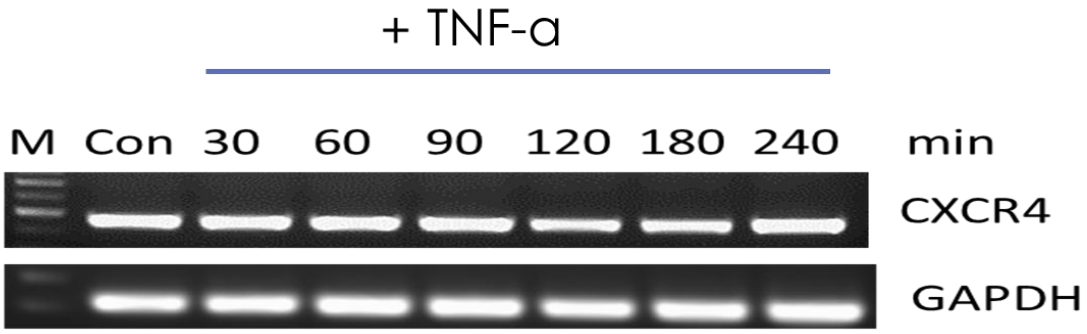

Figure 4.11: Expression level of mRNA of CXCR4 in HCASMCs. CXCR4 was found to be involved with CXCR7-induced signaling pathway, specifically through heterodimerization. Therefore, it is important to measure the level of CXCR4 in HCASMCs and investigate any involvement in the negative role of CXCR7 in HCASMCs. In this figure, the basal level of CXCR4 in HCASMCs is highly expressed.

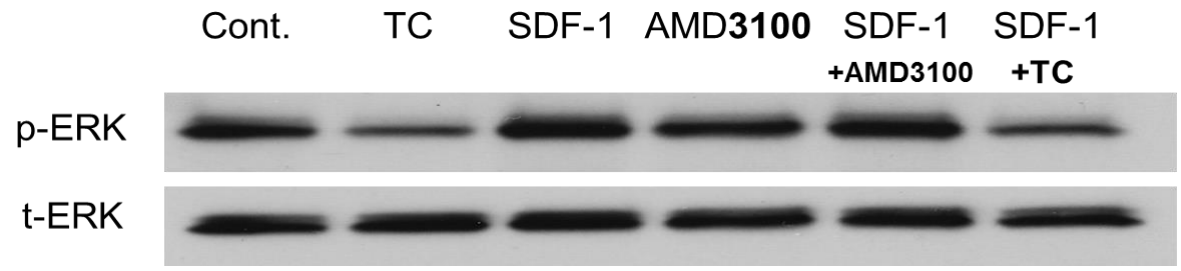

Figure 4.12: Using a CXCR4 inhibitor to investigate of any possible role of CXCR4 on ERK phosphorylation vs TC14012 effect. AMD3100, a CXCR4 antagonist, was used to block CXCR4 in HCASMCs. The ligand didn`t mimic the effect of TC14012, suggesting that CXCR7 is required for the negative effect of TC14012.

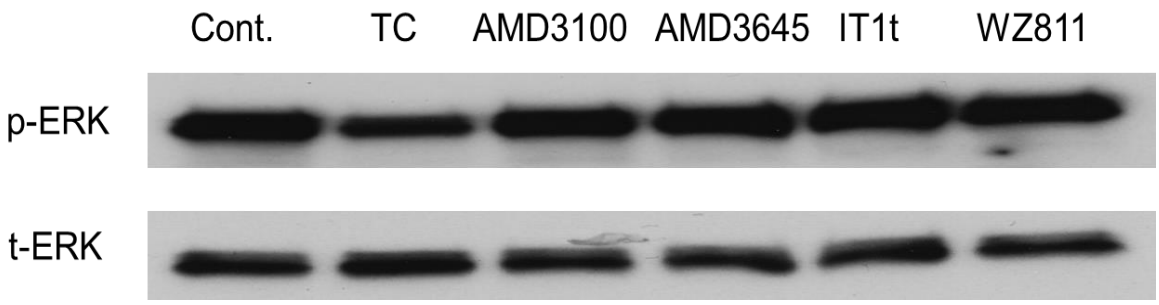

Figure 4.13: Comparison the effect of different CXCR4 inhibitors with TC14012 on ERK phosphorylation in HCASMCs. Different CXCR4 ligands were screened in HCASMCs. None of the ligands reduced the phosphorylation of ERK and thus mimicked the effect of TC14012, suggesting that CXCR7 is required for the effect of TC14012.

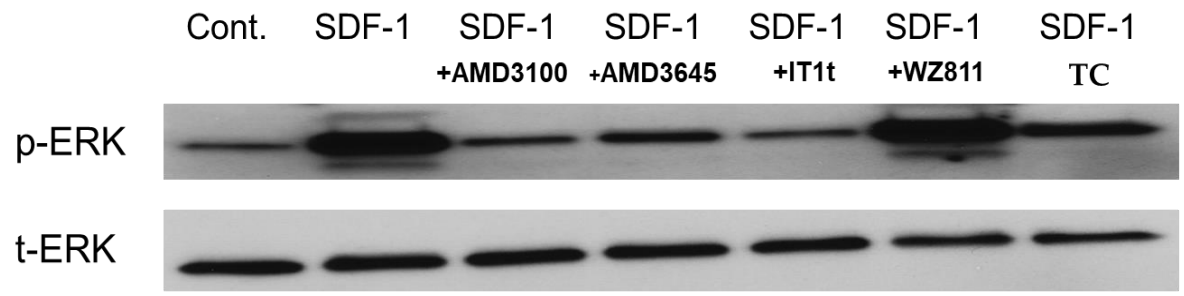

Figure 4.14: Comparison the effect of different CXCR4 and CXCR2 inhibitors with TC14012 on ERK phosphorylation in THP-1 cells. To make sure the appropriate doses of these ligands were used in HCASMCs to block CXCR4, THP-1, monocytic cells that expresses only CXCR4, was treated with the same ligands using the same doses. As expected, the ligands were able to reduce the phosphorylation of ERK in THP-1, suggesting that the doses were high enough to block CXCR4 in the previous experiment.

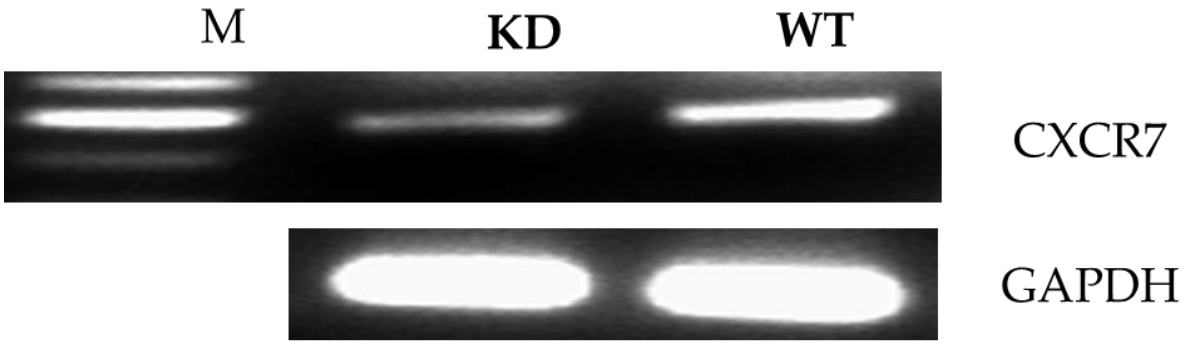

Figure 4.15: Real-time PCR analysis after Knocking down CXCR7 in HCASMCs. To evaluate the efficiency of shRNA in knocking down CXCR7 in HCASMCs, the mRNA level of CXCR7 was measured using RT-PCR. The expression of CXCR7 gene was significantly reduced in the knocked down cells compare to the wild type.

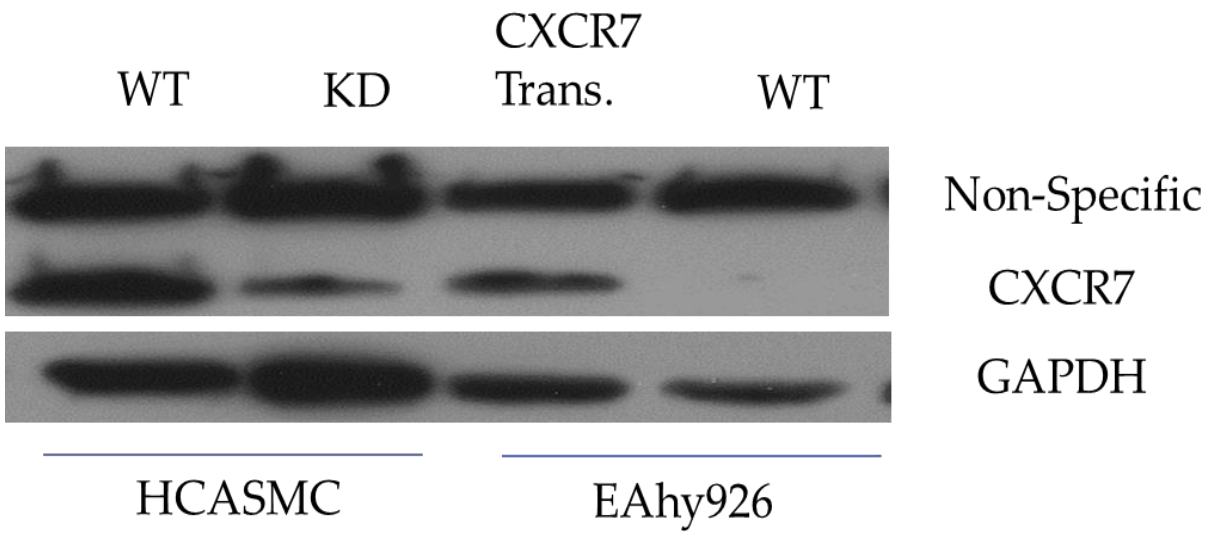

Figure 4.16: Western blot analysis after Knocking down CXCR7 in HCASMCs. The protein level of CXCR7 in HCASMCs was also analyzed to evaluate the effectiveness of shRNA assay using Western Blot. The protein level was significantly reduced in the knocked down compare to the wild type. Wild type and CXCR7 transfecting EAhy926 cell line was used as a positive and negative control.

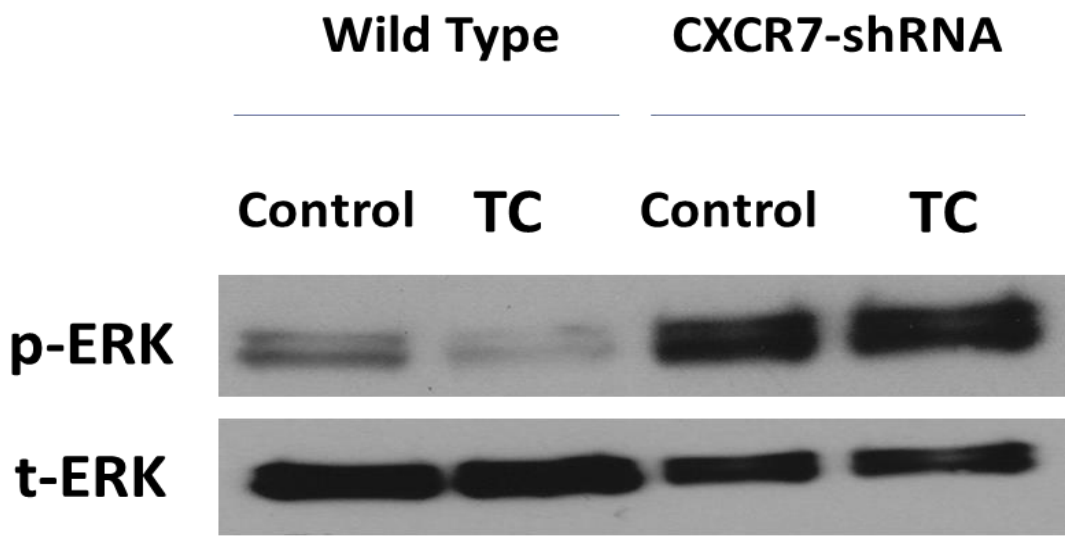

Figure 4.17: TC14012-inhibited ERK phosphorylation in wild type vs CXCR7-shRNA in HCASMCs. To investigate the requirement of CXCR7 for TC14012-inhibited ERK phosphorylation, the phosphorylation level of ERK was measured in both wild type and CXCR7-kncoked down cells. As expected, TC14012 was able to reduce the phosphorylation level of ERK in the wild type but lost its ability to reduce it in the CXCR7-knocked down cells. This data suggested that CXCR7 was required for the inhibitory effect of TC14012 on ERK phosphorylation in HCASMCs.

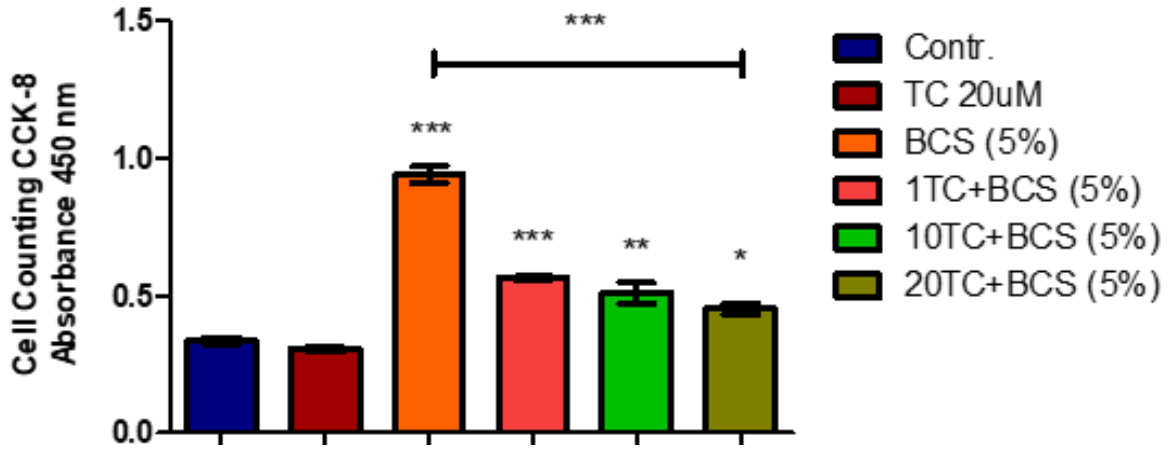

Figure 4.18: TC14012 functional significance on the proliferation of HCASMCs. To evaluate the functional significance of TC14012, CCK-8 assay was used to measure the proliferation of HCASMCs in the following groups: control, TC14012, BCS (5%), 1uM TC+BCS (5%), 10uM TC+ BCS (5%), and 20uM TC14012+ BCS (5%). The data shows that TC14012 was able to antagonize the proliferative effect of BCS (5%), suggesting that TC14012 might be a potential drug to reduce the incidence of in-restenosis.

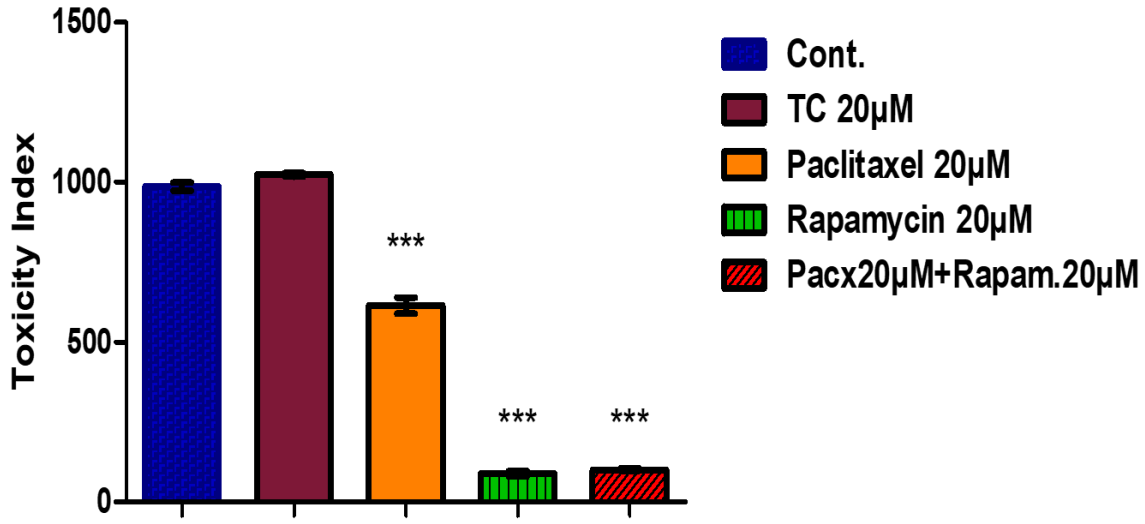

Figure 4.19: Cytotoxicity comparison between TC and current antiproliferative agents used in stent coating in HCASMCs. To evaluate the safety profile of TC14012 on HCASMCs, resazurin assay was used to measure the cell viability after treating HCASMCs with TC14012 and compare it with the current anti-proliferative drugs used to coat stents. Results showed that TC14012 was significantly safer than the other drugs, suggesting that TC14012 might not interfere with the natural body healing response after stenting-causing injury.

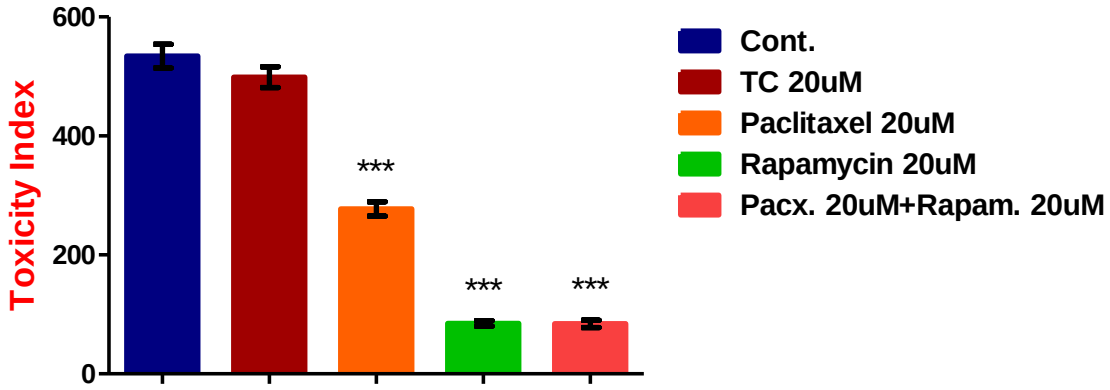

Figure 4.20: Cytotoxicity comparison between TC and current antiproliferative agents used in stent coating in vascular endothelial cells. To evaluate the safety profile of TC14012 on vascular endothelial cells, resazurin assay was used to measure the cell viability after treating the cells with TC14012 and compare it with the current anti-proliferative drugs used in stents coating. Results showed that TC14012 was significantly safer than the other drugs, suggesting that TC14012 might not interfere with the natural body healing response after stenting-causing injury.

5. Discussion:

Coronary atherosclerosis and its complications, such as stroke and myocardial infarction, represent critical contributors to cardiovascular diseases, which are the leading cause of death worldwide. Therefore, a large number of research projects have been conducted to improve the current therapeutic approaches for cardiovascular diseases.

Coronary stenting is the primary surgical approach for atherosclerotic coronary diseases. However, it was found to be ineffective in preventing occlusion in up to 40% of cases (a Kastrati, Schömig, Dietz, Neumann, & Richardt, 1993; A. Kastrati et al., 2001). Even though the approach has been greatly advanced by using DES and angioblasty, it is still ineffective in the long-term with more than 15% of restenosis incidences in most studies (Beijk et al., 2010; A. Kastrati et al., 2005). Therefore, it becomes a recognized clinical problem that needs further investigation. This problem has been shown to be mainly caused through the induction of HCASMC proliferation around the stent causing an in-stent restenosis (Stefanini & Holmes, 2013). During the insertion of the stent, the endothelial layer is denuded, exposing HCASMCs to circulated growth factors, which trigger the proliferative signaling pathway of HCASMCs (Clowes, Clowes, & Reidy, 1986). Therefore, there is a desperate need for discovering new strategies or enhancing the current atherosclerotic treatment approaches to lower restenosis incidences.

It is well known that the MAPK/ERK1/2 signaling pathway is required for HCASMC proliferation (Broberg et al., 2002; Cipolletta et al., 2010; Zalba, Beaumont, & José, 2000; Zhan et al., 2003). The preliminary data indicated that the CXCR7 receptor was upregulated during HCASMC inflammation and activating CXCR7 suppressed the ERK1/2 signaling pathway in HCASMCs. Thus, activation of CXCR7 may provide

therapeutic opportunity for coronary restenosis by attenuating the proliferation of the HCASMCs through the inhibition of the ERK1/2 pathway.

Different CXCR7 agonists were applied to HCASMCs and the phosphorylation level of ERK1/2 and its upstream signaling proteins were analyzed using the Western Blot technique. This approach helped us to understand the negative role of CXCR7 in controlling the ERK1/2 signaling pathway in HCASMCs and thus being able to attenuate the cell proliferation. TC14012 was found to be the only CXCR7 agonist to decrease the expression of p-ERK1/2, suggesting that TC14012 might be a potential drug used to inhibit the proliferation of HCASMCs. TC14012 was also found to decrease the phosphorylation of the upstream activator of ERK1/2, MEK4. Interestingly, TC14012 also antagonized the SDF-1-induced ERK1/2 phosphorylation. In addition, FBS-induced ERK1/2 phosphorylation was also antagonized by TC14012. These data suggested that TC14012 could potentially inhibit the proliferation of HCASMCs and thus prevent in-stent restenosis. Therefore, to investigate the functional significance and the efficacy of TC14012, CCK-8 proliferation assay was conducted on HCASMCs. As expected, TC14012 was significantly able to antagonize the proliferative effect of BCS 5%, suggesting that TC14012 could work against in-stent restenosis formation.

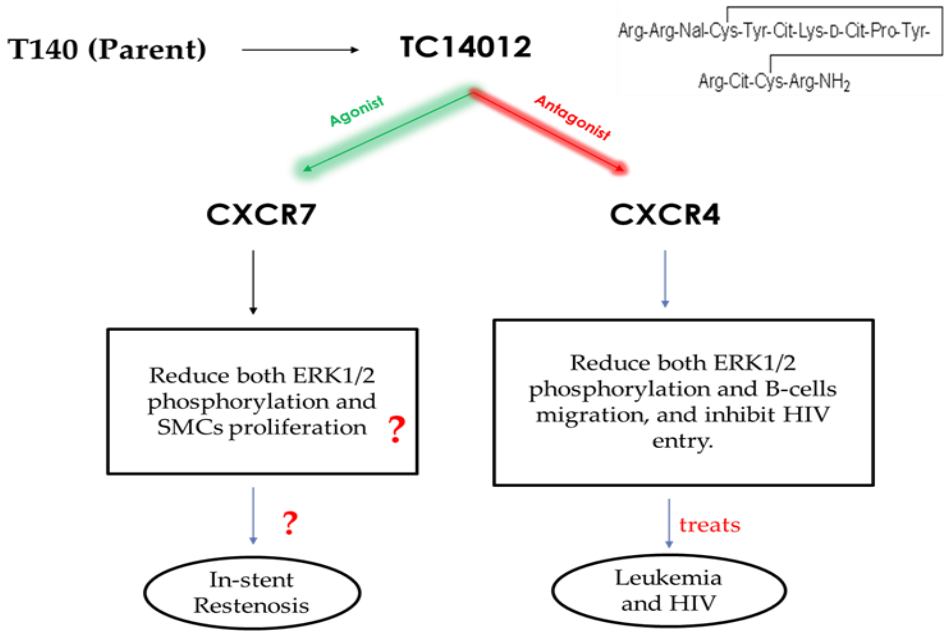

Figure 10. TC14012 compound and its effect. TC14012 was derived from its parent T140, which more toxic and less stable. TC14012 functions as CXCR7 agonist but as CXCR4 antagonist. CXCR4 inhibition helps in treating leukemia and preventing HIV from entering. Activating CXCR7 may help prevent in-stent restenosis by stopping the proliferation of vascular SMCs.

Our body respond naturally to the injury, caused by stent implantation, by triggering the growth of the endothelial cells trying to heal itself. Drugs used in DES has high cytotoxicity against the vascular endothelial cells and thus interfere with the body`s natural healing process. TC14012 was shown to have no cytotoxic effect on the vascular endothelial cells, suggesting that TC14012 would help our body to heal itself.

Since CXCR4 is involved with CXCR7 in many aspects, such as sharing the same ligand, SDF-1, and heterodimerization, it is important to study the involvement of CXCR4 in the effect of TC14012 on the ERK1/2 pathway. To investigate this, two methods were conducted, blocking CXCR4 receptor by using several CXCR4 antagonists, and shRNA

technology was used to knockdown CXCR7. After blocking the function of CXCR4, TC14012`s ability to inhibit ERK1/2`s pathway was analyzed. TC14012 still exerted its function and decreased ERK1/2 phosphorylation in the absence of CXCR4`s function. This finding partially suggested that CXCR4 was not involved in TC14012`s effect on ERK1/2 pathway. To confirm this finding, CXCR7 has to be knocked down to study the effect of TC14012 in the absence of CXCR7 receptor. As expected, TC14012 lost its ability to decrease the phosphorylation of ERK1/2 in the absence of CXCR7, indicating that CXCR7 was required for the effect of TC14012 on ERK1/2 pathway.

TC14012 was also investigated for the possibility of any off-target effects by measuring the activity of different major protein pathways, AKT, P-38, and JNK. The phosphorylation levels of these proteins were measured after treating HCASMCs with TC14012 for 20 mins using western blot. Results showed that TC14012 neither activated nor inhibited any of them, suggesting that TC14012 might function as a biased ligand toward the ERK pathway. However, when cells were pre-treated with TC14012 for 30 mins and then treated with SDF-1 for 10 mins, results showed that TC14012 was able to antagonize the effect of SDF-1 on JNK, AKT, and P-38 phosphorylation. This data suggested that TC14012 might antagonize the effect of the endogenous effect of SDF-1.

6. Future Plan:

In vitro results showed that CXCR7 worked as a break for the activity of ERK1/2 protein. After knocking down CXCR7, the ERK phosphorylation level was upregulated. Therefore, activating CXCR7 was shown to decrease the proliferation of HCASMCs, which is the main cause of in-stent restenosis.

Future studies will focus on whether activating CXCR7 is able to prevent in-stent restenosis *in vivo* using mouse model. Different atherosclerotic models are available. One of them, a wire-mediated vascular injury model, may effectively represent our model of interest in adult male16 to 24-week-old C57BL/6 mice. These mice are the most widely used and best-selling type of mouse strain, due to the availability of congenic strains, easy breeding, and robustness (Engber, 2011). It will be adopted from Masataka Sata`s work found in the following reference (Sata et al., 2000). The success rate of the wire insertion is expected to be more than 95% for all mice as examined in Masataka Sata`s article.

The mouse model will be created by a specific SMC-CXCR7 knock-out with atherosclerosis mechanically induced. This approach allows studying the role of CXCR7 in HCASMC-induced proliferation after vessel injury and how significantly CXCR7 may contribute to preventing in-stent restenosis. The absence of CXCR7 might allow the migration and proliferation and thus accumulation of HCASMCs in the injured region. Conversely, the activation of CXCR7, using TC14012 might prevent in-stent restenosis and thus the need of revascularization.

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